Università della Calabria

Expression of the K303R Estrogen Receptor α Mutation Induces Hormonal Resistance in Breast Cancer

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INDEX

Scientific Publication

1. Catalano S., Malivindi R., **Giordano C.,** Gu G., Panza S., Bonofiglio D., Lanzino M., Sisci D., Panno M.L. and Andò S. Farnesoid X Receptor, through the binding with steroidogenetic factor 1 responsive element, inhibits aromatase expression in tumor Leydig cells. The Journal Of Biological Chemistry 2009. In revision.

2. Catalano S., Barone I., **Giordano C.,** Rizza P., Qi H., Gu G., Malivindi R., Bonofiglio D. and Andò S. Rapid estradiol/ERα signaling enhances aromatase enzymatic activity in breast cancer cells. Mol Endocrinol, 23(10):1634–1645, Oct 2009.

3. Barone I., Cui Y., Herkyn MH., Corona-Rodriguez A., **Giordano C**., Selever J., Beyer A., Andò S., Fuqua S.A. Expression of K303R Estrogen Receptor α Breast Cancer Mutation Induces Resistance to an Aromatase Inhibitor via Addiction to the PI-3K/Akt Kinase Pathway. Cancer Research, 69(11):4724-32, Jun 2009.

4. **Giordano C**., Cui Y., Barone I., Andò S., Mancini M.A., Berno V., Fuqua S.A. Growth Factor-Induced Resistance to Tamoxifen is Associated with Mutation of Estrogen Receptor α and its Phosphorylation at Serine 305. Breast Cancer Res Treat, (Epub ahaed of print), Feb 2009.

5. Catalano S., **Giordano C**., Rizza P., Gu G., Barone I., Bonofiglio D., Giordano F., Malivindi R., Gaccione D., Lanzino M., De Amicis F., Andò S. Evidence that leptin through STAT and CREB signaling enhances Cyclin D1 expression and promotes human endometrial cancer proliferation. J Cell Physiol. John Wiley & Sons, Inc (USA), 218(3):490-500, Mar 2009.

6. Mauro L., Catalano S., Bossi G., Pellegrino M., Barone I., Morales S., **Giordano C**., Bartella V., Casaburi I. and Andò S. Evidences that leptin upregulates E-cadherin expression in breast cancer: effects on tumor growth and progression. Cancer Research, American Association for Cancer Research, Philadelphia (USA), 67: 3412-3421, 2007.

7. Catalano S., Rizza P., Gu G., Barone I**.**, **Giordano C.**, Marsico S., Casaburi I., Middea E., Lanzino M., Pellegrino M., Andò S. Fas Ligand expression in TM4 Sertoli cells is enhanced by estradiol "in situ" production. Journal of Cellular Physiology, John Wiley & Sons, Inc (USA), 211: 448-456, 2007.

INTRODUCTION

Estrogens play a crucial role in regulating the growth and differentiation of normal breast epithelium and also of breast cancers, with approximately two-thirds of all breast cancers dependent for their growth on a functional estrogen receptor α (ERα). ERα is a member of the nuclear hormone receptor superfamily that regulates transcription of ER target genes by binding with specific estrogen response elements (ERE) (classical action), or without directly interacting with DNA, via tethering to other transcription factors such as activator protein-1 (1), SP-1 (2) and others (nonclassical action) (3, 4). Furthermore estrogens also clearly have some nongenomic actions (fig.1), which are manifested in the rapid induction of kinase signalling cascade and are probably mediated by the receptors' activity in the vicinity of the cytoplasic membrane (5, 6).

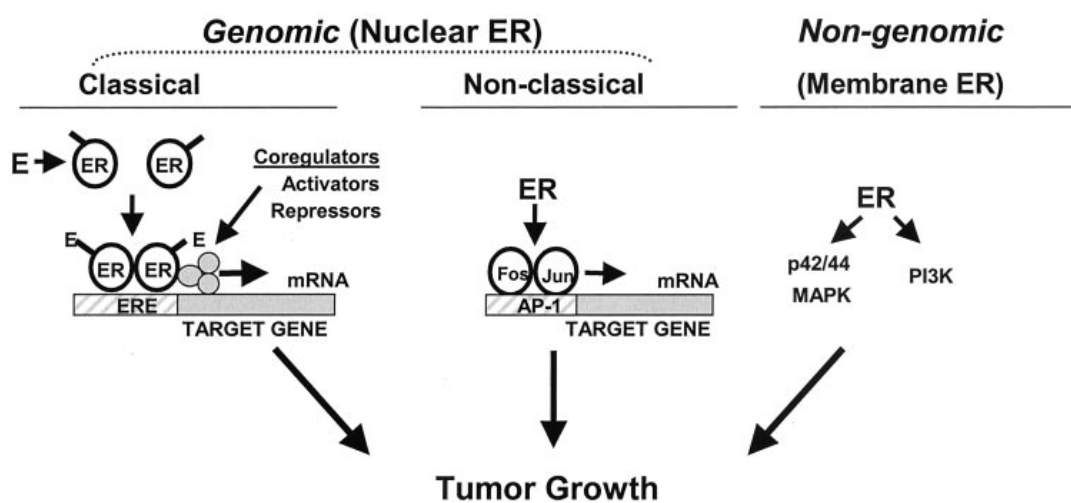**Fig.1 The multifaceted mechanisms of estrogen receptor signalling.**

Current endocrine therapies offered to women with ERα-positive breast cancer are based mainly on targeting the ER signalling pathway by either: antagonizing ER function with antiestrogens such as tamoxifen (Tam), or down-regulating ER levels with pure antiestrogens such as fulvestrant (Flasodex); or reducing levels of estrogens

using aromatase inhibitors. At present, a nonsteroidal SERM (Selective Estrogen Receptor Modulator) Tamoxifen, remains the most frequently prescribed drug for the treatment of all stages of breast cancer. ERα posses tow major trancriptional activation domains residing in their NH2 and the COOH termini which harbour, respectively, the constitutively active, ligand-independent AF-1 and the ligand-dependent AF-2 functions (Fig.2).

ERα
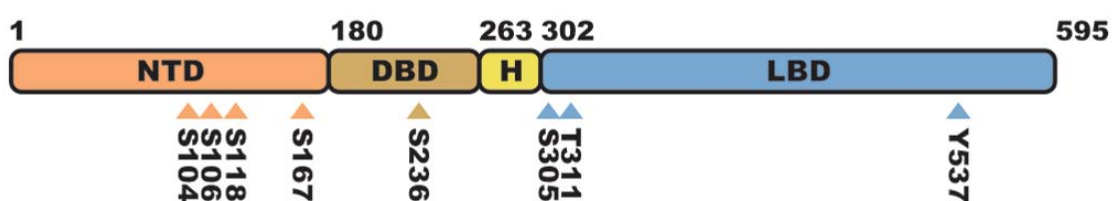
Fig. 2 Structure and functional domains of ERα. Domain structures of steroid receptors. The numbers of the amino acids found at the boundaries in the individual receptors between the NTD (AF-1, amino-terminal domain), DBD (DNA binding domain), hinge region (H), and ligand binding domain (LBD, AF-2) are indicated in the figure. Also shown are the best characterized phosphorylation sites in the human steroid receptors.

A key feature of the estrogen agonist-receptor structure is the ability of the ligand to be enveloped in a hydrophobic pocket that is closed by helix 12 in the ligand-binding site of ERα. Helix 12 positioning over the hydrophobic pocket is critical for the recruitment of coactivators to the AF-2 site and subsequent initiation of RNA polymerase activity. The repositioning of helix 12 after binding has been proposed as an important mechanisms for full estrogen action at ERα (7). SERMs like as 4-hydroxitamoxifen (4-OHT) fit into the hydrophobic pocket of the ligand binding domain, but the antiestrogen side chain prevents the reorientation of helix 12 that must seal the ligand into the receptor before coactivators can bind and produce a transcriptional complex. Tamoxifen treatment, then, inhibits breast cancer growth mainly through competitive blocking of

the ER activity, thereby inhibiting estrogen-induced growth. However, although tamoxifen is initially effective in many patients, and in general is very tolerated, a major obstacle to its use is tumor resistance. Almost 50% of breast cancers, despite the presence of ERα, fail to respond to tamoxifen (termed *de novo* resistance); furthermore, even patients who initially respond eventually acquire tamoxifen resistance, leading to tumor progression and death. In general, acquired resistance to tamoxifen is not attributable to loss of or alteration in the ERα, and resistant tumors often respond to second-line endocrine therapy (8).

In previous work Fuqua *et al.* identified an A to G somatic mutation at ERα nucleotide 908 (A908G) from several usual ductal hyperplasias, which are early premalignant breast lesions (9). This transition introduces a lysine to arginine substitution at residue 303 (K303R ERα) within exon 4, at the border between hinge domain D and the beginning of hormone-binding domain E of the ERα. Growth studies in breast cancer cells stably transfected with the mutated ERα, demonstrated that the K303R ERα mutation confers increased sensitivity to subphysiological levels of estrogen (9). To date, no other ERα mutation has been identified in more that a few invasive breast cancers (10). Others have reported that the K303R ERα mutation was not present in invasive tumors (11, 12), but Fuqua *et al.* have demonstrated that the detection method used by these other investigators, standard dye-terminator sequencing, was not sensitive for detection of this specific mutation (13). In addition, Conway *et al* have identified this mutation in 6% of breast cancers using a different detection method (14). The mutation resides at major post-translational modifications sites (acetylation, ubiquitination, methylation) adjacent to a protein kinase A (PKA) phosphorylation site at serine residue 305 (S305). There are four potential sites for phosphorylation by PKA in ERα (serine S236, S305, S338, and S518), with S236 being the best characterized

site regulating receptor dimerization and DNA binding (15). These serines are all within the cAMP- dependent protein kinase phosphorylation sequences XRRXSX or SKKXSX. Interestingly, it has been shown that the mutation of the SK*K*XSX recognition sequence to SK*R*XSX increases the kinetics of PKA phoaphorylation (16) and predicts that the K303R mutation in ERα may generate a more efficient substrate for PKA phosphorylation at S305. Recently, Cui *et al.* have demonstrated that this naturally-occurring mutation is a more efficient substrate for phosphorylation by PKA, and is hypoacetylated which subsequently alters estrogen sensitivity (17). Michalides *et al.* have suggested that phosphorylation of ERα S305 by PKA induces a switch from antagonistic to agonistic effects of Tam, which induces resistance to this antiestrogen (18). It has also been shown that the ERα S305 site can be an *in vivo* substrate for p21-activated kinase 1 (PAK 1)-mediated phosphorylation (19), and its overexpression correlates with resistance to tamoxifen in breast cancer patients (20). These findings identify S305 as a crucial site in ERα that upon phosphorylation by either PKA or PAK-1 is responsible for resistance to tamoxifen. Therefore, this phosphorylation affects conformational changes in ERα following binding to tamoxifen, which alters its orientation toward the co-activator SRC-1 resulting in the recruitment of RNA polymerase II, thereby stimulating estrogen receptor-driven transcription by the anti-estrogen tamoxifen (19, 21).

We know that estrogens regulation of breast cancer cell growth can also be modulated by complex interactions with a variety of peptide growth factors. A large body of evidence supports the idea that rapid membrane effects of ERα (also called non-genomic effects of ER) may activate various components of growth factor tyrosine kinase signaling, such as that from insulin-like growth factor-IR (IGF-IR), epidermal growth factor receptor (EGFR), and c-erbB2/HER2 (22-24). Furthermore, the kinase

cascade signaling initiated by growth factor receptors can activate ERα (termed ligand-independent effects) and its coregulatory proteins, causing an interdependent loop of cross-talk that leads to enhanced tumor cell survival and proliferation (8, 25-27). The genomic and non-genomic mechanisms of action of ERα are not mutually exclusive, and many interactions between these two pathways exist. For example, ER induces the expression of transcripts for both transforming growth factor α (TGFα) and amphiregulin (AR) (28, 29). TGFα and AR are both able to bind and activate EGFR thus laeding to activation of MAPK and AKT signalling (30). In addition, ERα binds to caveolin-1 at the cell membrane and activates specific G protein (23), this phenomenon leads to activation of src, which in turn activates matrix metalloproteinases that cleave transmembrane precursors of heparin binding-EGF (HB-EGF), an EGFR ligand (31). Therefore, both genomic and non-genomic activities of ERα can lead to increased activation of EGFR and its downstream effectors. Several preclinical and clinical studies suggest that overexpression of EGFR or HER2, and/or high levels of phosphorylated Akt and extracellular signal-regulated kinases (ERKs) in breast cancers contribute to Tam resistance (27, 32-36). Particularly, the membrane tyrosine kinase HER2 (c-ErbB2, HER2/neu) is gene-amplified in 20-25% of ER-positive breast cancer, and there is clinical evidence that tamoxifen is less effective in HER2-positive tumors (37). Furthermore, preclinical models show that HER2 overexpression can cause tamoxifen-stimulated growth as a mechanism of de novo resistance and that the HER family receptors are also implicated in acquired resistance to this drug (27, 38). The ErbB/HER family is composed of four growth factor receptors that share a high degree of sequence homology: EGFR (or erbB1 or HER1), erbB2 (or HER2; or neu in rodents), erbB3 (or HER3), and erbB4 (or HER4). Signaling through EGFR, HER2, and HER3 has been shown to promote tumor cell proliferation and survival in a variety of

epithelial malignancies (39). These receptors are transmembrane proteins with intracellular tyrosine kinase activity (except for HER3), and their activation results from the formation of homodimers and heterodimers, which is dependent on their ligands (except for HER2). Four subdomains have been identified in the extracellular domain of these proteins: subdomain I and III for ligand binding and the cystein-rich subdomains II and IV for receptor dimerization (40) (Fig. 3). The EGF family of ligand can be divided into three groups: EGF, transforming growth factor (TGF)-α, and amphiregulin, which bind exclusively to EGFR; betacellulin, heparin-binding EGF-like growth factor, and epiregulin, which bind to both EGFR and HER4; and the neregulin 1 and 2, which bind to both HER3 and HER4, and neregulin 3 and 4, which bind to HER4 (40). The extracellular domain of HER2 is unable to bind extracellular ligand and depends on heterodimerizing with other ligand-activated receptors.
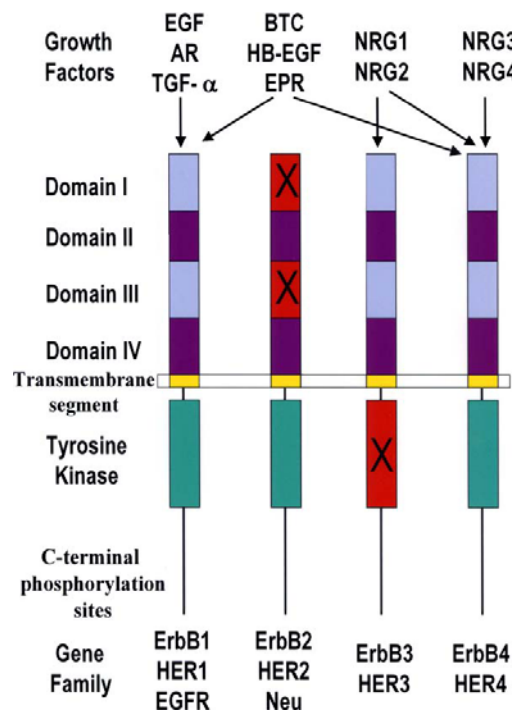

Fig. 3 Epidermal growth factor family of ligands and the ErbB gene family. The inactive ligand-binding domains of ErbB2 and the inactive kinase domain of ErbB3 are denoted with an X.

6

Overexpression of HER2 leads to activation of the HER2-mediated signalling pathway, even in the absence of ligand. Changes in HER2 auto-activated conformation might facilitate dimerization that includes the transmembrane and kinase domains but not extracellular domain homodimers. HER2 cooperating with other receptors mediates multiple downstream signalling pathways, including the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) pathways. These signaling pathways play a key role in tumorogenesis by promoting cell proliferation, differentiation, angiogenesis, invasion, and survaival (fig. 4). Advanced studies of ER biology have related new insights into ER action in breast cancer and have highlighted the role of an intimate crosstalk between the ER and the epidermal growth factor receptor (EGFR/HER1)/HER2 signaling pathways as a fundamental contributor to the development of resistance to endocrine therapies (37) (fig. 4).

We therefore hypothesized that the K303R mutation adapts ERα for enhanced reception of intracellular signal transduction, which leads to antiestrogen resistance. To test this hypothesis we used as an experimental model MCF-7 breast cancer cells stably transfected with either wild-type (WT) or the K303R mutant ERα. We found that cells expressing the estrogen hypersensitive K303R ERα mutant showed elevated levels of growth factor signaling, and enhanced crosstalk between the mutant and the HER2 growth factor receptor. Furthermore, in the second part of this study, we focused our attention on the possible role of K303R mutation in the resistance on aromatase inhibitors. It is well know that aromatase inhibitors (AI) are rapidly becoming the first choice for hormonal treatment of ERα-positive breast cancer in postmenopausal women. However, *de novo* and acquired resistance frequently occurs. To understand resistance mechanisms, several laboratories have developed in vitro cell line models to study the molecular changes associated with long-term estrogen deprivation (41-44).

Fig. 4 Interactions of estrogen and growth factor receptor signaling in human tumors.

AI resistance has also been esamine using aromatase-overexpressing MCF-7 breast cancer cell line models grown as xenografts in athymic nude mice (45), or breast cancer cells made resistant to AIs via long-term treatment with these drugs (46, 47). These models suggest the hypothesis that resistance to endocrine therapy may be through the acquisition of estrogen hypersensitivity, whereby low subphysiologic levels of estrogens remaining after estrogen deprivation are sufficient for maintenance of tumor growth. Thus, we questioned whether resistance to AIs could arise in breast cancer cells expressing the ERα mutation. Particularly, we hypothesized that such a mutant could provide a continuous mitogenic stimulus to the breast even during phases of low circulating hormone, such as menopause, thus affording a proliferative advantage especially during treatment with AIs. As preclinical models to directly test this

possibility, we generated K303R-overexpressing MCF-7 cells stably transfected with an aromatase expression vector. Cells were stimulated with the aromatase substrate, androstenedione, with or without the AI anastrozole (Ana). We found that Ana decreased androstenedione-stimulated growth of wild-type cells, whereas K303R-expressing cells were resistant to the inhibitory effect of Ana on growth.

Taken together, the results obtained in this study contribute to amplify the knowledge of the mechanisms by which breast cancer cells become resistant to endocrine therapy, and suggest that the presence of the A908G ERα somatic mutation may be useful as a predictive marker of hormonal response in breast cancer patients whose tumors exploit ERα and/or growth factor cross-talk to evade treatment.

MATERIALS AND METHODS

Reagents, hormones and antibodies

17β-Estradiol (E$_2$), 4-Hydroxytamoxifen (4-OH), Epidermal growth factor (EGF), Insulin like growth factor -1 (IGF-1), and Heregulin (H), 4-androstene-3,17-dione (Ana) were from Sigma (St. Louis, MO). Anastrozole was provided by AstraZeneca. Herceptin was form Genentech (San Francisco, CA). Antibodies used for immunoblotting were: ERα (6F11) from (Novocastra, Newcastle, United Kingdom); progesterone receptor (PR) from DAKO (Carpinteria, CA); Rho GDIα from Santa Cruz Biotechnology (Santa Cruz, CA); total MAPK, total Akt, total c-Src, phosphorylated p42/44 MAPK (Thr^{202}/Tyr^{204}), Akt (Ser^{437}), c-Src (Tyr^{416})and poly(ADP ribose)polymerase (PARP) from Cell Signaling Technology (Beverly, MA); total HER2 from NeoMarker (Fremont, CA); phosphorylated HER2 (Tyr^{1248}), phosphor-ER-S305 from UPSTATE (Temecula, CA); Living colors^{TM} Full Length polyclonal antibody (Clontech, Mountain View, CA); cytochrome P450 aromatase was from Serotec; Bax and Bcl-2 were from Calbiochem.

Plasmids

Generation of yellow fluorescent protein (YFP)-tagged expression construct, YFP-WT and K303R ERα, has been previously described (17).

Full-length human aromatase cDNA was amplified from the pCMV6-Arom plasmid (OriGene Technologies) by PCR using the following primers: forward 5'-ACACTAGTATGGTTTTGGAAATGCTGAACCC-3' and reverse 5'-ACGCGGCCGCCTAGTGTTCCAGACACCTGTCT-3'. This PCR product was subcloned into the Spe I/Not I sites of the pZeoSV2-vector (Invitrogen). The resulting

pZeoSV2-aromatase expression vector (pZeo- Arom) sequence was confirmed by DNA sequencing.

Cell culture

MCF-7 breast cancer cells, originally obtained from Dr. Benita Katzenellenbogen (University of Illinois, Urbana, IL), were maintained on plastic in minimal essential medium (MEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS; Summit Biotechnology, Fort Collins, CO), 0.1 nmol/L nonessential amino acid, 2 mmol/L L-glutamine, 50 units/ml penicillin/streptomycin, at 37°C with 5% CO_2/ 95% air. HeLa cells were obtained form American Type Culture Collection (Manassas, VA), and were maintained in the same media. Chinese Hamster Ovary (CHO) cells were cultured in Kaighn's Modification F-12 media (F-12K, Invitrogen) supplemented with 10% fetal bovine serum (FBS), 0.1 nmol/L nonessential amino-acid, 2 mmol/L L-glutamine, and 50 units/ml penicillin/streptomycin.

MCF-7 wild-type (WT) and K303R ERα-expressing cells were generated as following described. MCF-7 cells were stably transfected yellow fluorescent protein (YFP) ERα- and K303R ERα-tagged vectors, using Fugene 6 according to the manufacturer's instructions (Roche, Indianapolis, IN), and individual clones were isolated and expanded with G418 selection. MCF-7 parental and YFP-K303R ERα clones were stably transfected with the pZeo-Arom expression vector using Fugene 6 reagent according to the manufacturer, and individual clones were isolated and expanded with Zeocin selection. In some experiments we used a pools of stably transfected cells selected for one week with G418 or Zeocin antibiotics. Chinese hamster ovary (CHO) stably transfected with YFP-WT ERα and YFP-K303R ERα expression vectors, were also used. Stably transfected clones and pools of stably transfected cells were screened

for expression of exogenous and endogenous ERα using immunoblot analysis before each experiment.

Quantitative image analyses by high throughput microscopy (HTM)

High-throughput microscopy for biological purposes may be broadly defined as an automated image acquisition system coupled to cell identification and cell morphological detection/measurement algorithms. Particularly, HTM is an analytical tool to study steroid nuclear receptor and nuclear receptor coregulator biology at the single cell level. The molecular mechanisms of action for estrogens and antiestrogens at the single cell level are still being determined. Single cell studies of ERα show that, in the absence of ligand, its subcellular distribution is predominantly a diffuse nuclear pattern nuclear. The addition of ligand causes a redistribution of ERα into discrete foci. However, the nuclear redistribution of ERα does not always correlate well with transcription, for example, E_2 and tamoxifen both induce subnuclear redistribution, however, in HeLa, the latter does not induce transcription. Therefore, Berno et al. have developed a model system (PRL-HeLa cell line) that is conducive to HTM analysis in order to study ERα transcriptional properties at the single cell level.

PRL-Hela is a cell line specifically engineered for the single cell study of ER function (48). PRL-Hela cells contain multiple genomic integrations of a replicated prolactin (PRL) promoter/enhancer. The multiple integrations (PRL array) are spatially confined and are visualized by the accumulation of fluorescently tagged ERα. PRL-Hela cells transiently expressing YFP-WT ERα or YFP K303R ERα, pretreated with forskolin (10 μM) for 15 minutes, and then treated with increasing concentration of E_2 or Tam for 30 minutes, were fixed and DAPI-stained as previously reported (48, 49). The cells were imaged using the Cell Lab IC 100 Image Cytometer (Beckman Coulter, Fullerton, CA)

with a Nikon 40X Plan S flour 0.90 NA objective. Two channels were imaged: channel 0 (DAPI stain) was used to find the focus and nuclei, when channel 1 was used to image YFP ERα. A proprietary algorithm (GPRC) developed at Beckman Coulter was used to identify and quantify the YFP-ERα-targeted PRL array. The parameters for the GPRC algorithm were: object scale=30 and minimum peak height=10. Foci identified by the GPRC algorithm were masked. The area of the mask in pixels was the measure of PRL array size. Channel 1 was offset 2μm from DAPI focused for cells in all treatment conditions. After image acquisition and application of the GPRC algorithm, the total cell populations for each treatment were progressively filtered (gated) using the same criteria. Nuclei clusters, and mitotic cells were filtered from the total cell population using an intersection of DNA content and DNA clusters gates. In addition, low YFP ERα expression and low aggregate number gates were generated and applied to produce the final cell population to be analyzed. From the final population of cells, the array size was determined using the GPRC mask (50). The images and masks were visually inspected for accuracy. Unpaired student's t-test assuming equal variance was performed to determine statistical significance (two-tailed, $p<0.05$). Standard deviations are shown.

Immunoprecipitation and immunoblot analysis

Cells were starved in phenol red free MEM with 5% charcoal-stripped FBS for 48 h and treated as indicated before lysis [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 2% NP40, 0,25% deoxycholic acid, 1 mmol/L EDTA, 1 mmol/L Na_3VO_4, and 1:100 protease inhibitors cocktail; Calbiochem]. For coimmunoprecipitation experiments, we used 1 mg of total cellular protein and a 1:200 dilution of anti-Living colors Full Length polyclonal antisera (Clontech) that recognizes native and denatured forms of recombinant YFP fusion proteins expressed in mammalian cells, and 2 μg of

HER2 polyclonal antisera overnight, followed by protein A/G precipitation with rotation at 4°C for 2 h. Immunoprecipitated proteins were washed thrice with lysis buffer. Equal amounts of cell extract and immunoprecipitated proteins were resolved under denaturing conditions by electrophoresis in 8% to 10% polyacrylamide gels containing SDS (SDS-PAGE), and transferred to nitrocellulose membranes (Schleicher & Schuell, Keen, NH) by electroblotting. After blocking the transferred nitrocellulose membranes were incubated with primary antibodies overnight at 4°C, with secondary antibodies goat anti-mouse or goat anti-rabbit antisera (1:3000; Amersham Bioscences; Piscataway, NJ) for 1 h at room temperature and developed with enhanced chemi luminescence reagents (Alpha Innotech, San Leandro, CA).

Cell proliferation assays

Anchorage-independent soft agar growth assays: cells (5000 per well) were plated in 4 ml of 0.35% agarose with 5% charcoal-stripped FBS in phenol red-free MEM, in a 0.7% agarose base in six-well plates. Two days after plating, media containing control vehicle or hormonal treatments was added to the top layer, and the appropriate media was replaced every two days. In some experiments a pools of stably transfected cells were used. After 14 days, 150 µl of MTT was added to each well and allowed to incubate at 37°C for 4 h. Plates were then placed in 4°C overnight and colonies > 50 µm diameter from triplicate assays were counted. Data are the mean colony number of three plates and representative of two independent experiments analyzed for statistical significance ($p<0.05$) using a two-tailed student's Test, performed by Graph Pad Prism 5 (GraphPad Software, Inc., San Diego, CA). Standard deviations are shown.

MTT assay: Seven hundred cells were plated into 96-well plates in phenol red–free MEM containing 5% charcoal-stripped fetal bovine serum (FBS). After 24 h, cells

were treated as indicated. Nine days later, proliferation was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) reagent. Briefly, MTT was added to each well and incubated at 37°C and 5% CO_2 for 2 h followed by medium removal and solubilization in 100 µl DMSO. The resulting color change was read at 570 nm and calculated as absorbance above background.

Blocking peptide delivery

A blocking peptide of 13 residues (**IKRSKKNSLALSC**) from the sequence (residues 298-310) surrounding the S305 residue (in bold) of the human ERα was transferred into cells using a cationic amphiphile molecule, PULSin^{TM} delivery reagent (Polyplus transfection, Illkirch, France), as suggested by manufacturer. Briefly, cells were plated in a 6-well plate with regular growth media, and then starved for 48 h in a phenol red free MEM with 5% charcoal stripped FBS. After starvation cells were washed with PBS to remove all traces of serum, and fresh phenol-red free media without serum was added. The mixture containing the S305 blocking peptide (4 µg/well) diluted in 200 µl of 20 nM Hepes, and 16 µl of PULSin^{TM} was incubated for 15 min at room temperature, and then added to the cells. The media was changed after 4 hours of incubation, cells were treated as indicated, and cellular extracts were prepared. Delivery of R-phycoerythrin was used as a positive control and live cells were observed by fluorescence microscopy after 4 h.

Aromatase activity assay

Aromatase activity was evaluated using a ^{3}H water release assay using 0.5 µmol/L of [1β - ^{3}H]-androst-4-ene-3,17-dione as substrate (51). The incubations were performed at 37°C for 1 h. The results were expressed as fmol-pmol/h/mg protein.

Tumor xenografts

All animal studies were carried out according to the guidelines and with the approval of the Baylor College of Medicine Animal Care and Use Committee. Female nude ovariectomized athymic mice were injected with MCF-7 YFP-WT and YFP-K303R ERα–expressing cells or MCF-7 Arom 1 and K303R Arom 1–expressing cells as described (52). Animals were supplemented with estrogen tubing releasing ~80 pg/mL, or supplemented daily with injections of the aromatase substrate androstenedione (AD; 100 μg).

Apoptosis assays

Cells were seeded into six-well plates (10^5/well) in phenol red–free MEM with 5% charcoal-stripped FBS for 48 h and then incubated with different treatments for 72 h. Apoptosis was measured using the Cell Death Detection ELISA Kit (Roche). Data are representative of two independent experiments performed in triplicate.

Statistical analysis

Data were analyzed by Student's t test using the GraphPad Prism5 software program.

RESULTS

Ligand-independent signaling to the K303R ERα mutant reduces Tam sensitivity

We have previously shown that K303R ERα mutant-overexpressing cells display enhanced ligand-independent activity when stimulated with cyclic AMP, in part because the mutation generates a more efficient substrate for PKA-mediated signaling (17). It is well known that ligand-independent signaling can influence cellular responsiveness to Tam (53). For instance, PKA-mediated phosphorylation of ERα at S305 allows the antagonist Tam to behave as an agonist, which then results in ERα-dependent transactivation (18). Here we have utilized the stable cell line PRL-Hela containing a multi-copy integrated prolactin enhancer/promoter DNA array that is responsive to ER such that when the receptor is expressed as a YFP-fusion protein, the integration site can be easily visualized (PRL-array) (48). High-throughput microscopy has been used to identify ligand-independent changes in the size of the PRL-array which is an indicator of the chromatin condensation status at the promoter (50). Arrays rapidly decondense after E_2 treatment or condense after anti-estrogen treatment (both within minutes). Thus, in PRL-Hela cells, the array size is an indicator of receptor-mediated transcription function in response to different treatments and allows direct and live observation of ER-dependent chromatin remodelling. Therefore using a mammalian-based, stably transfected prolactin promoter array system, we analyzed live cell dynamics on an estrogen-responsive promoter, to visualize chromatin remodelling induced by E_2 and Tam. We transiently transfected PRL-Hela cells with YFP tagged-ERα WT or the YFP-ERα K303R mutant receptor expression vector, and examined E_2 (Fig 1a) and Tam's (Fig 1b) effect on chromatin remodelling in the presence of forskolin, an activator of PKA signaling. Using high through-put microscopy, the amount of chromatin remodelling was quantified (50). 24h after transfection cells were

pretreated with forskolin (FSK), and then treated with E_2 or Tam at different doses, as indicated, for 30 minutes. Over 60 nuclei in two separate experiments were analyzed to determine the size of the PRL-reporter array. Vehicle control showed similar values for the array size in the presence of the WT receptor and the mutant (Fig. 1). In PRL-Hela cells expressing the WT receptor, estrogen treatment increased array size in a dose-dependent manner in estrogen concentrations between 10^{-12} M and 10^{-9} M. Mutant-expressing cells showed a linear dose-response in the range of 10^{-17} M through 10^{-11} M of estrogen. Indeed, mutant ERα expressing cells exhibited a much lower EC50 (3 X 10^{-13} M) compared with WT ERα-expressing cells (5.1 X 10^{-11} M). These data demonstrate that K303R mutant-expressing cells are hypersensitive to estrogen and form larger arrays in the presence of low estrogen concentrations. With forskolin treatment, WT and mutant ERα cells stimulated with estrogen both displayed significantly increased array sizes. WT ERα-expressing cells demonstrated a one-log reduction in the EC50 (4.2 X 10^{-12} M $\pm$ 0.0038 compared to 5.1 X 10^{-11} M $\pm$ 0.0132 in the absence of forskolin) and a significantly increased average array size (Fig. 1a). Mutant-expressing cells also displayed a significantly increased average size, but without a significant change in the EC50 (8 X 10^{-13} M $\pm$ 612.5 compared to 3 X 10^{-13} M $\pm$ 0.0004 in the absence of forskolin). Thus, forskolin increased average array size, and reduced the EC50 of WT ERα cells thus enhancing estrogen sensitivity, whereas forskolin treatment of mutant-expressing cells only increased the array size, suggesting that these cells displayed an inherent hypersensitivity to estrogen. As shown in Fig. 1b, Tam treatment decreased the array size in WT ERα-expressing cells, however the mutant receptor was significantly less responsive to Tam ($p < 0.0001$). Forskolin treatment blocked Tam-induced promoter condensation (reduced size of the array), and the mutant receptor array size was not affected by physiological levels of Tam (100 nM)

in the presence of forskolin. All together these results suggest that hormone-independent kinase signaling to the mutant receptor K303R ERα may confer resistance to antiestrogen treatment in breast cancer cells.
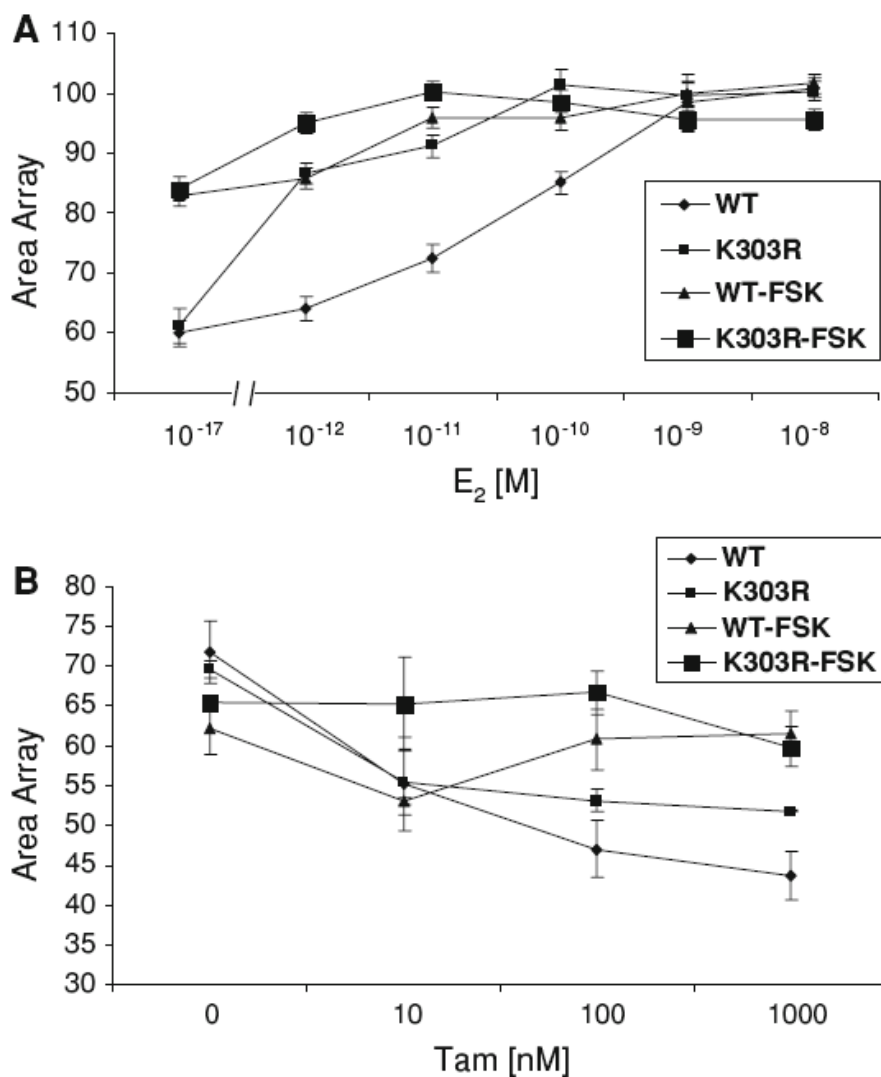

Fig. 1 E$_2$ and Tam effects on large-scale chromatin structure in PRL-Hela cells. Cells transiently expressing YFP-ERα WT (WT) or the YFP-K303R ERα (K303R) mutation were pretreated with forskolin (FSK) for 15' minutes and then were treated with E$_2$ (**a**) or Tam (**b**) at different doses for 30 minutes. After fixing and counter-staining with DAPI, cells were imaged and array size was quantified using HTM as described in Materials and Methods. Results are expressed as array size normalized to control cells obtained from three independent experiments.

MCF-7 K303R-ERα mutant expressing cells exhibit altered growth factor signaling

To further explore ligand-independent activation of signaling and estrogen hypersensitivity in mutant-expressing breast cancer cells, we developed MCF-7 ERα-positive human breast cancer cell lines overexpressing either WT or the K303R ERα mutant. We chose to introduce the mutant receptor into parental WT ERα-expressing cells to simulate the situation in invasive human breast tumors, where WT receptor is most frequently co-expressed along with the mutant (13). To differentiate the exogenously expressed receptor, we tagged the vector with YFP. Stably transfected clones were screened for expression of ERα using immunoblot analysis (Fig. 2a). Parental MCF-7 cells are shown along with one clone stably expressing YFP-WT, and three clones expressing YFP-K303R ERα (MCF-7 K303R-1-3). First, to directly demonstrate *"in vivo"* the biological consequences of the K303R ERα mutation, we examined growth of stably transfected cells as tumor xenografts in ovariectomized athymic nude mice. MCF-7 cells stably transfected WT and K303R ERα were injected into the number 4 glands of ovariectomized athymic nude mice supplemented with 80 pg/ml of estradiol, representing low premenopausal levels (Fig. 2b). K303R ERα tumors grew faster than the WT ERα tumors (P=0.0466). Thus, expression of the mutant generated an estrogen hypersensitive phenotype.

It is well accepted that the response to endocrine therapies in human breast cancer patients correlates with ERα and progesterone receptor (PR) levels. Several studies have shown that patients with ERα/PR-positive breast cancers derive greater benefit from adjuvant hormonal therapy than those patients whose tumors lack PR (54-56) however it must be noted that other studies have not always found this result (57). It has been hypothesized that high growth factor signaling activity in breast cancers may be

associated with decreased PR levels (for a review see (58)) and elegant studies have shown that up-regulation of pMAPK Erk1/2 leads to a loss of PR via degradation by the 26S proteasome (59). In clinical samples we have previously reported a borderline significant inverse correlation between the presence of the K303R ERα mutation and the PR-B isoform (13), and we have shown that a reduction in PR-B levels was associated with a poorer response to endocrine therapy (60). Therefore, we first evaluated the levels of PR-A and B in our parental MCF-7 cells, and two stably transfected clones (Fig. 2c). Parental MCF-7 cells expressed both PR isoforms, with PR-A as the predominant form in these cells. PR is a known estrogen-induced gene, and as expected we saw higher levels of both PR-A and B in MCF-7 cells after estrogen treatment. In comparison, MCF-7 WT-overexpressing cells exhibited slightly less PR induction with E_2. In contrast, in basal conditions, K303R ERα mutant-overexpressing cells demonstrated much lower levels of both PR isoforms, and less were induced with E_2 treatment. Lower levels of PR mRNA levels were also detected in estrogen-stimulated mutant cells using Real-Time polymerase chain reaction (qPCR), (p=0.0008, data not shown). These results suggest that growth factor signals might be altered in the mutant-overexpressing cells.

To test for altered intracellular signaling, we next examined the effects of short-term treatments with E_2, heregulin (H), EGF (E), and IGF-1 (I) on phosphorylation levels of downstream growth factors signaling components, such as HER2, Akt, and MAPK using immunoblot analysis (Fig. 3a) which is quantitatively represented in Fig. 3b. Cells were maintained under estrogen-depleted conditions for 2 days, and then treated for 10 minutes with E_2 or different growth factors as indicated, and cellular extracts were prepared. MCF-7 K303R mutant cells showed constitutively higher levels of phosphorylated HER2 as well as total HER2, compared to MCF-7 WT ERα-overexpressing cells (Fig. 3a). To begin to understand the mechanism associated with

higher levels of total HER2 in the mutant cells we analyzed the expression level of

HER2 mRNA in different clones of mutant ERα expressing cells, by qPCR; but we did

not found differences in HER2 mRNA levels between WT and K303R ERα expressing
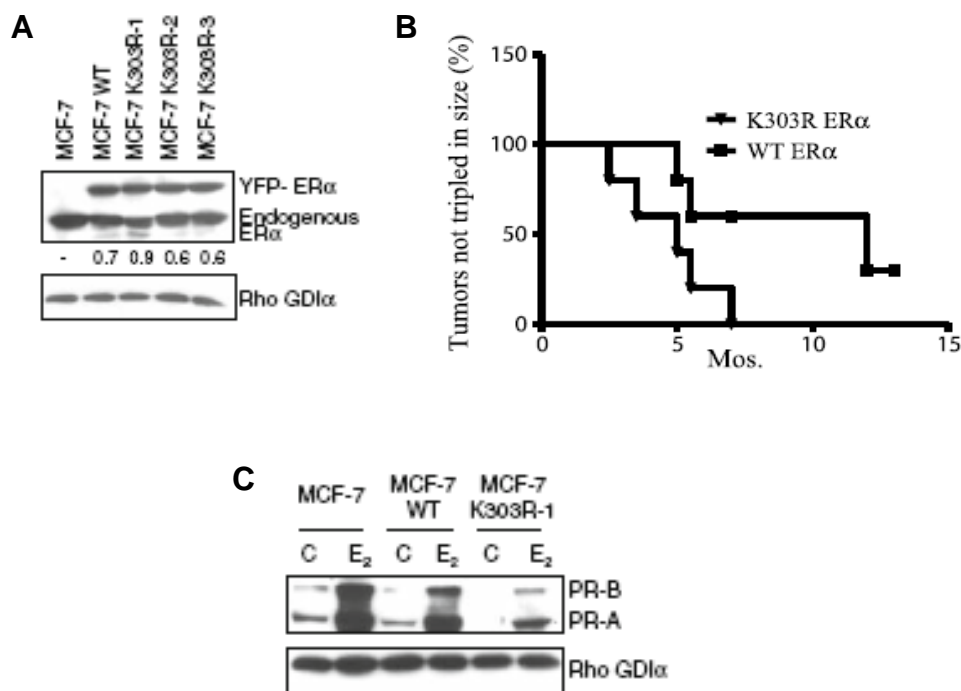
cells (data not shown).

Fig. 2 Characterization of MCF-7 K303R ERα-expressing cells. (**a**) MCF-7 cells stably transfected with a yellow-fluorescent protein (YFP)-tagged expression construct YFP-WT ERα (MCF-7 WT) and different clones transfected with the mutant YFP-K303R ERα construct (MCF-7 K303R-1, -2, -3) were screened for expression of exogenous (96 kDa) and endogenous ERα (66 kDa) by immunoblot analysis using an antibody against human ERα. Parental cells (MCF-7) were used as a control for endogenous ERα expression. Rho GDIα was used as a control for equal loading and transfer. Numbers below the blot represent the ratio between YFP-ERα and endogenous ERα protein expression. (**b**) WT ERα and K303R ERα cells were injected into mice (n = 5/group) supplemented with E_2. Tumor growth was plotted as the time for tumor tripling [months (mos)]. (**c**) MCF-7 parental, MCF-7 WT, and MCF-7 K303R-1 cells were serum-starved for 48h, and then treated with or without 1 nM E_2 for 24 hours before lysis. Equal amounts of total cellular extract were analyzed for progesterone receptor (PR-A and PR-B) levels by Western blotting.

This result suggests that post-translational modification and increase in protein

stability could be involved in the HER2 up-regulation that we found in mutant ERα-

expressing cells, and we are currently exploring this possibility. Treatment with E_2,

heregulin, EGF, and IGF-1 induced higher levels of pHER2 in mutant-overexpressing cells, but only a small induction was seen in MCF-7 WT cells which contain low endogenous levels of HER2. Treatment with heregulin or EGF led to increased phosphorylation of the downstream signaling molecules Akt and MAPK in mutant-overexpressing cells compared with MCF-7 WT cells. IGF-1 treatment also induced enhanced phosphorylation of Akt, but increased levels of phosphorylated MAPK were only detected in K303R ERα mutant-overexpressing cells. In both cell lines, no enhancement of Akt and MAPK phosphorylation was seen with estrogen treatment.
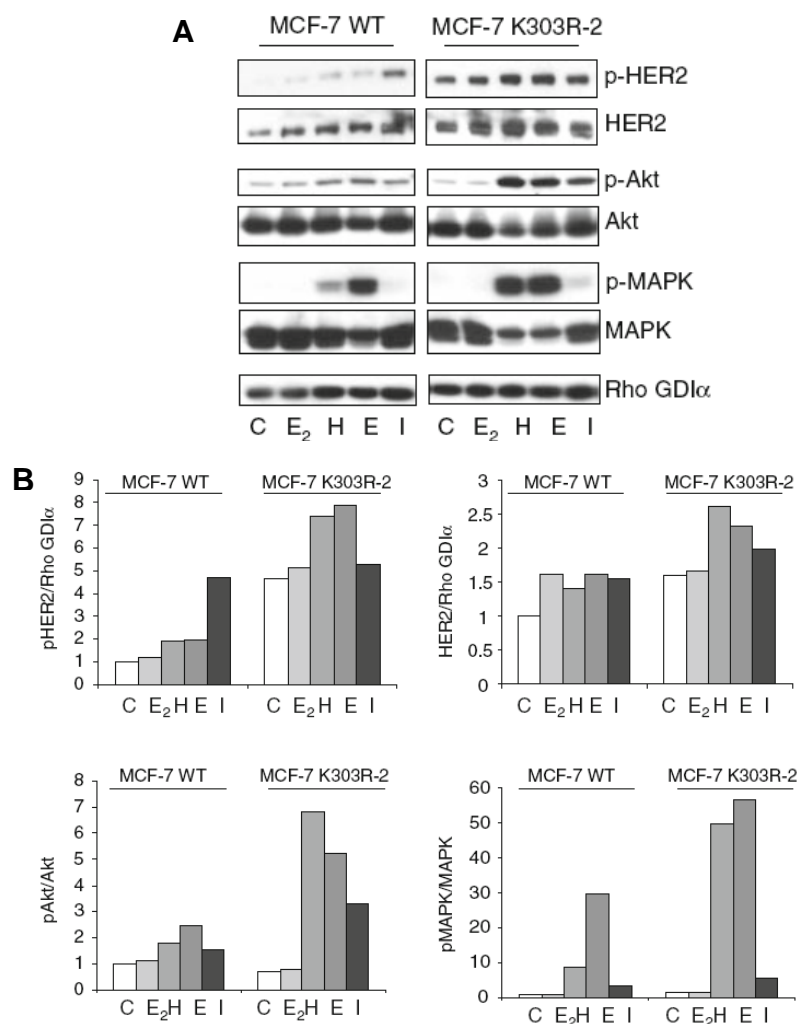

Fig. 3 **Growth factor signaling in MCF-7 WT-ERα and MCF-7 K303R ERα-expressing cells.** (**a**) MCF-7 WT and MCF-7 K303R-2 cells were treated with vehicle ethanol (C), 1 nM E$_2$, 2 ng/ml heregulin (H), 10 ng/ml EGF (E) and 10 ng/ml IGF-1 (I) for 10 min before lysis. Levels of phosphorylated (p) HER2 (Tyr^{1248}), Akt (Ser^{473}), and MAPK (Thr^{202}/Tyr^{204}), at the indicated residues, and total non-phosphorylated protein were measured in cellular extracts by immunoblot analysis. (**b**) Quantitative analysis of the blots shown above.

To investigate if the kinetics of growth factor signaling might be altered in mutant-expressing cells, we performed a time-course study with heregulin treatment in WT ERα and K303R ERα mutant cells (Fig. 4). After two days of starvation cells were treated for 1, 3, 5, 10, or 20 minutes with heregulin, and then lysed as described in Materials and Methods. As shown in Fig. 4 a rapid response to heregulin was seen within 1' in MCF-7 WT cells with activation of phospho-HER2, and phospho-AKT/phosphoMAPK within 10-20' (numerical quantitation relative to control WT is shown beneath each immunoblot). In contrast, mutant-expressing cells exhibited enhanced activation of pHER2, pAKT, and pMAPK. Compared with WT-expressing cells, these molecules were all stimulated at earlier time points in mutant cells; heregulin induced phosphorylation of MAPK and Akt by 5 minutes, but levels of these phosphorylated kinases were not detectable at this time-point in WT ERα cells. We saw that the non receptor tyrosine kinase c-Src also exhibited an earlier time-point of activation (by 3 min), and higher phosphorylation levels at the activating c-Src tyrosine residue 416 (Tyr[416]) was seen in K303R ERα cells.

These collective data demonstrate that the mutant-overexpressing cells are hypersensitive to growth factor signal transduction, and suggest that the mutant receptor could impact on ligand-independent signaling pathways commonly known to be employed to evade anti-hormonal therapeutic strategies in breast cancer.
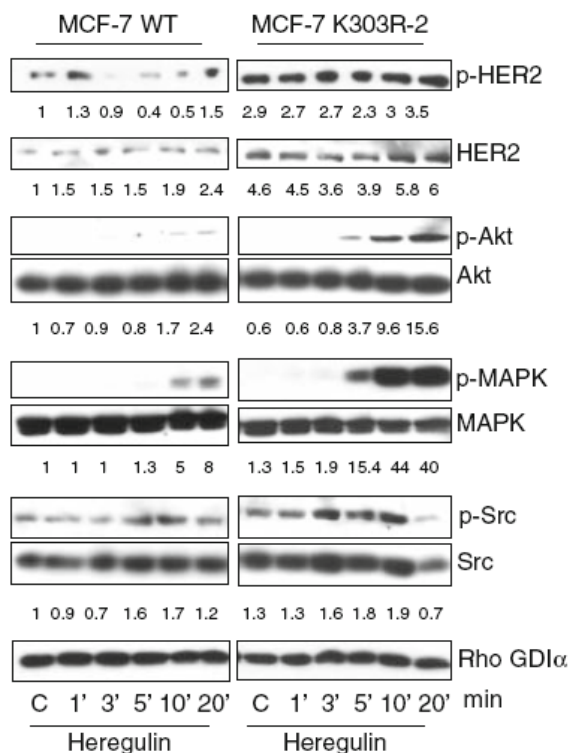
Fig. 4 Time-course study on growth factor signaling in MCF-7 WT-ERα and MCF-7 K303R ERα-expressing cells. MCF-7 WT and MCF-7 K303R-2 .cells were treated with heregulin (H) at 2 ng/ml for different times before lysis. Cellular extracts were analyzed as in panel **c**, and for p-Src (Tyr^{416}) at the indicated residues. Numbers below the blot represent fold change in protein expression of MCF-7 K303R-2 cells compared to MCF-7 WT cells.

Previous reports have shown that ERα and growth factor pathways can interact at different levels, through a direct association or complex formation of ERα with key signaling molecules such as c-Src, Shc, and the p85α regulatory subunit of PI3K (61-64). C-Src family tyrosine kinases are involved in signaling of a number of different growth factor receptors, including EGFR/HER2, in breast cancer cells. In previous work we have reported that MCF-7 cells stably expressing the K303R ERα mutant receptor exhibited increased c-Src kinase activity and c-Src tyrosine phosphorylation, when compared with WT ERα-expressing cells (65). Therefore we next addressed whether the mutation might alter the ability of the receptor to bind with the HER2 tyrosine kinase receptor, which is expressed at higher levels in mutant cells (Fig. 3a). To evaluate potential protein-protein interactions between WT and mutant receptors with

HER2 in our model system, we treated MCF-7 cells overexpressing WT or the mutant for 5' with EGF 100 ng/ml and then lysates were prepared (Fig. 5). Equal amounts of protein were immunoprecipitated with either anti-YFP antisera or anti-HER2 antisera followed by immunoblot for HER2 and YFP-ERα. As shown in Fig. 5, similar amounts of input from the whole-cell lysates were used. In the absence of treatment, both WT ERα and HER2 resided in a protein complex; and EGF treatment slightly increased the amount of the protein in the complex. The levels of mutant receptor bound to HER2 under basal conditions were higher, and EGF treatment did not further increase the amount of receptor in the complex. The bottom panel shows that equal amounts of YFP-ERα and HER2 were immunoprecipitated under all conditions tested. These results suggest that the K303R ERα mutant may be constitutively associated with HER2, which could be involved in the enhanced bidirectional crosstalk that we report between ERα and growth factor receptor signaling pathways.
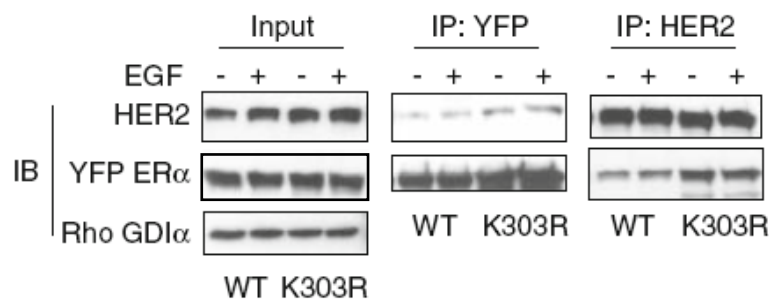

Fig. 5 Protein-protein study interactions between WT and mutant receptors with HER2. MCF-7 WT (WT) and MCF-7 K303R-3 (K303R) cells after 48h of starvation were treated with or without EGF 100 ng/ml for 5 min before lysis. YFP-WT ERα and YFP-K303R ERα proteins were immunoprecipitated using an anti-YFP polyclonal antibody (IP:YFP), or an anti-HER2 polyclonal antibody (IP:HER2), and immunoblotted (IB) with HER2 and anti-ERα antibodies, respectively. Whole-cell lysates (Input) were used as input controls. Rho GDIα was used as a control for equal loading and transfer. Immunoblots are representative of three separate experiments.

Tamoxifen fails to inhibit anchorage-independent growth induced by heregulin in

MCF-7 K303R ERα-overexpressing cells

It is already appreciated that altered crosstalk between several receptor tyrosine

kinases and ERα may contribute to endocrine resistance (8), and it has been shown that

elevated levels of HER2 contribute to resistance in a tamoxifen-resistant subline of

MCF-7 cells compared to unselected parental cells (66). Since the K303R ERα

mutation conferred enhanced heregulin-mediated signaling, we next addressed whether

this alteration might confer a decrease in response to antiestrogen therapy. To test this

question we next performed anchorage-independent growth assays using our two ERα-

overexpressing models. Cells were plated in soft agar and then treated with estrogen

(E_2, 1 nM) or heregulin (H, 2 ng/ml), EGF (10ng/ml, E), IGF-1 (10ng/ml, I) in the

presence or absence of tamoxifen (Tam, 100 nM). After 14 days of growth colonies
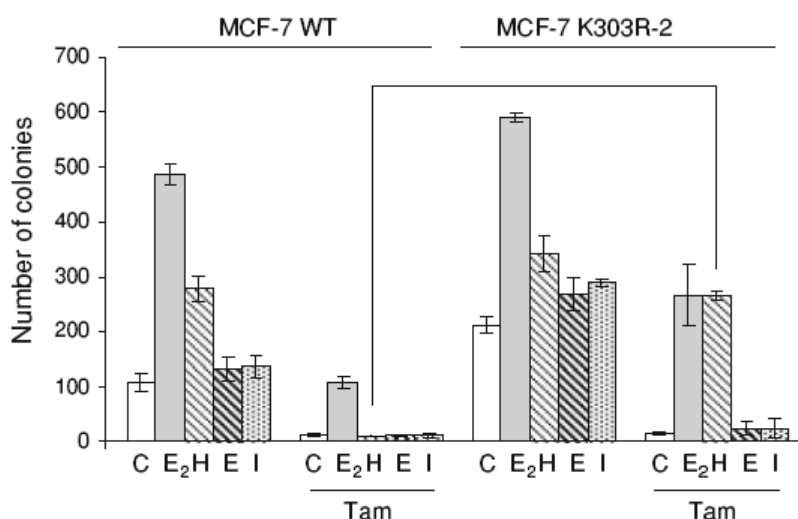
>50 μM in diameter were counted (Fig. 6).

Fig. 6 Heregulin treatment reduced the ability of tamoxifen to inhibit anchorage-independent growth of MCF-7 K303R cells. MCF-7 WT and MCF-7 K303R-2 stably transfected cells were seeded (5000/well) in 0.35% agarose and then treated with vehicle (C), E_2 (1 nM), heregulin (2 ng/ml, H), EGF (10ng/ml, E), IGF-1 (10ng/ml, I) with or without Tam (100 nM). Cells were allowed to grow for 14 days and the number of colonies >50 μm were quantified and the results were graphed. *p=0.0001 vs control (C) of MCF-7 WT cells.

Control (C) basal growth of mutant-expressing cells was higher compared to WT-expressing cells (p=0.001); as expected treatment with estrogen as well as heregulin increased the number of colonies in both cell lines, while EGF and IGF-1 slightly increased proliferation but only in MCF-7 K303R ERα-overexpressing cells. Tam treatment induced a significant reduction in the number of colonies in MCF-7 WT cells under control conditions, and was able to inhibit growth of cells stimulated with estrogen or heregulin (reduction in colonies 78% and 96%, respectively). In contrast Tam treatment was less efficient at inhibiting estrogen or heregulin-stimulated proliferation in mutant-overexpressing cells (reductions were only 54% and 21%, respectively), but effectively reduced EGF and IGF-1 stimulated growth in these cells Thus, response to Tam was greatly affected by the enhanced growth factor signaling by heregulin in mutant cells; this enhanced sensitivity prevented the major antagonistic activity of tamoxifen on the proliferation of mutant-expressing cells.

Next, we examined whether the HER2 pathway found to be up-regulated in the mutant-expressing cells, was responsible for the higher growth of mutant cells compared to WT ERα cells. We therefore evaluated anchorage-independent growth of either mutant or WT ERα cells after herceptin treatment. Herceptin is a humanized monoclonal antibody directed against the extracellular domain of HER2, and was developed as an agent to inhibit the growth of HER2-overexpressing tumor cells (67, 68). Cells were plated in soft agar and then treated with heregulin in the presence or absence of herceptin (10 μg/ml). After 14 days of growth, colonies were counted (Fig 7). As expected since HER2 levels are low in WT cells, herceptin had no effect on their growth either under basal conditions or with heregulin treatment. In contrast, herceptin significantly inhibited anchorage-independent growth of K303R mutant cells in control untreated conditions, and with heregulin treatment (*p=0.0002 vs control; **p=0.03 vs heregulin treated cells). These data confirm that the HER2 pathway may be responsible

for the higher constitutive growth of mutant-overexpressing cells rendering these cells

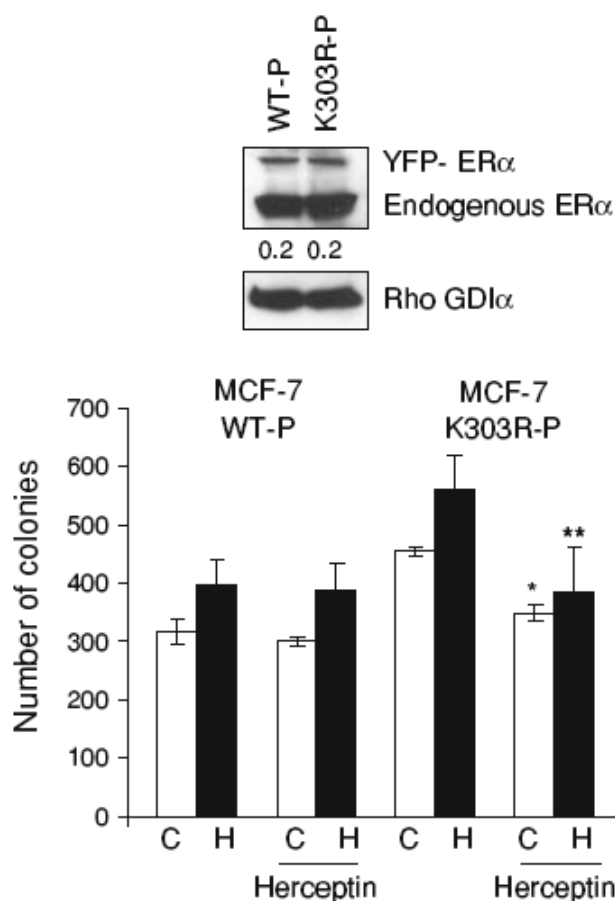more sensitive to the inhibitory effect of this selective HER2-targeted agent.

Fig. 7 Anchorage-independent growth of either mutant or WT ERα cells after herceptin treatment. Immunoblot analysis showing YFP-ERα and endogenous ERα protein expression (upper panel) in the pool of stable transfectants. Numbers below the blot represent fold change in protein expression of MCF-7 K303R-2 cells compared to MCF-7 WT cells. MCF-7 WT-P and MCF-7 K303R-P pool of transfected and overexpressing cells were plated in soft agar and then untreated or treated with heregulin 2 ng/ml (H) in the presence or absence of Herceptin (10 μg/ml). *p=0.0002 vs control (C); **p=0.03 vs heregulin (H) treated cells (bottom panel); standard deviations are shown.

Phosphorylation at serine residue 305 (S305) of ERα K303R mutant is involved in growth factor signaling up-regulation

ERα is a known target of several post-translational modifications, such as phosphorylation, sumoylation, and acetylation (17, 69-71). For instance, receptor phosphorylation, which regulates receptor affinity, coregulator protein binding, and transcriptional activity, can be induced in the absence of ligand via cross-talk with various signal transduction pathways (72). We previously have reported that the K303R ERα mutant is a more efficient substrate for phosphorylation by PKA at S305 which enhanced hormone sensitivity and stimulated cellular growth (17). We hypothesize that the phosphorylation status of S305 in the mutant receptor may control receptor activity, and be a conduit for enhanced downstream cross-talk with growth factor signaling networks. To explore this possibility, we first evaluated the phosphorylation status of the S305 residue in either WT ERα or K303R mutant cells after estrogen treatments between 5 minutes to 2 hours. Cellular extracts were subjected to immunoblot assay using a specific anti-phospho-S305 ERα antibody (Fig. 8a). Estrogen treatment enhanced the phosphorylated levels of S305 within 15' in YFP-WT-expressing cells, and these levels remained elevated at 2 hours. In contrast, K303R ERα mutant-expressing cells exhibited elevated levels of pS305 under basal control conditions, and this elevated phosphorylation remained constant with longer estrogen treatments. Since, it is well established that ERα can be activated in a ligand-independent manner by MAPK (73) at serine 118 (S118), we also evaluated the levels of phospho-S118 in WT ERα or K303R ERα-overexpressing cells under the same conditions described above. We did not detect significant changes in S118 phosphorylation patterns between the two cell lines (data not show). These results suggest that constitutively higher phosphorylation of S305 in the mutant receptor might play a role in the ligand-

independent activation of the receptor itself. It is possible that this enhanced S305 phosphorylation within the mutant might play a key role in the observed up-regulation of growth factor signaling cascades seen in these cells as well. To test this hypothesis we performed immunoblot analysis to evaluate the phosphorylation levels of a number of growth factor signaling components after incubation with an S305 blocking peptide. To block phosphorylation at S305 we delivered a peptide (S305 peptide, residues 298 to 310) to the cells. After peptide delivery, the cells were subjected to short term treatments (10 min) with heregulin and then growth factor signaling molecules were analyzed by immunoblot analysis (Fig. 8b). Heregulin enhanced S305 phosphorylation in K303R mutant-overexpressing cells, but had no effect WT receptor. Phosphorylation of the mutant receptor was abrogated by the S305 peptide. Addition of the S305 blocking peptide also inhibited heregulin-induced phosphorylation of HER2, Akt, and MAPK in both cell lines. Interestingly, reduction in phospho-HER2 and MAPK levels were more pronounced in mutant-expressing cells compared with WT, suggesting that the mutant cells were more sensitive to the inhibitory effect of S305 blocking peptide. These data indicate that phosphorylation of the S305 residue may be crucial in mediating enhanced cross-talk between HER2 and mutant ERα, and suggest that phosphorylation blockade might be a potential therapeutic strategy to block mutant function.
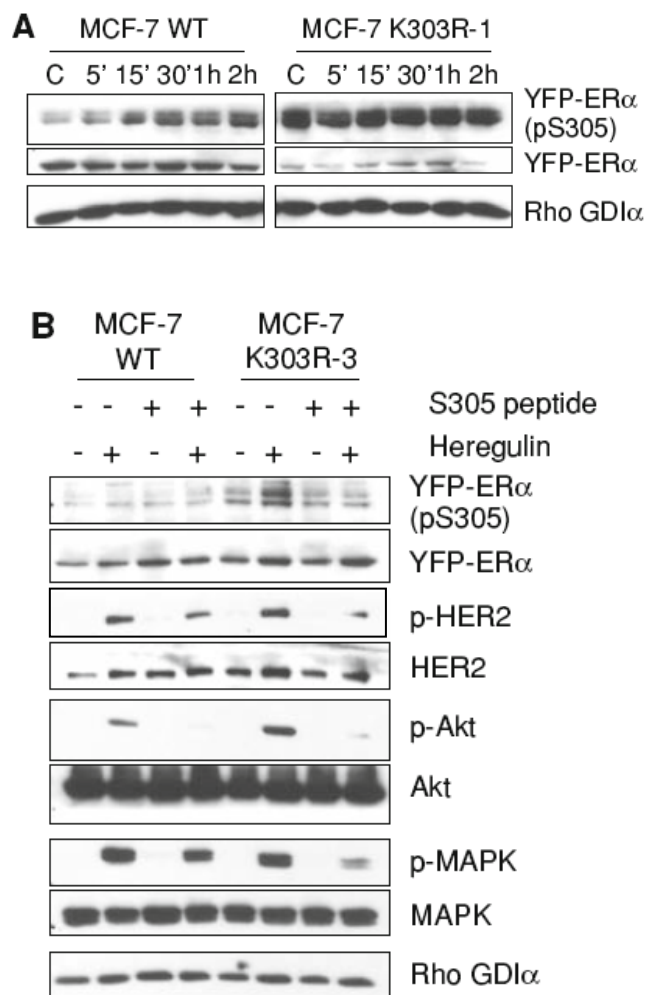
Fig. 8 Serine residue 305 (S305) in the K303R ERα mutant is involved in growth factor signal up-regulation. (**a**) MCF-7 WT and MCF-7 K303R-1 cells were treated for different times with E_2 (1nM) before lysis. Cellular extracts were analyzed for phosphorylation levels of S305 YFP ERα (pS305) and total non-phosphorylated YFP-ERα. (**b**) Cells were incubated with the S305 peptide (4 μg/well) for 4 hours in serum-free media and then treated with or without heregulin (2 ng/ml) for 10 min before lysis. Levels of phosphorylated (p) S305 YFP-ERα, HER2 (Tyr[1248]), Akt (Ser[473]), and MAPK (Thr[202]/Tyr[204]), and total non-phosphorylated proteins were measured in cellular extracts by immunoblot analysis. Blots are representative of three separate experiments. Rho GDIα was used as a control for equal loading and transfer

Expression of the mutation and AI resistance

Estrogen-deprived MCF-7 cell lines (41-43, 74), and breast cancer cells resistant to aromatase inhibitors (AI^R) have been generated (45, 46); one hypothesis emerging is that resistance may result from adaptive estrogen hypersensitivity or estrogen-independent ERα activation. This hypothesis led us to question if the K303R ERα mutant might play a role in AI^R due to its estrogen-hypersensitive phenotype. To test for AI^R, cells were stably transfected with an aromatase cDNA expression vector. Figure 9a shows aromatase protein levels in a vector control clone (MCF-7 V), one clone stably expressing aromatase (MCF-7 Arom 1), and two clones coexpressing the YFPK303R mutant ERα along with aromatase (K303R Arom 1 and Arom 2). These cells overexpressed aromatase protein and activity at ~1,000 times more than vector control cells (0.032 versus 29–65 pmol/h/mg protein) (data not shown).

We next evaluated proliferative responses in anchorage-independent assays (Fig. 9b). K303R Arom 1–expressing cells exhibited significantly enhanced control growth compared with WT ERα MCF-7 Arom1–expressing cells, and growth was further increased by AD treatment.
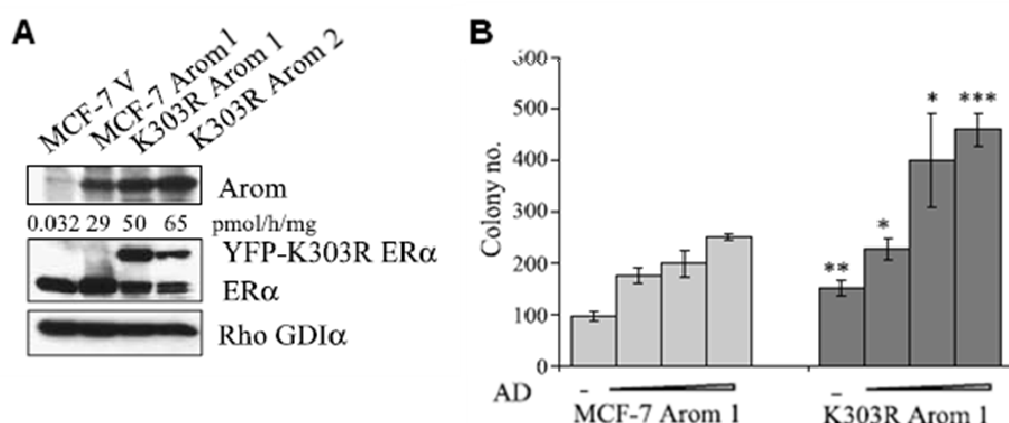

Fig. 9 Characterization of K303R Aromatase-expressing cells. (a) immunoblotting showing aromatase (Arom), ERα and Rho GDIα. Numbers below blots represent aromatase activity**. (b)** anchorage-independent growth assay in cells treated with vehicle or 4-androstene-3,17-dione (AD, 1, 10, and 25 nmol/L). Bars, SD (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0005$). K303R Arom 1 group versus the respective treatment in MCF-7 Arom 1 group.

MCF-7 Arom 1 and K303R Arom 1 cells were also injected into mice to monitor xenograft growth; a significant increase in the growth of AD-treated mutant tumors was seen (Fig. 10).
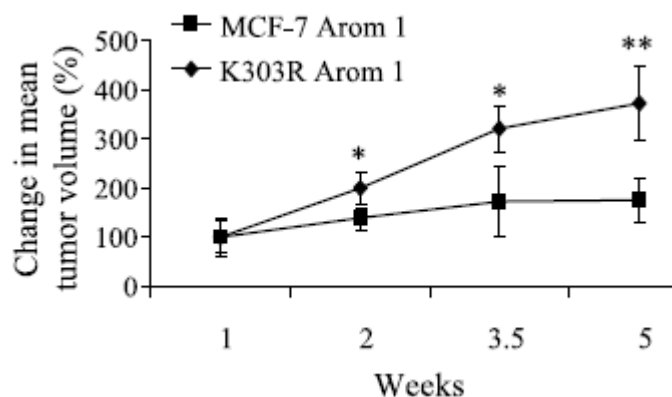

Fig. 10 Tumor xenograft studies. MCF-7 Arom 1 and K303R Arom 1 cells were injected into mice, and supplemented with AD. Tumor growth was plotted as the percentage of change in mean tumor volume/animal (n = 5) compared with day 0 of treatment ± SD (*, $P < 0.05$; **, $P < 0.01$ K303R Arom 1 group versus MCF-7 Arom 1 group).

To investigate whether the mutant hypersensitive phenotype could cause AI^R, we examined the effects of the nonsteroidal aromatase inhibitor Ana on cell growth using MTT assay (Fig. 11a). Growth of MCF-7 vector (V) cells was significantly enhanced by E_2, but as expected, they did not respond to AD treatment. AD treatment enhanced growth to the same extent as E_2 in MCF-7 Arom 1 cells, as well as K303R Arom 1–expressing cells. Ana treatment decreased AD-stimulated growth at ~40% in MCF-7 Arom 1–expressing cells, but had no effect on the growth of mutant cells. To extend the MTT data, we performed anchorage-independent soft agar growth assays (Fig. 11b). E_2 and AD treatments enhanced colony numbers, and treatment with Ana completely abrogated AD-stimulated growth of MCF-7 Arom 1 cells. In contrast, basal colony number was increased 10-fold in K303R Arom 1 and Arom 2 clones, and Ana was unable to inhibit AD-induced colony growth.
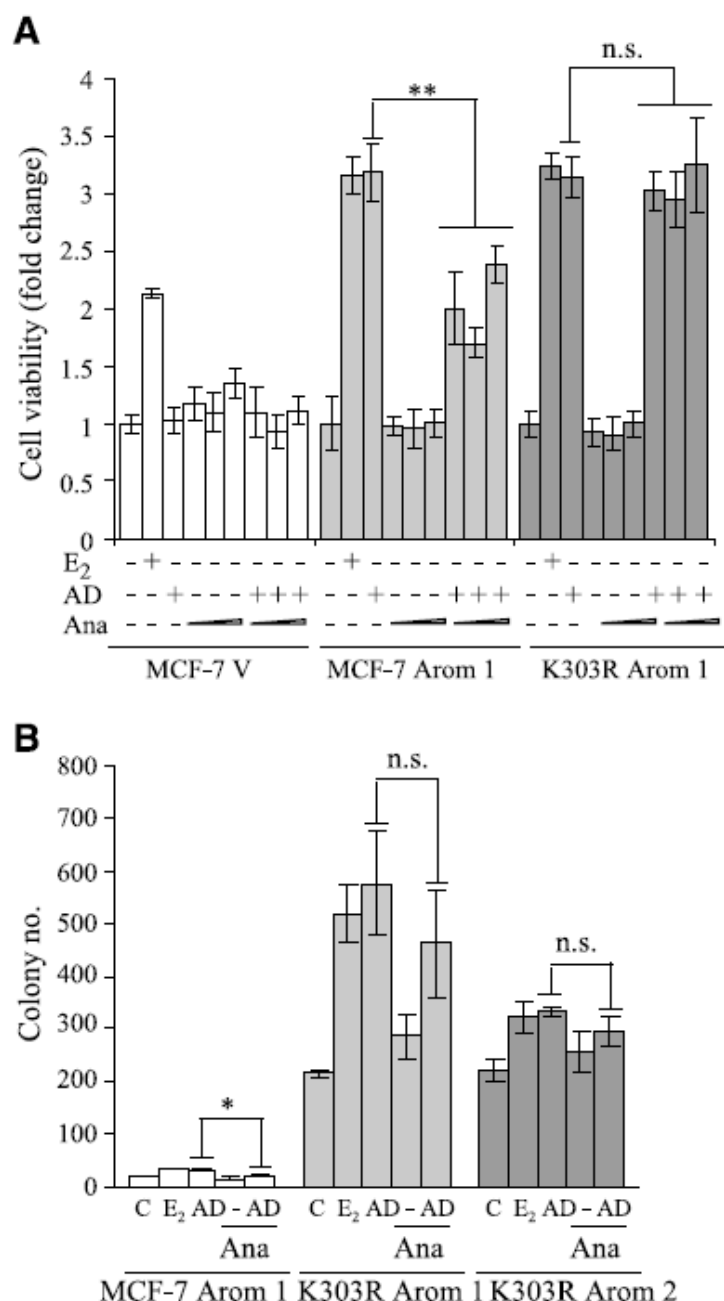
Fig. 11. K303R ERα mutation confers resistance to anastrozole. (a) MTT growth assays in cells treated with vehicle, E_2 (1 nmol/L), AD (10 nmol/L), and/or anastrozole (Ana 100 nmol/L, 1 μmol/L, 10 μmol/L). Cell proliferation is expressed as fold change relative to vehicle treated cells. Data are representative of three independent experiments, performed in quadruplicate. Columns, mean; bars, SD (**, P < 0.005; n.s., nonsignificant AD versus Ana + AD). **(b)** anchorage-independent growth assay in cells treated with vehicle, E_2 (1 nmol/L), AD (10 nmol/L) ± Ana (1 μmol/L). Bars, SD (*, P = 0.01; n.s., nonsignificant AD versus Ana + AD).

Because it was possible that overexpression of exogenous ERα alone might stimulate cell growth and contribute to the AI[R] phenotype, we also transfected MCF-7 Arom 1–expressing cells with an expression vector for YFP-WT ERα. Aromatase

activity levels and exogenous YFP-ERα expression levels are shown in Fig. 12a, top panel. Pools expressing exogenous WT or mutant receptor were then evaluated in soft agar assays (Fig. 13a, bottom panel). The basal growth of K303R ERα-Arom pools (P) was significantly higher than WT pools. Inhibition of aromatase activity by Ana caused a significant reduction in AD-stimulated growth in WT ERα Arom cells, but Ana was unable to reduce AD-stimulated mutant cell growth, confirming our stably transfected clones results. In the mutant pool AD + Ana stimulated growth compared with AD treatment alone, which may be a variable clonal effect. We also generated stable pools of YFP-tagged ERα in ER-negative, aromatase-positive CHO cells (Fig. 12b). Ana did not reverse AD-stimulated growth in CHO mutant cells, confirming that the AI^R phenotype was associated with expression of the mutant in a number of different cellular backgrounds.
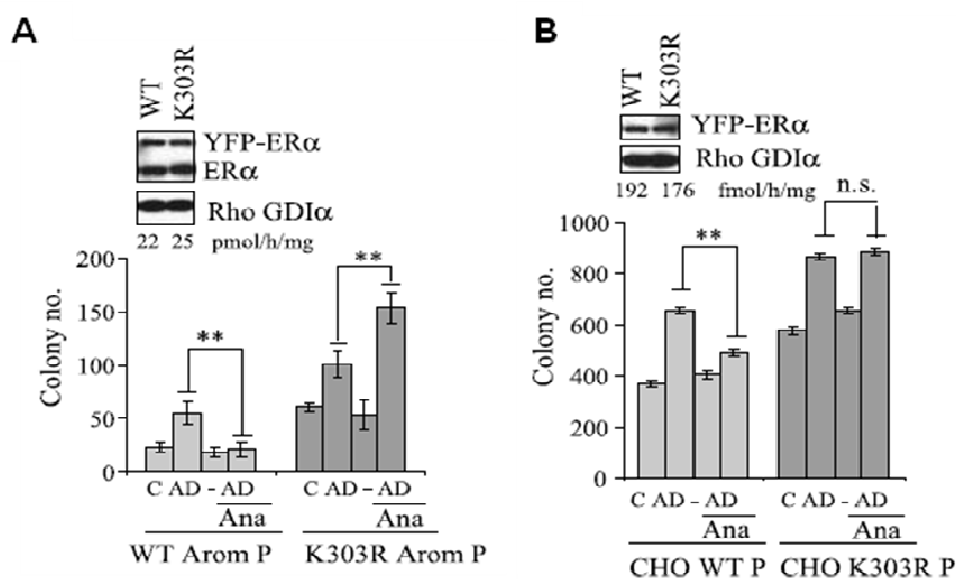

Fig. 12 AI^R phenotype in stably transfected pools. Anchorage-independent growth assay in MCF-7 Arom P (**a,** bottom) and CHO P (**b,** bottom) pools treated with vehicle, AD (10 nmol/L) ± Ana (1 μmol/L). Bars, SD (**, P < 0.01; n.s., nonsignificant AD versus Ana + AD). **a** and **b**, ERα and Rho GDIα (top). Numbers below blots represent aromatase activity

K303R ERα-aromatase cells exhibited altered apoptotic responses

It is well known that AIs induce cell death by apoptosis (75). This suggestion led us to question if the K303R ERα mutation could protect cells from apoptosis induced by Ana, providing these cells with a potential survival advantage. We first evaluated PARP proteolysis, and found a reduction in the basal levels of the proteolytic form of PARP (85 kDa) in mutant cells under control conditions (Fig. 13a). AD treatment reduced its levels in a time-dependent fashion in WT and mutant cells, but this reduction was less pronounced in WT cells. E_2 was able to reduce PARP-cleavage levels only in mutant cells, suggesting that reduced apoptosis may underlie their hypersensitivity. Ana treatment of MCF-7 Arom 1–expressing cells increased proteolysis compared with AD treatment; cleavage was unchanged in mutant cells (Fig. 13b).
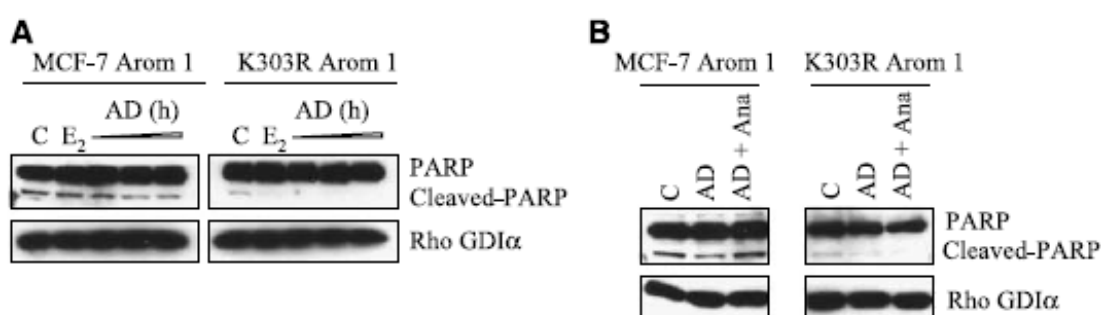

Fig. 13 PARP-cleavage in mutant cells. a and **b**, immunoblotting showing PARP and Rho GDIα in cells treated with vehicle, E_2 1 nmol/L (24 h), AD 10 nmol/L (12, 24, and 48 h, **a**), or AD 10 nmol/L and AD + Ana 1 Amol/L (24 h, **b**).

In addition, we found an increase in the Bcl-2/Bax ratio in K303R Arom 1–expressing cells, which was further increased with AD and Ana treatments (Fig. 14a and b). To determine the levels of cellular apoptosis, we also used ELISA cell death detection assays (Fig. 14c), and found that AD-stimulated mutant cells exhibited a lower apoptosis compared with MCF-7 Arom 1–expressing cells, suggesting that the proliferative advantage provided by the mutation may be achieved by a decreased

apoptotic response of these cells. In addition, Ana treatment induced an increase in cell

apoptosis only in MCF-7 Arom 1 cells. We hypothesize that mutant-expressing cells are

resistant to AI-induced cellular apoptosis.
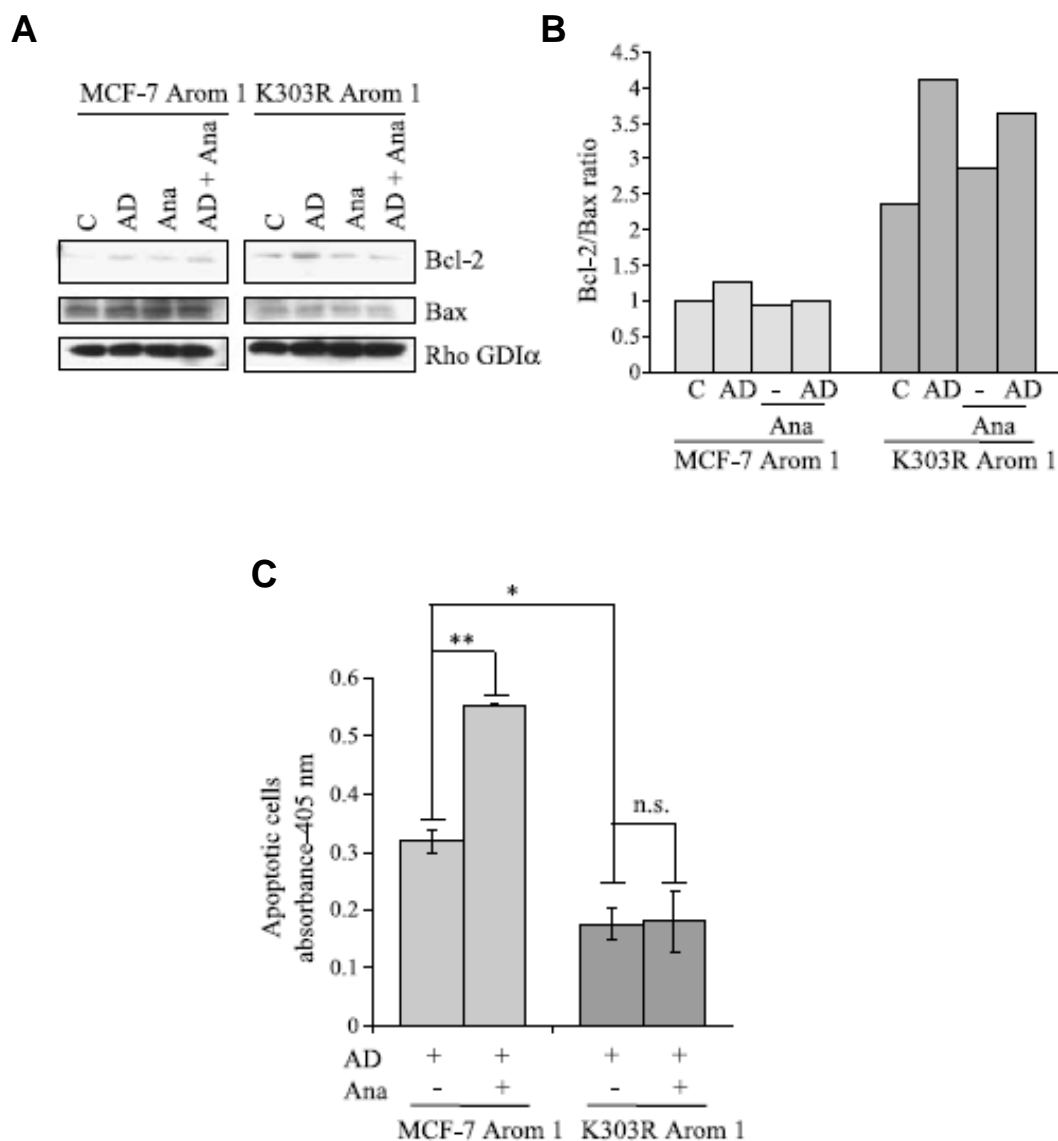
Fig. 14 Altered apoptotic response in mutant cells. (a) Bcl-2, Bax, and Rho GDIα in cells treated with vehicle, AD 10 nmol/L ± Ana 1 μmol/L (24 h). (b) quantitative analysis is the fold difference in Bcl-2/Bax ratio relative to vehicle-treated MCF-7 Arom 1 cells. (c) ELISA cell detection assay in cells treated with AD 10 nmol/L ± Ana 1 μmol/L. Columns, mean; bars, SD (*, $P < 0.05$, **, $P < 0.005$, n.s., nonsignificant AD versus Ana + AD).

DISCUSSION

Despite the improvements in the efficacy of hormonal therapies for the treatment of breast cancer patients with ER-positive tumors, *de novo* and acquired resistance remain major clinical problems that limit the efficacy of these therapies. In most cases, ERα remains essential to the problem of resistance due to its intimate cross-talk with growth factor signaling pathways (76, 77). In this study, we show that expression of K303R ERα mutant in ERα-positive MCF-7 breast cancer cells confers a decreased sensitivity to tamoxifen treatment in the presence of growth factor stimulation and confers AI[R] in MCF-7 K303R cells overexpressing aromatase. Furthermore, this naturally-occurring mutant is constitutively phosphorylated at S305, and shows an enhanced bidirectional cross-talk with the HER2 signaling pathway.

The A to G somatic mutation of ERα at nucleotide 908 (A908G) was previously identified in about 30% of premalignant breast lesions, but at a higher frequency (50%) in invasive breast tumors (9, 13). The mutation was found to be associated with biologic measures of poor outcome, including elevated HER2 protein, larger tumor size and axillary lymph node positivity. To date no other somatic ERα mutation has been identified in more than a few invasive breast cancers (10), making this mutation novel. Recently, in a study using a population-based, case-control study design, the A908G mutation was detected, but at a low frequency (7%), in invasive breast tumors (78), confirming our identification of the mutant in cancer. In the present study we focused on the K303R ERα mutation and its potential role in modulating hormonal response in breast cancer cells, and on the molecular pathways that could be involved in its hormone action.

It is clear that breast cancer cells can adapt in response to the selective pressures exerted by exposure to estrogen deprivation therapy (i.e., AIs or ovarian ablation), by

developing enhanced sensitivity to the proliferative effects of low levels of estrogens. The initial requirement for premenopausal levels of estradiol (i.e. 50–600 pg/ml) for tumor growth, but later the utilization of only 10–15 pg/ml, provides clinical evidence for "adaptive estrogen hypersensitivity" (79). To explore mechanisms underlying adaptive hypersensitivity, a number of different laboratories have developed *in vitro* models to study the molecular changes associated with long-term estrogen deprivation. Although different mechanisms of resistance have been reported, a unifying feature is that ERα signaling remains an integral part of the mechanisms driving proliferation of these cells. Here we show that the growth of mutant-expressing cells was enhanced compared to the WT MCF-7 cells, when they were injected into nude mice in the presence of low levels of estrogens. We also demonstrated that K303R aromatase-overexpressing cells, simulating the postmenopausal breast cancer where the source of non-ovarian estrogen is not under gonadotropin regulation, exhibited enhanced growth in basal conditions as well as following AD treatment both *in vitro* (soft agar) and *in vivo* (xenograft) growth. Furthermore, we found that the hypersensitive phenotype associated with expression of the ERα mutation resulted in the acquisition of resistance to the AI anastrozole. Anastrozole treatment specifically decreased AD-stimulated growth of WT cells, but had no effect on mutant-expressing cells. One of the mechanisms of AIs is to induce cell death by apoptosis (75). In this study we found a lower frequency of apoptosis in AD-stimulated K303R Arom-expressing cells, suggesting an altered apoptotic response in these cells. In addition, Ana treatment induced a significant increase in cell apoptosis in WT cells, but no changes were observed in the apoptotic response of mutant-expressing Arom- cells. It has been proposed that Bcl-2/Bax protein ratios may determine whether a cell will undergo apoptosis (80). We found an increased Bcl-2/Bax ratio in the mutant cells that was

further increased after AD and Ana treatments, confirming a potential survival advantage for the mutant receptor cells.

The mutation, indeed, conferred resistance to an AI, and the potential mechanisms of resistance include cellular strategies to evade apoptosis and enhance cellular proliferation possibly trhough enhanced cross-talk with growth factor pathways and reception of downstream signalling networks.

Recent research into the mechanisms associated with tamoxifen resistance suggest that some of the same growth factor receptor pathways implicated in adaptive hypersensitivity, such as Akt and MAPK, or specific oncogenes involved in intracellular signal transduction, become activated and are used to bypass normal endocrine controls and responsiveness. Our studies showed that growth factor signaling pathways were up-regulated in MCF-7 K303R ERα-expressing cells. In particular, MCF-7 mutant ERα expressing cells showed constitutively higher levels of total and phosphorylated HER2, the tyrosine kinase receptor that belongs to the epidermal growth factor receptors family. Preliminary quantitative RT-PCR analysis demonstrated that HER2 mRNA levels were not increased in mutant expressing cells compared to WT ERα-expressing cells. Thus, transcriptional regulation may not be the major mechanism for the observed increased levels of HER2 in mutant cells. In the present study we did not investigate the possible post-transcriptional mechanisms associated with higher levels of HER2, but instead focused on the effects that HER2 overexpression induced on downstream cell signaling and hormone responsiveness in mutant-expressing cells. It is well known that HER2 catalytic activity can amplify the signal of other c-erbB family receptors by the formation of HER2-containing heterodimers, which increases ligand binding affinity and receptor stability (81, 82). Moreover, it has been shown that c-neu, the mouse homolog of HER2, is able to multimerize, be phosphorylated, and thus activated when present at high density on the cell surfaces (83). Both mechanisms result in the

amplified activation of downstream signaling cascade, such as Akt and MAPK, which are involved in cell survival and proliferation.

We found that the peptide growth factors heregulin and EGF strongly enhanced phosphorylation of the two major downstream signaling cascades Akt and MAPK, in mutant-expressing cells compared to WT ERα-expressing cells. Furthermore, analysis of rapid kinetics showed that many of these downstream molecules, as well as the c-Src non receptor tyrosine kinase, were stimulated at earlier time points in mutant-expressing cells. The rapid responses of these downstream kinase cascades to heregulin suggest that the presence of K303R ERα mutation could modify the responsiveness of the cells to the growth factor signaling possibly through enhanced non-genomic activity of the mutant receptor.

Several reports indicate that the up-regulation of HER2 tyrosine kinase signaling in breast cancer plays an important role in the development of endocrine resistance (37). Preclinical studies have demonstrated that HER2 overexpression in ERα positive MCF-7 human breast cancer xenografts rendered them resistant to Tam (84), and markedly increased levels of EGFR and HER2 were found in some sublines of MCF-7 cells with acquired Tam resistance (85, 86). Tam resistance in these cells was reversed by EGFR/HER2 tyrosine kinase inhibitors, and combined treatment with these inhibitors and Tam was effective in reversing resistance in xenograft models (87), thereby strongly implicating this signaling network in resistance. Although HER2 overexpression occurs only in a minority of ERα-positive patients (88), clinical studies confirm that HER2-overexpressing tumors are less responsive to Tam treatment (89, 90). In a previous retrospective study we found an association between the K303R ERα mutation and elevated HER2 levels in invasive breast cancer (13). Here we observed increased HER2 levels in K303R mutant-expressing cells that was concomitant with an altered response to Tam with growth factor stimulation.

We also demonstrated that mutant-expressing cells exhibited a higher level of growth under all conditions tested. Importantly, Tam sensitivity was significantly affected with estrogen and heregulin treatments. Our data indicate that the Tam-resistant phenotype associated with the mutant was most pronounced in the presence of growth factor activation; in the presence of heregulin Tam inhibited soft agar growth only 21% compared with a 96% inhibition in WT ERα-expressing cells. We speculate that bidirectional cross-talk between the HER2 and mutant receptors could play a role in conferring a selective advantage in terms of growth to those patients that express the K303R ERα mutant that are treated with Tam. We are currently examining for the presence of the mutant in a retrospective cohort of patients treated with Tam who have long-term clinical follow-up.

Different therapeutic agents targeting the activity of the c-erbB family of receptors have been recently developed and tested in patients. For instance, herceptin (Trastuzumab[TM]), a monoclonal antibody against HER2, was approved for therapeutic use in patients with HER2-overexpressing breast cancer (91, 92). By binding to the juxtamembrane domain of HER2 (93), this agent blocks HER2 homo- and heterodimerization with the other members of the c-erbB family, and thereby interrupts the activation of downstream proliferative signaling. Here we show that herceptin elicited its antiproliferative effects either under basal conditions or with hergulin treatment in K303R ERα mutant cells, but did not affect growth of the WT cells. These findings confirm our hypothesis that the up-regulation of HER2 pathway become prominent in the control of cell growth in breast tumor cells bearing the mutation, probably through increased crosstalk between HER2 and ERα signalling.

The existence of bidirectional cross-talk between ERα and growth factor receptor pathways, and its involvement in the development of endocrine resistance has been well documented (25, 27). Several studies have demonstrated direct or indirect activation of

growth factor signaling via ERα. For example, ligand-independent activation of serine 118 ERα by EGFR/MAPK-mediated phosphorylation regulates growth of tamoxifen-resistant MCF-7 breast cancer cells (94). Chung *et al*. have also demonstrated that HER2 and ERα can directly interact at the cell membrane (38), and this interaction protected breast cancer cells from Tam-induced apoptosis. Moreover, membrane or cytoplasmic ERα can induce phosphorylation of EGFR through activation of G-proteins, c-Src, and matrix metalloproteinases (95), and can directly interact with adaptor proteins such as c-Src, Shc and the p85α regulatory subunit of PI3K (61-64). These processes activate downstream kinases that in turn activate ERα and its coregulatory proteins, thus also enhancing genomic activities of the receptor (96, 97). All together these effects amplify the bidirectional crosstalk which multiplies signals between and downstream of the growth factor receptors and ERα, thus sustaining survival and proliferative signals in breast cancer cells.

The present findings suggest an enhanced hormone-independent physical association/complex between the mutant receptor and HER2 compared to WT receptor. This suggests that the mutation, present in the hinge region of the receptor, may increases the ability of ERα to interact with HER2 or other components of the complex. As yet, the interaction surface between HER and ERα has not been defined. Altered interactions as we describe may imply that Tam may not antagonize the mutant receptor because the HER2 pathway may be dominant and non-genomic action predominates. This is consistent because we have already demonstrated that the mutation can alter coregulator protein binding. The mutation demonstrates enhanced binding ability to bind to the TIF-2 coactivator (9), and the AIB coactivator at very low and physiological levels of estradiol, but decreased binding to the corepressor NCoR1. Mutant receptor binding to BRCA-1 has also been shown to be enhanced. These collective data suggest

that altered affinity for ER coregulators, and possibly signaling molecules such as HER2, could be one mechanisms by which the K303R mutation confers hypersensitivity to low levels of estrogen, and reduced sensitivity to Tam. The mutant receptor appears to exhibit increased ligand-independent activity which bypasses antiestrogen treatment.

To explore why the K303R ERα mutation has increased cross-talk with the HER2 pathway, we focused on the differences in post-translational modifications between WT and mutant receptor. It is known that ERα activity can be modulated by several post-translational modifications, such as protein phosphorylation (69), acetylation (70), ubiquitination (98), and sumoylation (71). The majority of studies in this field have focused their attention on the phosphorylation status of the receptor, and its effect on receptor activity. For instance, receptor phosphorylation by different kinases such as c-Src, PKA, MAPK, and Akt can all regulate receptor affinity, coregulator protein binding, and transcriptional activity (69). We have previously shown that the K303R ERα mutation renders the receptor a more efficient substrate for PKA-induced phosphorylation at residue S305 which has distinct biological results—enhanced hormone sensitivity for growth (17). Phosphorylation at the S305 residue can also be mediated by both protein kinase A (PKA) and p21-activated kinase-1 (PAK-1) signaling network (17, 20).

Several reports have identified the serine residue at 305 as a physiologically important site that modifies response to Tam. In particular it has been demonstrated, using fluorescence resonance energy transfer (FRET) analysis, that PKA signaling to ERα S305 causes a conformational arrest in the ERα and switches Tam from an antagonist to an agonist (18). Michalides *et al*. have also demonstrated that PKA phosphorylation at S305 ERα induces Tam resistance through an altered orientation of ERα towards the co-activator SRC-1 (99). In addition, S305 ERα phosphorylation by

PAK-1 up-regulates cyclin D1 expression in breast cancer cells (100). Here we show that K303R mutant cells have elevated phosphorylation levels of S305 compared with WT-expressing cells suggesting that the mutant have constitutive ligand-independent activity. The contribution of the S305 site in enhanced cross-talk with HER2 was established using a blocking Peptide. We show that heregulin-stimulation enhanced S305 phosphorylation in mutant expressing cells, but it did not significantly influence the phosphorylation status of WT receptor. Interestingly, we found that the S305 peptide affected heregulin-induced ERα phosphorylation, and prevented downstream phosphorylation events, such as activation of MAPK and Akt. These effects were even more prominent in K303R mutant cells possibly due to prominent role of the S305 site in ligand-independent activity of the mutant receptor. A similar experimental approach was used by Varricchio *et al.* (101). They demonstrated that a six aminoacid peptide surrounding the phosphotyrosine residue 537 was able to block ER/c-Src interactions, cyclin D1 expression, and growth of MCF-7 and LNCaP cells.

We have previously shown that transcriptional activity of the mutant receptor was induced at very low concentrations of estradiol (10^{-12} M), and only the specific lysine to arginine substitution at 303 residue resulted in a receptor with enhanced sensitivity to estrogen. We have also demonstrated that cAMP-dependent signaling can enhance the receptor's intrinsic sensitivity to hormone, and that blocking PKA activity reversed the hypersensitive proliferative phenotype in mutant-expressing cells (17). The data obtained herein using live cell dynamics agrees with these earlier results. The live cell high through-put analyses allowed us to study mutant receptor/promoter interaction and chromatin remodelling (48, 50). Our results confirm that the K303R mutant is inherently hypersensitivity to estrogen, and only WT receptor showed increased sensitivity to estrogen after forskolin treatment. We used the same experimental approach to test for altered Tam activity in the presence of forskolin. Tam treatment

inhibited transcriptional responses in WT, but at statistically significant lower levels in mutant cells. Forskolin treatment blocked Tam-induced promoter condensation, suggesting that ligand-independent kinase signaling to the mutant receptor decreased tamoxifen sensitivity.

Clinical studies have reported that breast tumors with HER2 amplification show reduced levels of PR (102), and the absence of PR is a marker of a more aggressive phenotype (103). Patients whose tumors lack PR derive less benefit from adjuvant hormonal therapy (54-56). *In vitro* studies suggest that amplified growth factor signaling may underlie the reduction of PR levels in breast cancer cells (104). In this report we found that PR protein expression was almost undetectable in K303R cells under basal conditions, and estrogen induced only a small increase in PR content, compared to the induction elicited in WT-expressing cells. The lack of PR expression observed in K303R mutant-expressing cells may be the consequence of altered growth factor signaling that contributed to the Tam-resistant phenotype observed in our model system.

We conclude that the K303R ERα hypersensitive phenotype involves an integration of post-translational modification events, such as phosphorylation at S305, with enhanced bidirectional cross-talk between the mutant and growth factor receptors such as HER2, and that genomic and nongenomic mechanisms probably contribute to Tam resistance.

Because our molecular and biological data demonstrate that the cells expressing ERα mutation may be resistant to Tam and to AI treatments, we suggest that the genetic assays for the mutation might offer a new predictive marker for hormonal response in breast cancer tumors. Moreover, our molecular studies suggest that use of a specific blocking peptide to prevent S305 phosphorylation of the mutant may reduce ligand-

independent activity, and be a new therapeutic approach to treat patients with mutation-positive tumors which are resistant to Tam therapy.

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FARNESOID X RECEPTOR, THROUGH THE BINDING WITH STEROIDOGENIC FACTOR 1 RESPONSIVE ELEMENT, INHIBITS AROMATASE EXPRESSION IN TUMOR LEYDIG CELLS

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Running head: FXR regulates aromatase expression in Tumor Leydig cells.
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The Farnesoid X Receptor (FXR) is a member of the nuclear receptor superfamily that regulates bile acid homeostasis. It is expressed in the liver and the gastrointestinal tract, but also in several non-enterohepatic tissues including testis. Recently, FXR was identified as a negative modulator of the androgen-estrogen-converting aromatase enzyme in human breast cancer cells.

In the present study we detected the expression of FXR in Leydig normal and tumor cell lines and in rat testes tissue. We found, in rat Leydig tumor cells, R2C, that FXR activation by the primary bile acid chenodeoxycholic acid (CDCA) or a synthetic agonist GW4064, through a SHP-independent mechanism, down-regulates aromatase expression in terms of mRNA, protein levels and its enzymatic activity. Transient transfection experiment, using vector containing rat aromatase promoter PII, evidenced that CDCA reduces basal aromatase promoter activity. Mutagenesis studies, electrophoretic mobility shift and chromatin immunoprecipitation analysis reveal that FXR is able to compete with SF-1 in binding to a common sequence present in the aromatase promoter region interfering negatively with its activity. Finally, the FXR activator CDCA exerts anti-proliferative effects on tumor Leydig cells at least in part through an inhibition of estrogen-dependent cell growth.
In conclusion our findings demonstrate that FXR ligands as aromatase inhibitors may represent a promising new therapeutic approach for Leydig cell tumors.

The Farnesoid X Receptor (FXR, NR1H4) is a member of the nuclear receptor superfamily of ligand-dependent transcription factors, normally produced in the liver and the gastrointestinal tract, where it acts as a bile acid sensor (1-3). FXR regulates the expression of a wide variety of target genes involved in bile acid, lipid and glucose metabolism by binding either as monomer or as a heterodimer with the Retinoid X Receptor (RXR) to FXR response element (FXREs) (4-7). FXR induces the up-regulation of nuclear receptor SHP (Small Heterodimer Partner) which interacts with other nuclear receptors preventing their activation (8-10).

Recently, new functions of FXR beyond its roles in metabolism were discovered in several nonenterohepatic tissues, including its control in regulating cell growth and carcinogenesis (11-14). For instance, it has been demonstrated that FXR activation inhibits breast cancer cell proliferation and negatively regulates aromatase activity reducing local estrogen production which sustains tumor growth and progression (13).

Estrogen dependency is also a feature of testicular tumor which is the most frequent solid malignant tumour diagnosed in young men (20-40 years old) accounting for up to 20% of all malignancies diagnosed at this age. Ninety-five percent of all human testicular neoplasms arise from germinal cells whereas Leydig cell tumors are the most common tumors of the gonadal stroma (15). The molecular basis of testicular cell malignant transformation is poorly defined. It has been reported that estrogen serum levels are elevated in patients with testicular germ cell cancer as a consequence of increased local estrogen production reflecting an higher aromatase activity present in Sertoli and Leydig cells (16). Several studies on both rodents and humans indicate that prenatal, early post-natal and adult exposure to an excess of estrogens might have a central role in the mechanism leading to male reproductive tract malformations

such as testicular and prostatic tumors (17). The biological significance of estrogen-induced testicular tumorogenesis has been suggested by transgenic mice overexpressing aromatase and exhibiting enhancement of 17β-estradiol (E2) circulating levels (18). About half of these male mice were infertile and/or had enlarged testis and showed Leydig cell hyperplasia and Leydig cell tumors (18). Recently, we demonstrated aromatase and ERs expression in testis from patients affected by Leydigioma in which high estradiol levels in the presence of ERα could significantly contribute to tumor cell growth and progression (19). Besides, we also reported that one of the molecular mechanisms determining Leydig cell tumorogenesis is an excessive estrogen production that stimulates a short autocrine loop determining cell proliferation (20).

Aromatase activity is regulated primarily at the level of gene expression by tissue-specific promoters and is present in testicular somatic cells and along the maturative phases of male germ cells (21, 22). A promoter proximal to the translation start site, called promoter II (PII) regulates aromatase expression in fetal and adult testis, R2C and H540 rat Leydig tumor cells, and in purified preparations of rat Leydig, Sertoli, and germ cells (23, 24). Specific sequences seem to be mainly involved in aromatase expression: cyclic AMP (cAMP)-responsive element (CRE)-like sequences binding CREB/ATF protein families (25, 26) and a sequence containing an half-site binding nuclear receptors (AGGTCA) in position -90 binding steroidogenic factor 1 (SF-1) (27) which is essential for sex differentiation and development of gonads (28).

On the basis of all these observations, in this study we investigated in rat tumor Leydig cells, R2C whether FXR activation by specific ligand chenodeoxycholic acid (CDCA) or a synthetic agonist GW4064 may modulate aromatase expression and antagonize estrogen signalling, inhibiting testicular tumor growth and progression. We, for the first time, demonstrated that the molecular mechanism by which FXR ligands inhibit aromatase gene expression in R2C cells is mediated by a direct binding of FXR to SF-1 response element present in the aromatase promoter region.

Experimental Procedures

Reagents- Nutrient Mixture F-10 Ham, Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12), Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine, penicillin, streptomycin, fetal bovine serum (FBS), horse serum (HS), phosphate-buffered saline, aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), bovine serum albumin (BSA) and sodium orthovanadate were purchased by Sigma (Milan, Italy). TRIzol, Lipofectamine 2000 by Invitrogen (Carlsbad, CA, USA) and FuGENE 6 by Roche Applied Science (Indianapolis, IN, USA). TaqDNA polymerase, RETROscript kit, 100-bp DNA ladder, Dual Luciferase kit, TNT master mix and TK Renilla luciferase plasmid were provided by Promega (Madison, WI, USA). Antibodies against FXR, β-actin, GAPDH, Cyclin D1, Cyclin E and Lamin B by Santa Cruz Biotechnology (Santa Cruz, CA, USA), antibody against Aromatase by Serotec (Raleigh, NC, USA) and antibody against SF-1 kindly provided from Dr. K. Morohashi (National Institute Basic Biology, Myodaiji-cho, Okazaki, Japan). ECL system and Sephadex G-50 spin columns from Amersham Biosciences (Buckinghamshire, UK). [1β-^{3}H]androst-4-ene-3,17-dione, [γ^{32}P]ATP, and [^{3}H]thymidine from PerkinElmer (Wellesley, MA, USA). Salmon sperm DNA/protein A agarose by UBI (Chicago, IL, USA).

Plasmids- The plasmids containing different segments of the rat aromatase PII sequence ligated to a luciferase reporter gene [-1037/+94 (p-1037), -688/+94 (p-688) and -688/+94 mut (p-688m) (SF-1 site mutant)] were previously described (27). FXR responsive reporter gene (FXRE-IR1) and FXR-DN (dominant negative) expression plasmids were provided from Dr. T.A. Kocarek (Institute of Environmental Health Sciences, Wayne State University, USA) (29). FXR expression plasmid was provided from Dr. D.J. Mangelsdorf (Southwestern Medical Center, TX, USA). SF-1 expression plasmid and hCYP17 gene reporter were obtained from Dr. W. E. Rainey (Medical College of Georgia, USA). XETL plasmid is a construct containing an estrogen-responsive element from the *Xenopus* vitellogenin promoter, driving expression of the luciferase gene.

Cell Cultures and animals- Rat Leydig tumor cells (R2C) were cultured in Ham/F-10 supplemented with 15% HS, 2.5% FBS, and antibiotics. Mouse Leydig cells (TM3) were cultured in DMEM/F-12 supplemented with 5% HS, 2.5% FBS, and antibiotics. Human Cervix

tumor cells (HeLa) and Hepatoma cells (HepG2) were cultured in DMEM supplemented with 10% FBS, 1% L-glutamine and antibiotics. The cells were starved in serum free medium (SFM) 24 hours before treatments. Male Fisher 344 rats (a generous gift of Sgma-Tau), 6 (FRN) and 24 (FRT) months of age, were used for studies. Twenty-four-month-old animals presented spontaneously developed Leydig cell tumors, which were absent in younger animals. Testes of all animals were surgically removed by qualified, specialized animal care staff in accordance with the Guide for Care and Use of Laboratory Animals (NIH) and used for experiments.

Aromatase Activity Assay - The aromatase activity in subconfluent R2C cells culture medium was measured by the tritiated water release assay using 0.5 µM [1β-^{3}H]androst-4-ene-3,17-dione as substrate (30). The incubations were performed at 37°C for 2 h under an air/CO_2 (5%) atmosphere. The results obtained were expressed as pmol/h and normalized to mg of protein (pmol/h/mg protein).

Total RNA extraction and reverse transcription-PCR assay- Total RNA was extracted from R2C and TM3 cells using TRIzol reagent and the evaluation of genes expression was performed by the reverse transcription-PCR method using a RETROscript kit. The cDNAs obtained were amplified by PCR using the following primers: forward 5'-CAGCTATACTGAAGGAATCCACACTGT-3' and reverse 5'-AATCGTTTCAAAAGTGTAACCAGGA-3' (P450 aromatase) forward 5'-TTTCTACCCGCAACAACCGGAA-3' and reverse 5'-GTGACAAAGAAGCCGCGAATGG-3' (FXR), forward 5'-CAGCCACCAGACCCACCACAA-3' and reverse 5'-GAGGCACCGGACCCCATTCTA-3' (rat-SHP), forward 5'-CGTCCGACTATTCTGTATGC-3' and reverse 5'-CTTCCTCTAGCAGGATCTTC-3'(mouse-SHP) or forward 5'-GAAATCGCCAATGCCAACTC-3' and reverse 5'- ACCTTCAGGTACAGGCTGTG -3' (L19). The PCR was performed for 25 cycles for P450 aromatase (94°C 1 min, 58°C 1 min, 72°C 2 min), 35 cycles for FXR (94°C 1 min, 65°C 1 min, 72°C 2 min), 28 cycles for SHP (94°C 1 min, 65°C 1 min, 72°C 2 min) and 25 cycles for L19 (94°C 1 min, 60°C 1 min, and 72°C 2 min) in the presence of 1µl of first strand cDNA, 1 µM each of the primers, 0.5 mM dNTP, *Taq*

DNA polymerase (2 units/tube) and 2.2 mM magnesium chloride in a final volume of 25 µl. DNA quantity in each lane was analyzed by scanning densitometry.

Immunoblot analysis- R2C, TM3, HepG2 cells or total tissue of FRNT and FRTT were lysed in 500 µl of 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM EDTA, 0.1% SDS, containing a mixture of protease inhibitors (aprotinin, PMSF, sodium ortho-vanadate) for protein extraction. Nuclear extracts were prepared as previously described (31). Equal amount of proteins were resolved on 11% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and probed with FXR, Aromatase, Cyclin D1 and Cyclin E antibodies. To ensure equal loading all membranes were stripped and incubated with anti Lamin B antibody for nuclear extracts or anti-GADPH and anti-β-actin antibodies for total extracts. The antigen-antibody complex was detected by incubation of the membranes with peroxidase-coupled goat anti-mouse, goat anti-rabbit, or donkey anti-goat IgG and revealed using the ECL System. The bands of interest were quantified by Scion Image laser densitometry scanning program.

Immunofluorescence- R2C cells seeded on glass coverslips were treated with CDCA 50 and 100 µM for 24 h, washed with PBS and then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Next, cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min, blocked with 5% BSA for 30 min, and incubated overnight with anti-aromatase antibody (1:100) in PBS overnight at 4°C. The day after the cells were washed three times with PBS and incubated with the secondary antibody anti-mouse IgG-FITC (1:200) for 1 h at room temperature. To check the specificity of the immunolabelling the primary antibody was replaced by normal mouse serum (negative control). Immunofluorescence analysis was carried out on a OLYMPUS BX51 microscope using a 40x objective.

Transient transfection assay- R2C cells were transiently transfected using the FuGENE 6 reagent with FXR reporter gene (FXRE-IR1) or XETL plasmid. A set of experiments was performed transfecting rat aromatase PII constructs p-1037, p-688 and p-688m. HeLa cells were transiently cotransfected with CYP17 gene promoter and FXR or SF-1 expression plasmids. After transfection, R2C and Hela cells

were treated with CDCA 50 µM for 24 h. Empty vectors were used to ensure that DNA concentrations were constant in each transfection. TK Renilla luciferase plasmid was used to normalize the efficiency of the transfection. 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.

RNA interference (RNAi)- R2C cells were transfected with RNA duplex of stealth RNAi targeted rat SHP mRNA sequence 5'-ACUGAACUGCUUGAAGACAUGCUUU-3' (Invitrogen, Carlsbad, CA, USA), or with a stealth RNAi control to a final concentration of 50nM using Lipofectamine 2000 as recommended by the manufacturer. After 5 h the transfection medium was changed with SFM and 24 after transfection the cells were exposed to CDCA 50µM or 100µM for further 24 h. These transfected cells were used to examine the effects of silencing SHP gene expression on the aromatase mRNA and protein content.

Electrophoretic mobility shift assay (EMSA)- Nuclear extracts from R2C cells were prepared as previously described (31). The probe was generated by annealing single-stranded oligonucleotides, labeled with [γ^{32}P] ATP using T4 polynucleotide kinase, and purified using Sephadex G50 spin columns. The DNA sequences used as probe or as cold competitors are the following (nucleotide motifs of interest are underlined and mutations are shown as lowercase letters): SF-1, CAGGACCTGAGTCTCCCA<u>AGGTCA</u>TCCTT GTTTGACTTGTA; mutated SF-1, TCTCCCA<u>AtaTCA</u>TCCTTGT. *In vitro* transcribed and translated SF-1 and FXR proteins were synthesized using the T7 polymerase in the rabbit reticulocyte lysate system. The protein-binding reactions were carried out in 20µL 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 R2C nuclear protein or an appropriate amount of SF-1 or FXR proteins 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. For experiments involving anti-SF-1 and anti-FXR antibodies, the reaction mixture was incubated with these antibodies at 4°C for 12 h before addition of

labeled probe. The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel in 0.25x Tris borate-EDTA for 3 h at 150 V.

Chromatin immunoprecipitation and ReChip assays- R2C cells were treated with CDCA 50 µM for 1 h and then cross-linked with 1% formaldehyde and sonicated. Supernatants were immunocleared with salmon sperm DNA/protein A agarose for 1 h at 4°C. The precleared chromatin was immunoprecipitated with specific anti FXR or anti polymerase II antibodies and re immunoprecipitated with anti SF-1 antibody. A normal mouse serum IgG was used as negative control. Pellets were washed as reported, eluted with elution buffer (1% SDS, 0.1 M NaHCO$_3$) and digested with proteinase K. DNA was obtained by phenol/chloroform/isoamyl alcohol extractions and precipitated with ethanol; 3 µl of each sample were used for PCR amplification with the following primers flanking SF-1 sequence present in the P450arom PII promoter region: 5'-ATGCACGTCACTCTACCCACTCAA -3' and 5'-TAGCACGCAAAGCAGTAGTTTGGC -3'. The amplification products were analyzed in a 2% agarose gel and visualized by ethidium bromide staining.

[^{3}H]thymidine incorporation- R2C cells were treated with CDCA 50 and 100 µM for 24h and 48 h. For the last 6 hours, [^{3}H]thymidine (1 µCi/ml) was added to the culture medium. After rinsing with PBS, the cells were washed once with 10% and three times with 5% trichloroacetic acid, lysed by adding 0.1 N NaOH and then incubated for 30 min at 37 °C. Thymidine incorporation was determined by scintillation counting. In a set of experiment R2C cells were transiently transfected with FXR-DN expression plasmid 24 h before starting with the same treatments mentioned above.

Anchorage-independent soft agar growth assays- R2C cells were plated in 4 ml of Ham/F-10 with 0.5% agarose and 5% charcoal-stripped FBS, in 0.7% agarose base in six-well plates. Two days after plating, media containing hormonal treatments (androst-4-ene-3,17-dione, CDCA), was added to the top layer, and the appropriate media was replaced every two days. After 14 days, 150 µl of MTT was added to each well and allowed to incubate at 37° for 4 h. Plates were then placed at 4°C overnight and colonies > 50 µm diameter from triplicate assays were counted. Data are the mean colony number

of three plates and representative of two independent experiments.

Statistical Analysis- Each datum point represents the mean ± S.D. of three different experiments. Statistical analysis was performed using ANOVA followed by Newman-Keuls testing to determine differences in means. $P<0.05$ was considered as statistically significant.

RESULTS

FXR expression in normal and tumor testicular cells. We first aimed to evaluate, by Western Blotting analysis, the expression of FXR receptor in Leydig normal (TM3) and tumor (R2C) cell lines and in testes tissue from younger (FRNT) and older (FRTT) Fisher rats. The latter group have a high incidence of spontaneous Leydig cell neoplasma (32,33), a phenomenon not observed in younger animals. Immunoblot analysis revealed the presence of a FXR-immunoreactive protein band at ~ 60kDa in all samples examined, particularly, FXR receptor seems to be more expressed in R2C cells with respect to TM3 and in FRTT with respect to its control FRNT (Fig. 1A). Human hepatocyte cells (HepG2) were used as a positive control for FXR expression. In R2C cells, incubation for 24 h with CDCA 50 and 100 μM, a natural ligand of FXR, increased the level of the receptor at both mRNA and protein levels (Figs. 1B & 1C). Moreover, to assess the ability of CDCA to transactivate endogenous FXR, we transiently transfected R2C cells with FXR responsive reporter gene (FXRE-IR1). As reported in Figure1D, CDCA treatment for 24 h induced a significant enhancement in transcriptional activation of the reporter plasmid.

Inhibitory effects of FXR agonists on aromatase expression in R2C cells. Starting from previous findings showing that FXR activation represses aromatase expression in breast cancer cells (13) we investigated the ability of FXR agonists to modulate aromatase enzyme in R2C cells which have been shown to have high aromatase expression and activity (27). Treatment with CDCA 50 and 100 μM for 24 h showed a down-regulation of aromatase mRNA and protein content in a dose related manner (Figs. 2A & 2B). Because CDCA may also exert FXR-independent effects (34), the influence of GW4064, a synthetic FXR agonist, on aromatase gene was also investigated. We observed that GW4064 (3μM) reduced aromatase mRNA and protein levels to a similar

order of magnitude as CDCA (Figs 2A & 2B). The down-regulatory effects of CDCA on the expression of aromatase was further confirmed by immunofluorescence analysis. The strong P450 aromatase immunoreactivity was detected in the cytoplasm as well as in the perinuclear region of untreated R2C cells and it was drastically decreased upon CDCA at the doses of 50 and 100 μM for 24 h (Fig. 2C). Next, we evaluated the effects of CDCA on aromatase enzymatic activity by tritiated water release assay. As reported in Figure 2D, exposure to CDCA 50 and 100 μM for 24 h reduced enzymatic activity in a dose dependent manner in R2C cells.

SHP is not involved in the down-regulatory effects induced by FXR ligand on aromatase. Induction of SHP expression is considered one of the canonical features of FXR transactivation. SHP has been shown to be expressed in the interstitial compartment of the adult testis, including steroidogenic Leydig cells (35).

We evidenced that SHP mRNA expression was significantly higher in R2C cells compared with very low levels detected in TM3 cell line, but administration of CDCA or GW4064 did not induce an increase of SHP mRNA in both cell lines (data not shown). However, to explore the role of SHP in CDCA-mediated repression of aromatase gene, we knocked SHP by siRNA. SHP mRNA expression was effectively silenced as revealed by RT-PCR after 24, 48 and 72 h of siRNA transfection (Fig. 3A). As shown in Figure 3B and 3C, silencing of the SHP gene failed to reverse the inhibition of aromatase expression induced by the specific FXR ligand in R2C cells ruling out any SHP involvement in the inhibitory effects of CDCA on aromatase expression.

CDCA down-regulates aromatase promoter activity through SF-1 site. The aforementioned observations led us to ascertain if the down-regulatory effects of CDCA on aromatase expression were due to its direct inhibitory influence in regulating aromatase gene transcriptional activity. Thus, we transiently transfected in R2C cells plasmids containing different segments of rat PII aromatase (Fig. 4A). A significant reduction of promoter activity was observed in cells transfected with p-1037 and p-688 exposed to CDCA 50 μM for 24 h. It is worth to note that construct p-688m bearing SF-1 mutated site displays significantly lower basal activity

compared with the p-688 plasmid while no inhibitory effects were noticeable upon CDCA treatment (Fig. 4B). This latter result highlights the importance of the SF-1 binding site in the regulation of aromatase expression in the R2C cells and suggests that the inhibitory effect of CDCA requires AGGTCA sequence motif.

To further demonstrate the functional interaction of FXR with SF-1 binding site, we transiently cotransfected Hela cells which do not express significant levels of SF-1 (36) with hCYP17 promoter construct containing multiple SF-1 response elements (37) with or without SF-1 plasmid in the presence of increasing amount of FXR expression plasmid. SF-1 expression vector strongly increased the CYP17 promoter activity, which was progressively reduced by FXR overexpression (Fig. 4C). We observed a similar results also in HeLa cells overexpressing FXR and treated with CDCA (data not shown). These data support the competitive role of FXR in binding SF-1 site.

FXR protein binds to SF-1 RE in vitro and in vivo. On the basis of the evidence that the inhibitory effect of CDCA on aromatase requires the crucial presence of SF-1 RE, EMSA experiments were performed using the SF-1 motif present in aromatase promoter as probe. We observed the formation of a complex in nuclear extract from R2C cells (Fig. 5A, *lane 1*), which was abrogated by 100 fold molar excess of unlabeled probe (Fig. 5A, *lane 2*) demonstrating the specificity of the DNA binding complex. This inhibition was not longer observed when mutated oligodeoxyribonucleotide was used as competitor (Fig. 5A, *lane 3*). CDCA 50 μM for 6 h induced an increase in DNA binding complex compared with control samples (Fig. 5A, *lane 4*). The inclusion of anti-SF-1 and anti-FXR antibodies in the reactions attenuated the specific bands suggesting the presence of SF-1 and FXR proteins in the complex (Fig. 5A, *lane 5 and 6*). Using SF-1 and FXR proteins transcribed and translated in vitro, we obtained complexes migrating at the same level as that of R2C nuclear extracts (Fig. 5A, *lane 7 and 8*).

The interaction of FXR with the aromatase gene promoter was further investigated by ChIP assay. Using specific antibody against FXR and RNA-POL II, formaldehyde cross-linked protein-chromatin complexes were immunoprecipitated from R2C cells cultured with or without CDCA 50 μM. The resulting genomic DNA precipitated by using anti-FXR

was then reprecipitated with the anti SF-1 antibody. The results analyzed by PCR indicated that FXR was weakly constitutively bound to the aromatase promoter in untreated cells and this recruitment was increased upon CDCA treatment, which was correlated with a reduced association of RNA polymerase II (Fig. 5B). Interestingly, by Re-ChIP assay, we observed upon CDCA stimulation a significant reduction in SF-1 recruitment to the aromatase promoter (Fig. 5B).

CDCA inhibits R2C cell proliferation through FXR activation. Finally, we evaluated the effect of CDCA on the growth of R2C cells by measuring changes in the rate of DNA synthesis (3H thymidine incorporation). As shown in Figure 6A, treatment with CDCA for 24 and 48 h reduced R2C cells proliferation in a dose and time dependent manner. The specific involvement of FXR in the antiproliferative response of R2C cells to CDCA was demonstrated by the evidence that such inhibitory effects were completely reversed in the presence of FXR dominant negative plasmid (Fig. 6B).

It is well known that aromatase overexpression in tumor Leydig cells leads to a consequent excess of in situ estradiol production that sustains tumor cell growth and proliferation (18). Since we demonstrated the ability of CDCA to down-regulate aromatase expression and activity in R2C cells, we wondered if CDCA was able to antagonize the effect of an aromatizable androgen androst-4-ene-3,17-dione (AD) on estradiol/ERα signaling in R2C cells. To this aim we performed transient transfection experiment using XETL plasmid, which carries firefly luciferase sequences under the control of an estrogen response element upstream of the thymidine kinase promoter. As shown in Figure 6C we observed that the exposure to CDCA (50 μM) per se did not elicit any changes in luciferase activity but it completely reversed XETL activation induced by AD. Moreover, we examined if CDCA was able to inhibit the effect of AD on R2C cell proliferation using two experimental approaches thymidine incorporation and anchorage independent soft agar growth assay. As expected, treatment with 100 nM of AD, through its conversion into estradiol, increased thymidine incorporation as well as the number of colonies present in soft agar (Figs 6D & 6E) concomitantly with an increased levels of cell cycle regulators cyclin D1 and cyclin E (Fig. 6F). All these events were

completely reversed by CDCA exposure (Figs 6D & 6E & 6F). These data demonstrated that FXR ligand, through an inhibition of aromatase activity, is able to reduce the estrogen dependent tumor Leydig cells proliferation.

DISCUSSION

FXR is highly expressed in the enterohepatic system where it drives bile acid absorption and secretion, lipid, glucid metabolism, and immunological response to intestinal bacterial overgrowth (2,4,38-40). In hepatocytes, activation of FXR causes both feedback inhibition of cholesterol 7a-hydroxylase (CYP7A1), the rate-limiting enzyme in bile acid biosynthesis from cholesterol, and activation of intestinal bile acid-binding protein (41). In addition, several observations suggest that FXR may also be involved in the control of steroid metabolism (13,42). Indeed, FXR activation results in the modulation of genes encoding androgen precursor-synthesizing enzymes, namely dehydroepiandrosterone sulfotransferase (SULT2A1), 5α-reductase and 3β-HSD (3β-hydroxysteroid dehydrogenase) in the liver (43,44). Recently, FXR was shown to inhibit androgen glucuronidation in prostatic cancer cell lines (45) and to suppress the activity of the aromatase in human breast cancer cells (13). The enzyme aromatase coded by the gene CYP19, converts androgens in estrogens and is involved in the progression and growth of various estrogen hormonal-induced neoplasms. For instance, overexpression of aromatase plays a significant role in the excessive estrogen production sustaining tumorogenesis in Leydig cells (18).

Here, we have documented that FXR is expressed in tissues of normal and tumor Fisher rat testis and in Leydig normal and tumor cell lines. In R2C cells, the FXR activators CDCA and GW4064, downregulate aromatase expression at both mRNA and protein level, together with the inhibition of its enzymatic activity.

One of the well-characterized mechanism by which FXR down-regulates gene expression is through induction of SHP (10) an atypical nuclear receptor lacking both a DNA-binding domain and the NH2-terminal ligand-independent activation domain (8). This receptor interacts with other nuclear receptors, including Peroxisome Proliferator Activated Receptor (PPAR), RXR, Estrogen Receptor (ER) and Liver Receptor Homolog-1 (LRH-1), preventing their activation of gene transcription (8-10). In preadipocytes of cancerous breast tisuue, LRH-1 can regulate via an alternate promoter (II) the expression of aromatase induced by prostaglandin E2 (46, 47). Moreover, SHP can inhibit LRH-1 induction of aromatase (48). LRH-1 is most homologous to SF1, which is essential for sex differentiation and development of gonads (28), since they share a highly conserved DBD (DBD>90% identity) and a moderately conserved LBD (LBD 56% identity). SHP is detected in the interstitial cells of the adult testis and its expression has been shown to be induced by FXR (35).

Our current study revealed that FXR activation does not induce SHP expression in Leydig tumor cells in which the inhibition of aromatase protein by CDCA occurs even when this nuclear receptor was knocked down. These results suggest that SHP is not required for the effect of FXR ligand to down-regulate aromatase expression, at least in R2C cells. On the basis of these observations, we focused our attention on the direct effect of FXR on the transcriptional activity of aromatase gene.

Distinctive tissues specific promoters are employed to direct the expression of aromatse mRNA driving from a single aromatase gene. The promoter located immediately upstream of the transcriptional initiation site (PII) regulates aromatase expression in rat Leydig, Sertoli and germ cells and in R2C Leydig tumor cells (23,24). A number of functional motifs have been identified in the PII aromatase promoter: three motifs resembling cAMP response elements (CRE) and an SF-1 binding site (27,28).

We demonstrated by functional studies, using constructs containing different 5'-deleted regions of rat PII aromatase promoter, that CDCA treatment induces a decreased transcriptional activity. The observed inhibitory effect of CDCA was abrogated when a promoter fusion containing a mutated SF-1 element was employed. These results clearly suggest that the integrity of SF-1 sequence is a prerequisite for the down-regulatory effects of FXR ligand on aromatase promoter activity. These findings raise the possibility that FXR and SF-1 are competing for binding to a common site within this regulatory region. This assumption is further supported by the observation that FXR expression vector is able to abrogate the

induction of SF-1 on human CYP17 promoter which contains multiple SF-1 response elements. As a transcription factor, FXR binds to a specific consensus sequence (inverted repeat of 2 AGGTCA half-sites) either as a monomer or as a heterodimer with a common partner for NRs, as RXR to regulate the expression of various genes (4).

Location of an AGGTCA sequence at the -90 position supports a possible binding of FXR to this promoter region, which we verified by EMSA experiments. Nuclear extracts from R2C cells treated with CDCA revealed an increase in DNA binding complex which was immunodepleted by both anti-SF-1 and anti-FXR antibodies suggesting how the two proteins are able to bind the AGGTCA sequence located in PII aromatase promoter. In addition, the *in vivo* iinteraction between FXR and aromatase promoter was further supported by ChIP assay, where upon CDCA treatment we observed a reduced recruitment of RNA-POLII to this promoter addressing a negative transcriptional regulation mediated by FXR. All together these data suggest that FXR is able to compete with SF-1 in binding to a common sequence within the PII promoter of aromatase interfering negatively with its activity.

Finally, in our study we demonstrated that FXR activator CDCA induces growth inhibition in R2C cells which was reversed in the presence of FXR dominant negative addressing a FXR dependency of this event.

However it is worth to mention, on the basis of our recent findings, that aromatase overexpression, in Leydig tumor cells, determines an excessive local estradiol production that is able to stimulate the expression of genes involved in cell cycle regulation sustaining cell proliferation (20).

Here, we evidenced the ability of CDCA to reverse the stimulatory effects of an aromatizable androgen androst-4-ene-3,17-dione (AD) at three different levels: 1) E2/ERα signaling; 2) an anchorage dependent and independent R2C cell growth proliferation; 3) expression of cell cycle regulators cyclin D1 and cyclin E. The latter finding bring us to emphasize how the intrinsic property of FXR to inhibit R2C cell proliferation sound to be not linked to any substantial effect on cyclin D1 and cyclin E expression.

In conclusion, our results elucidate, for the first time, a new molecular mechanism through which FXR antagonizes estrogen signalling and inhibits Leydig tumor growth and progression addressing FXR ligands as potential pharmacological tools to be implemented in the novel strategies for testicular tumoral treatment.

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FOOTNOTES

The abbreviation used are: FXR, Farnesoide X Receptor; RXR, Retinoid X Receptor; FXREs, FXR response element; SHP, Small Heterodimer Partner; E2, 17β-estradiol; CDCA, chenodeoxycholic acid; DMEM/F12, Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; HS, horse serum; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; RNAi, RNA interference; EMSA, Electrophoretic mobility shift assay; FXRE-IR1,FXR responsive reporter gene; AD, androst-4-ene-3,17-dione; PPAR, Peroxisome Proliferator Activated Receptor; ER, Estrogen Receptor; LRH-1, Liver Receptor Homolog-1; CRE, cAMP response elements.

FIGURE LEGENDS

Fig. 1. FXR expression and activation in R2C cells. *A*, Western blot analysis of FXR was done on 50 μg of total proteins extracted from normal (TM3), tumor Leydig cells (R2C) and human hepatocytes cells (HepG2) or from tissues of normal (FRNT) and tumor (FRTT) Fisher rat testes. β-actin was used as a loading control. *B*, Total RNA was extracted from R2C cells treated with vehicle (-) or CDCA 50 and 100 μM for 24 h and reverse transcribed. cDNA was subjected to PCR using primers specific for FXR or L19 (ribosomal protein). NC: negative control, RNA sample without the addition of reverse transcriptase. The histograms represent the means ± S.D. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control, which was assumed to be 100%. *$p<0.05$, **$p<0.01$ compared to vehicle. *C*, Nuclear proteins were extracted from R2C cells treated with vehicle (-) or CDCA 50 and 100 μM for 24 h and then western blotting analysis was performed using anti-FXR antibody. Lamin B was used as loading control. The histograms represent the means ± S.D. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control, which was assumed to be 100%. *$p<0.05$ compared to vehicle. *D*, R2C cells were transiently transfected with FXR reporter gene (FXRE-IR1) and treated with vehicle (-) or CDCA 50 μM and 100 μM for 24 h. The values represent the means ± S.D. of three different experiments performed in triplicate. * $p<0.01$ compared to vehicle.

Fig. 2. Effects of CDCA on aromatase expression and activity in R2C cells. *A*, Total RNA was extracted from R2C cells treated with vehicle (-), CDCA 50 and 100 μM or GW4064 3 μM for 24 h and reverse transcribed. cDNA was subjected to PCR using primers specific for P450 aromatase or L19. NC: negative control, RNA sample without the addition of reverse transcriptase. The histograms represent the means ± S.D. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control which was assumed to be 100%. *$p<0.05$, **$p<0.01$ compared to vehicle. *B*, Total proteins extracted from R2C cells treated with vehicle (-), CDCA 50 and 100 μM or GW4064 3 μM for 24 h were used for immunoblot analysis of aromatase. GAPDH was used as a loading control. The histograms represent the means ± S.D. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control which was assumed to be 100%. *$p<0.01$ compared to vehicle. *C*, R2C cells were treated with vehicle (-) or CDCA 50 and 100 μM for 24 h and aromatase expression was determined by immunofluorescence analysis. DAPI staining was used to visualized the cell nucleus. Each experiment is representative of at last 4 tests. *D*,

R2C were cultured in the presence of vehicle (-) or 50 and 100 μM of CDCA for 24 h. Aromatase activity was performed as described in Materials and Methods. The results obtained were expressed as pmol [^{3}H] H$_2$O/h release and were normalized for mg protein (pmol/mg proteins/h). The values represent the means ± S.D. of three different experiments each performed with triplicate samples. *p<0.01 compared to vehicle.
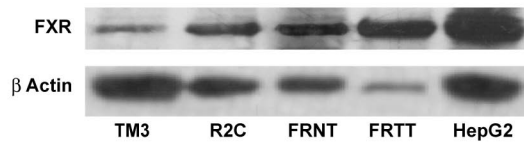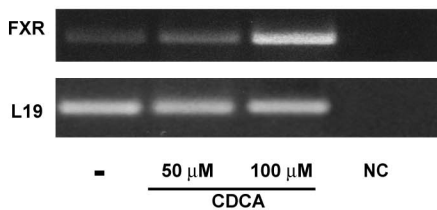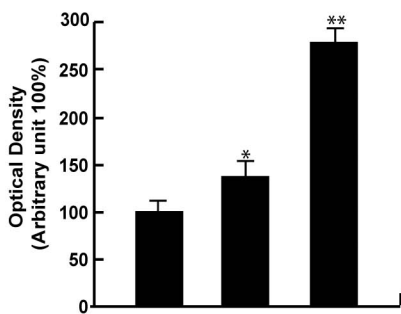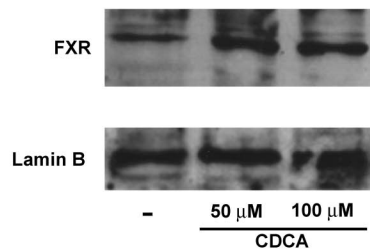
Fig. 3. SHP is not involved in CDCA-mediated down-regulation of aromatase. *A*, SHP mRNA expression in R2C cells which were not transfected (–) or transfected with RNA interference (RNAi) targeted rat SHP mRNA sequence as reported in Materials and Methods for 24, 48 and 72 h. L19 was used as loading control. NC: negative control, RNA sample without the addition of reverse transcriptase. The histograms represent the means ± S.D. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control, which was assumed to be 100%. *p<0.01 compared to vehicle. *B*, R2C cells were transfected with control siRNA or SHP siRNA for 24 h, and then treated with vehicle (-) or CDCA 50 and 100 μM for 24 h. Total RNA was extracted and RT-PCR analysis was performed to evaluate the expression of aromatase. L19 was used as loading control. NC: negative control, RNA sample without the addition of reverse transcriptase. The histograms represent the means ± S.D. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control, which was assumed to be 100%. *p<0.01 compared to vehicle. *C*, In the same experimental condition of B, total proteins were extracted and western blotting analysis was performed. GAPDH was used as loading control. The histograms represent the means ± S.D. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control which was assumed to be 100%. * p<0.05, ** p<0.01 compared to vehicle.
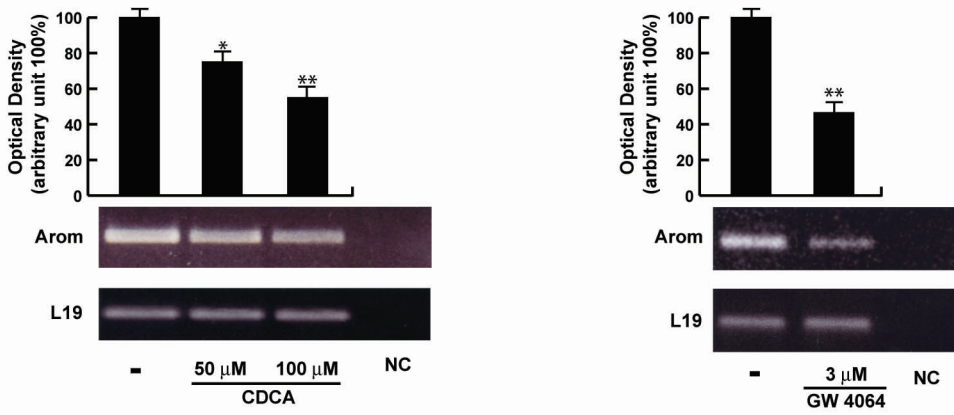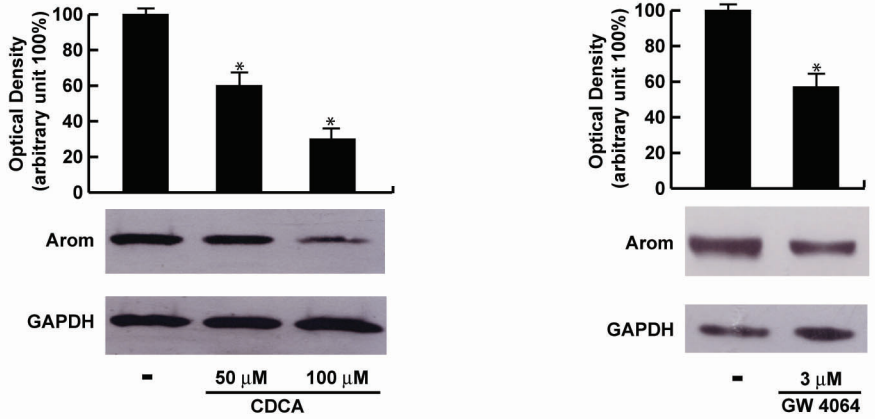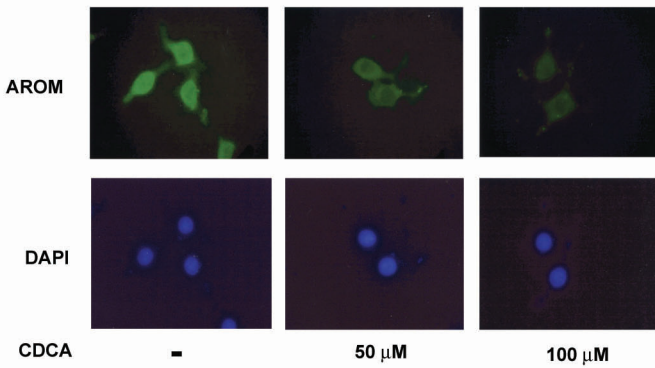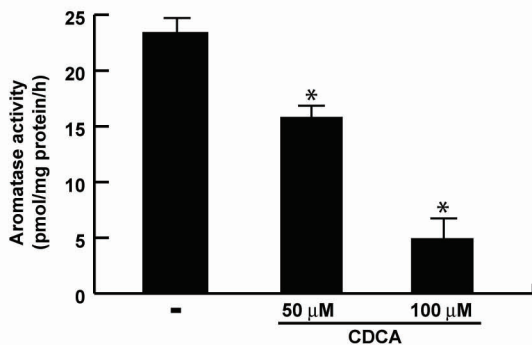
Fig. 4. Functional interaction between FXR and SF-1 site. *A*, Schematic map of the P450arom proximal promoter PII constructs used in this study. All of the promoter constructs contain the same 3' boundary (+94). The 5' boundaries of the promoter fragments varied from -1037 to -688. Three putative CRE motifs (5'-CRE at -335; 3'-CRE at -231; XCRE at -169) are indicated as square. The AGGTCA site (SF-1 RE at-90) is indicated as rectangle. A mutated SF-1 binding site (SF-1 mut) is present in p-688m (black rectangle). *B*, Aromatase transcriptional activity of R2C cells transfected with promoter constructs are shown. After transfection, cells were treated in the presence of vehicle (-) or CDCA 50 μM for 24h. These results represent the means ± S.D. of three different experiments performed in triplicate. *p<0.01 with respect to the vehicle, **p<0.01 with respect to the the control of p688. *C*, HeLa cells were transiently cotransfected with CYP17 promoter and with SF-1 plasmid or empty vector (EV) in the presence of increasing amount of FXR expression plasmid. These results represent the means ± S.D. of three different experiments performed in triplicate. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. *p<0.01 with respect to the EV, **p<0.01 with respect to the SF-1 alone.
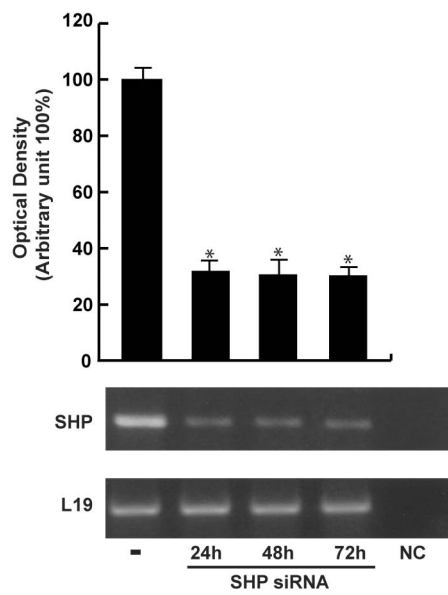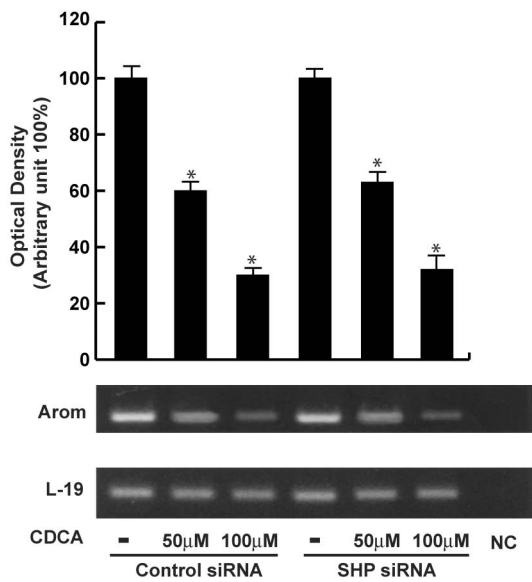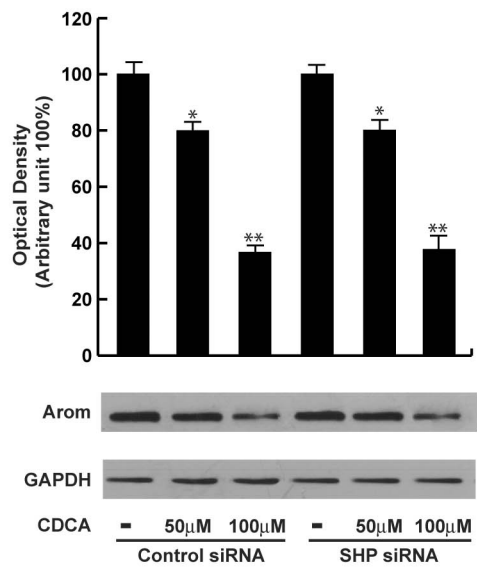
Fig. 5. FXR binds to SF-1 site within aromatase promoter region. *A*, Nuclear extract from R2C cells were incubated with a double-stranded SF-1-specific sequence probe labeled with [γ^{32}P]ATP and subjected to electrophoresis in a 6% polyacrylamide gel (lane 1). Competition experiments were performed adding as competitor a 100-fold molar excess of unlabeled probe (lane 2) or a 100-fold molar excess of unlabeled oligonucleotide containing a mutated SF-1 RE (lane 3). Lane 4, nuclear extracts from CDCA (50 μM) treated R2C cells. Lanes 5 and 6, CDCA-treated nuclear extracts were incubated with anti-SF-1 or anti-FXR antibodies respectively. We used as positive controls transcribed and translated in vitro SF-1 (lane 7) and FXR (lane 8) proteins. Lane 9 contains probe alone. *B*, R2C cells were treated in the presence of vehicle (-) or CDCA 50 μM for 1 hour, then cross-linked with formaldehyde, and lysed. The precleared chromatin was immunoprecipitated with anti-FXR, and anti-RNA Pol II antibodies and normal mouse serum (NC) as negative control. Chromatin immunoprecipitated with the anti-FXR antibody was re-immunoprecipitated with anti-SF-1 antibody. The PII promoter sequence containing SF-1 site was detected by PCR with specific primers as detailed in the Materials and Methods. To determine input DNA, the PII promoter fragment was
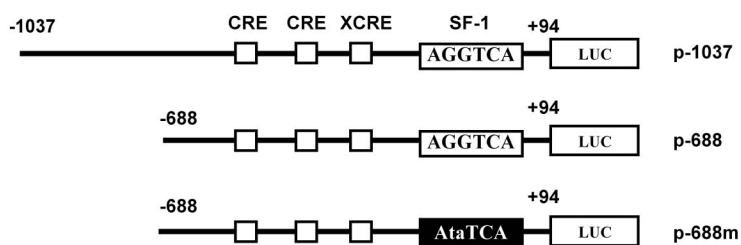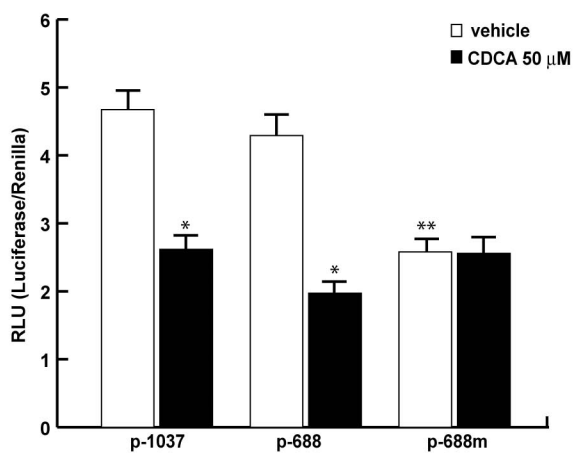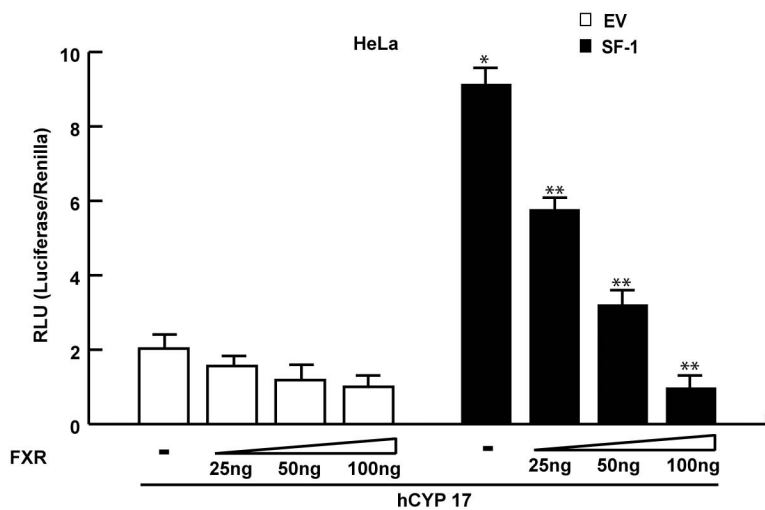
amplified from 30 μl initial preparations of soluble chromatin before immunoprecipitations. Similar results were obtained in multiple independent experiments.

Fig. 6. CDCA effects on R2C cell proliferation. *A*, R2C cells were treated with vehicle (-) or CDCA 50 and 100 μM for 24 and 48 h or *B*, transiently transfected with FXR dominant negative (FXR-DN) for 24 h, and then treated as above reported. Thymidine incorporation assay was performed. The results represent the means ± S.D. of three different experiments each performed with triplicate samples, and expressed as percentage of growth vs control which was assumed to be 100%. *C*, R2C cells were transiently transfected with XETL promoter plasmid. Cells were treated with CDCA 50 μM in the presence or not of androst-4-ene-3,17-dione (AD) 100 nM for 24h. These results represent the means ± S.D. of three different experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. *p<0.01 with respect to the vehicle. **p<0.01 CDCA+AD treated vs AD alone. *D*, R2C cells were treated with androst-4-ene-3,17-dione (AD) 100nM in the presence or not of CDCA 50 μM for 24h. Thymidine incorporation assay was performed. The results represent the means ± S.D. of three different experiments each performed with triplicate samples. *p<0.01 AD treated compared to vehicle. **p<0.01 CDCA+AD treated vs AD alone. *E*, R2C cells were seeded (10,000/well) in 0.5% agarose and the treated as described above. Cells were allowed to grow for 14 days and then the number of colonies >50μm were quantified and the results graphed. The results represent the means ± S.D. of three different experiments each performed with triplicate samples. *p<0.01 AD treated compared to vehicle. **p<0.01 CDCA+AD treated vs AD alone. *F*, Total proteins extracted from R2C cells treated with vehicle (-), Ad 100 nM, CDCA 50 μM and AD*CDCA for 24 h were used for immunoblot analysis of cyclin D1 and cyclin E. β-actin was used as a loading control. The histograms represent the means ± S.D. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control which was assumed to be 100%. *p<0.01 AD treated compared to vehicle. **p<0.01 CDCA+AD treated vs AD alone.

A

FXR

β Actin

TM3   R2C   FRNT   FRTT   HepG2

B

Optical Density (Arbitrary unit 100%)

300
250
200
150
100
50
0

**

*

FXR

L19

−   50 μM   100 μM   NC
       CDCA

C

Optical Density (Arbitrary unit 100%)

200

150

100

50

0

*   *

FXR

Lamin B

−   50 μM   100 μM
       CDCA

D

RLU (Luciferase/Renilla)

0.5

0.4

0.3

0.2

0.1

0.0

*

*

−   50 μM   100 μM
       CDCA
     FXRE IR1

Fig.1

Fig. 2

A

Optical Density (Arbitrary unit 100%)

SHP

L19

-    24h 48h 72h   NC

SHP siRNA

B

Optical Density (Arbitrary unit 100%)

Arom

L-19

CDCA   -   50μM 100μM   -   50μM 100μM   NC

Control siRNA   SHP siRNA

C

Optical Density (Arbitrary unit 100%)

Arom

GAPDH

CDCA   -   50μM 100μM   -   50μM 100μM

Control siRNA   SHP siRNA

Fig. 3

Fig. 4

A

	1	2	3	4	5	6	7	8	9
Nuclear extract	+	+	+	+	+	+	–	–	–
Competitor (100 fold excess)	–	+	Mut	–	–	–	–	–	–
CDCA 50 μM	–	–	–	+	+	+	–	–	–
Ab SF-1	–	–	–	–	+	–	–	–	–
Ab FXR	–	–	–	–	–	+	–	–	–
SF-1 protein	–	–	–	–	–	–	+	–	–
FXR protein	–	–	–	–	–	–	–	+	–

B

ChIP: FXR

ChIP: RNA pol II

ChIP: FXR / ReChIP: SF-1

Input

-      CDCA 50 μM   NC

Fig. 5

A

Thymidine uptake (%)

CDCA   −   50 μM   100 μM   50 μM   100 μM
         24h   48h

B

Thymidine uptake (%)

CDCA   −   50 μM   100 μM   50 μM   100 μM
         24h   48h
              FXR DN

C

XETL

RLU (Luciferase/Renilla)

−   CDCA 50μM   −   CDCA 50μM
                    AD 100nM

D

Thymidine uptake (%)

−   −   CDCA 50 μM
     AD 100nM

E

Soft Agar

Number of Colonies

−   −   CDCA 50μM
     AD 100nM

F

■ Cyclin D1
□ Cyclin E

Optical Density (arbitrary unit 100%)

Cyclin D1
Cyclin E
β Actin

−   AD 100 nM   CDCA 50 μM   AD + CDCA

Fig. 6

Rapid Estradiol/ERα Signaling Enhances Aromatase Enzymatic Activity in Breast Cancer Cells

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In situ estrogen production by aromatase conversion from androgens plays an important role in breast tumor promotion. Here, we show that 17β-estradiol (E_2) can rapidly enhance aromatase enzymatic activity through an increase of aromatase protein phosphorylation in breast cancer cell lines. *In vivo* labeling experiments and site-directed mutagenesis studies demonstrated that phosphorylation of the 361-tyrosine residue is crucial in the up-regulation of aromatase activity under E_2 exposure. Our results demonstrated a direct involvement of nonreceptor tyrosine-kinase c-Src in E_2-stimulated aromatase activity because inhibition of its signaling abrogated the up-regulatory effects induced by E_2 on aromatase activity as well as phosphorylation of aromatase protein. In addition, from our data it emerges that aromatase is a target of cross talk between growth factor receptors and estrogen receptor α signaling. These findings show, for the first time, that tyrosine phosphorylation processes play a key role in the rapid changes induced by E_2 in aromatase enzymatic activity, revealing the existence of a short nongenomic autocrine loop between E_2 and aromatase in breast cancer cells. (**Molecular Endocrinology** 23: 1634–1645, 2009)

Estrogens play a crucial role in the development and progression of breast cancer. The biosynthesis of estrogens from androgens is catalyzed by the enzyme complex termed "aromatase," which is composed of two polypeptides, an ubiquitous nonspecific flavoprotein, reduced nicotinamide adenine dinucleotide phosphate-cytochrome P450 reductase, and a specific microsomial form of cytochrome $P450_{arom}$ encoded by the cytochrome P450 (CYP)19 gene (1).

Aromatase expression in breast cancer tissue as well as in breast cancer cell lines has been shown by enzyme activity measurement, immunocytochemistry, and RT-PCR analysis (2–4). Cell culture (5) and nude mouse experiments (6) using aromatase-transfected MCF-7 cells have shown that aromatase expressed in breast cancer cells can promote tumor growth in both an autocrine and a paracrine manner. In addition, overexpression of aromatase in mammary gland of transgenic mice causes pre-

malignant lesions, such as atypical ductal hyperplasia (7, 8). $P450_{arom}$ is found to be expressed at higher levels in cancer than in normal breast tissue (9, 10). Thus, induction of aromatase within the breast tumor can result in high levels of 17β-estradiol (E_2) production that, in turn, stimulate tumor growth. Indeed, intratumoral aromatase of breast carcinoma has been extensively studied for its potential clinical significance as a target for endocrine therapy using aromatase inhibitors (11, 12).

It is well known that aromatase is regulated at the transcriptional level through the alternative use of tissue-specific promoters (13, 14), whereas posttranscriptional regulation of this protein remains poorly understood. Balthazart *et al.* (15, 16) demonstrated that phosphatases modulate the activity of brain aromatase and that the phosphorylation status of the enzyme is critical for its activity. In addition, several studies have suggested that aromatase activity could be modulated at the posttrans-

lation level in different cell types upon the addition of growth factors and kinase inhibitors (17–20). Recently, Miller *et al.* (21) demonstrated that aromatase serine (S) 118 is a potential phosphorylation site in mammalian cells, and mutation of S118 blocked phosphorylation and increased aromatase activity.

The classic effects of estrogens are mediated through binding to estrogen receptors (ERα and ERβ) and stimulation of transcription at nuclear levels. Recently, the nongenomic actions of estrogens have been reported through binding to membrane-associated ER (22, 23), which resides in or near the cell membrane and cross talks with the signal transduction pathways, including the c-Src/Ras/MAPK and cAMP pathway (24–26). Signaling from membrane ER induces posttranslational modification of many proteins. This includes the phosphorylation and regulation of enzymes, such as kinases or phosphatases, that impact cell physiology (27).

In the present study we demonstrated, in estrogen-dependent MCF-7 breast cancer epithelial cells, that 17β-estradiol (E$_2$) is able to rapidly up-regulate aromatase enzymatic activity, and this may occur through an enhanced tyrosine phosphorylation levels of aromatase protein. Our results provide a new insight into the regulation of aromatase through posttranscriptional modulation in human breast cancer cells.

Results

Rapid increase of aromatase activity induced by E$_2$ treatment

We first aimed to evaluate the effects of estrogens on aromatase activity by tritiated water assay in MCF-7 cells incubated for 10 and 120 min in the presence of 0.1, 1, and 10 nM of E$_2$. As reported in Fig. 1A, E$_2$ enhanced enzymatic activity at both times and doses investigated, even though to a higher extent under 1 and 10 nM E$_2$. The E$_2$ induction was also observed in MCF-7 cells transiently transfected with the aromatase gene (CYP19), that displayed a 6-fold increase in enzymatic activity (95.36 ± 0.92 fmol/h · mg protein) compared with parental MCF-7 cells (15.16 ± 0.47 fmol/h · mg protein) (Fig. 1B). To evaluate whether the E$_2$ effects on aromatase activity were transient, MCF-7 cells were treated with E$_2$ 1 nM for different times (10 min; 6, 12, and 24 h; and 2, 4, and 6 d). We found that aromatase activity doubled upon E$_2$ exposure ranging from 10 min to 12 h and remained moderately high up to 6 d (data not shown). The ER antagonists, ICI 182,780 (ICI) and tamoxifen (TAM) were able to abrogate the up-regulation induced by E$_2$, whereas these treatments alone had no agonist activity (Fig. 1C). This

suggests that estrogens can increase aromatase activity by binding to ERs.

It has been shown that the rapid actions of estrogen could be mediated by membrane-associated ER (22, 23). Thus, we cotransfected in ER-negative Chinese hamster ovary (CHO) cells CYP19 vector with ERα wild-type (wt) plasmid or membrane ERα (mERα) construct. The mERα construct consists solely of the AF-2/ligand binding domain (E) of ERα cloned into the membrane-enhanced cyan fluorescent protein (Mem-ECFP) vector that encodes a fusion protein called GAP-43 (N-terminal 20 amino acids of neuromodulin) containing a signal that targets this portion of the receptor to the plasma membrane (28, 29). This construct is a well-established mutant ERα able to discriminate the nongenomic to the genomic actions of E$_2$. As reported by Razandi *et al.* (30), expression of the E domain of ERα to the plasma membrane allowed the activation of ERK but did not result in the transactivation of an estrogen response element/luciferase reporter by E$_2$ treatment. As revealed in Fig. 1D, 1 and 10 nM E$_2$ for 10 min up-regulated enzymatic aromatase activity in CHO ectopically expressing mERα as well as ERα wt plasmids, suggesting that the expression of mERα is sufficient for E$_2$ induction.

We also evaluated the effects of E$_2$ on aromatase activity in ER-negative breast cancer cell line SKBR3 and in R2C rat Leydig tumor cells that express ERα and high levels of aromatase protein. No changes were observed in SKBR3 parental cells whereas E$_2$ treatment enhanced aromatase activity both in SKBR3, ectopically expressing ERα, and in R2C cells (Fig. 1, E and F). R2C cells displayed an elevated aromatase activity that is 1 order of magnitude higher than that detected in the other cell types investigated. This may probably explain the lack of E$_2$ dose-dependent stimulation of aromatase activity in the E$_2$ range concentration tested.

Because we observed an enhanced aromatase activity under E$_2$ treatment in a number of different cell lines, it could suggest that this regulation may underlie a general mechanism not related to cell specificity. However, this effect assumes a great importance in breast cancer cells, which are strongly dependent on estrogens for their growth.

E$_2$ increases tyrosine phosphorylation levels of aromatase protein

One mechanism by which E$_2$ might increase aromatase activity would be an enhancement in the transcription of aromatase mRNA and thus in the concentration of the enzyme. We performed RT-PCR and Western blotting analysis in MCF-7 cells treated with 1 nM E$_2$ for 10 and 120 min. We did not observe any change on aromatase
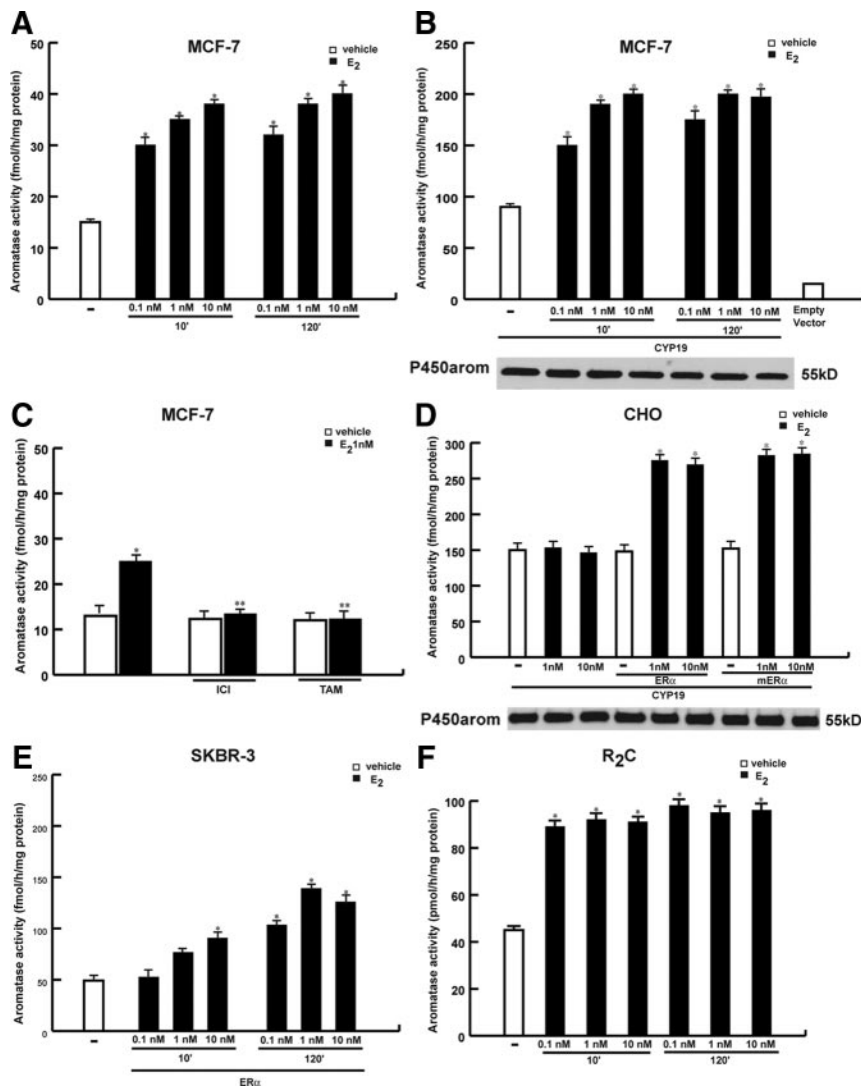
FIG. 1. Rapid effects of E_2 on aromatase activity. MCF-7 (A) or MCF-7 cells transiently transfected with CYP19 vector (B) were treated with vehicle (-) or 0.1, 1, and 10 nM E_2 for 10 and 120 min. Western blotting shows the expression of CYP19 vector used in the experiment. C, MCF-7 cells were pretreated with 1 μM ICI 182,780 (ICI) and 1 μM TAM for 30 min and then exposed or not to 1 nM E_2 for 10 min. D, CHO cells were transiently transfected with CYP19 vector and ERα wt or mERα or empty vector and treated with 1 and 10 nM E_2 for 10 min. Western blotting shows the expression of CYP19 vector used in the experiment. E, SKBR3 cells transiently transfected with ERα wt, and R2C cells (F) cells were treated as reported. Aromatase activity was performed as described in *Materials and Methods*. Empty vector: aromatase activity measured in cells transfected with pUC19 vector. The values represent the means ± SE of three different experiments, each performed with triplicate samples. *, $P < 0.01$ compared with vehicle; **, $P < 0.01$ compared with E_2-treated samples.

mRNA and protein level compared with the control (supplemental Fig. 1, A and B, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). These results suggest that rapid changes in E_2-induced aromatase enzymatic activity are due to ERα action at the nongenomic level.

It is well known that the activity of many enzymes can be modulated rapidly by phosphorylation processes inducing conformational changes in the enzyme molecule. Previous analyses of the aromatase gene in a variety of mammalian and avian species demonstrated several con-

sensus sites of phosphorylation on aromatase cDNA and deduced amino acid sequence (31–33). Thus, to evaluate the phosphorylation status of aromatase protein, we performed *in vivo* labeling experiment in MCF-7 cells transiently transfected with His$_6$-arom, a plasmid coding for the entire human aromatase sequence with six tandem histidine residues on the carboxyl terminus, as described in *Materials and Methods*. The His$_6$-tagged protein had the advantage to allow a higher yield of purified aromatase due to the specificity of Ni-NTA (nitrilotriacetic acid) agarose beads and avoid interference with the band of 55 kDa from heavy chains of antibodies used for immunoprecipitation. MCF-7 cells were transiently transfected with His$_6$-arom, metabolically labeled with radioactive orthophosphate and then treated with 1 and 10 nM E_2 for 10 min. Equal amounts of proteins were incubated with Ni-NTA agarose beads for isolation of recombinant P450$_{arom}$, and the eluates were run on SDS-PAGE. Autoradiography of the membrane revealed that aromatase protein was efficiently phosphorylated *in vivo* upon E_2 treatment (Fig. 2A). The membrane then was probed with an anti-aromatase antibody to visualize the input levels of the samples.

To determine which type of amino acid is phosphorylated, we performed Western blotting analysis with antibodies directed against phosphotyrosine and phosphoserine residues using cell lysates from MCF-7 cells transfected with His$_6$-arom and treated with 0.1, 1, and 10 nM E_2 for 10 min. Our results showed that E_2 was able to increase phosphotyrosine levels of purified aromatase protein, whereas no changes were detectable on serine phosphorylation status (Fig. 2B). This enhancement on tyrosine phosphorylation of aromatase was ERα-dependent because pretreatment with ICI reduced the E_2-associated tyrosine phosphorylation (Fig. 2C). We obtained similar results after pretreatment with TAM (data not shown). Moreover, in the presence of a specific inhibitor of tyrosine phosphatases, sodium orthovanadate, we observed an increase of aro-
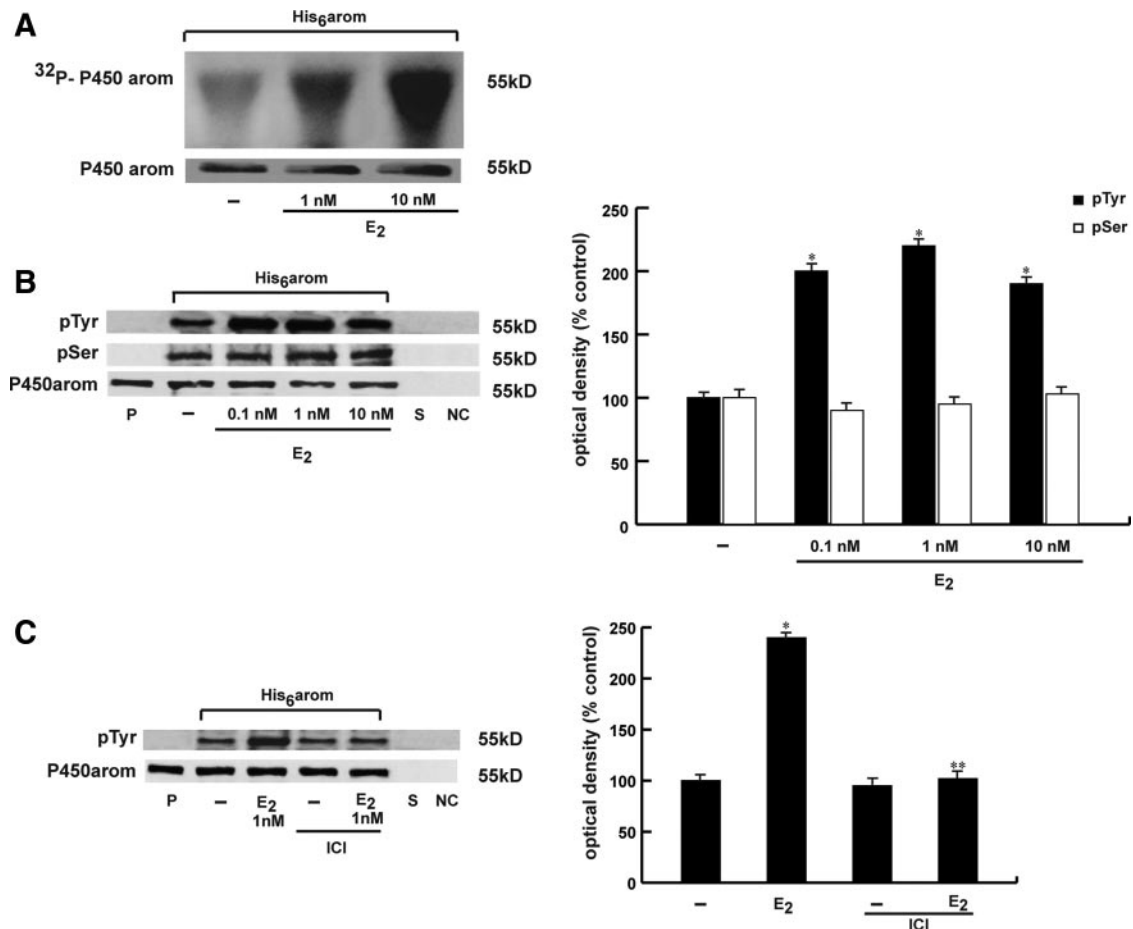
FIG. 2. Tyrosine phosphorylation levels of aromatase protein is enhanced by E_2. A, MCF-7 cells were transiently transfected with His$_6$-arom, labeled with [^{32}P]orthophosphate, and then treated with vehicle (-) or 1 and 10 nm E_2 for 10 min. Aromatase was purified using Ni-NTA agarose beads after which the complexes were resolved in SDS-PAGE. The *top panel* shows autoradiography of the SDS-PAGE, and the *bottom panel* shows immunoblot analysis with antiaromatase antibody (P450$_{arom}$) as a control for expressed protein. B, MCF-7 cells transiently transfected with His$_6$-arom were treated with vehicle (-) or 0.1, 1, and 10 nm E_2 for 10 min. Aromatase was purified using Ni-NTA agarose beads after which the complexes were resolved in SDS-PAGE. Immunoblotting was performed using the antiphosphotyrosine (pTyr) and antiphosphoserine (pSer) antibodies. C, MCF-7 cells transiently transfected with His$_6$-arom were pretreated with 1 μM ICI and then exposed or not to 1 nm E_2 for 10 min. To verify equal loading, the membrane was probed with antiaromatase antibody. Microsomal extracts from placenta (P) were used as positive control. As negative controls we used the supernatant removed after incubation with Ni-NTA agarose beads (S) and vector-transfected MCF-7 cell lysates incubated with Ni-NTA agarose beads (NC). The *side histograms* represent the means ± SE of three separate experiments in which band intensities were evaluated in terms of OD density arbitrary units and expressed as percentages of the control, which was assumed to be 100%. *, $P < 0.01$ compared with vehicle; **, $P < 0.01$ compared with E_2-treated samples. kD, Kilodaltons.

matase enzymatic activity as well as enhanced phosphotyrosine levels of purified aromatase protein, which were slightly increased by E_2 cotreatment (data not show).

All these data indicate that E_2 exposure is able to rapidly phosphorylate *in vivo* aromatase protein and increase tyrosine phosphorylation status of the enzyme.

Identification of tyrosine residue involved in the E_2 activation

Consensus phosphorylation sites of human aromatase protein were analyzed using the public domain software (netphos 2.0 server) available on the web site of the Center for Biological Sequence Analysis at http://www.cbs.dtu.dk. Based on a deduced amino acid sequence and on a previously encoded database of potential phosphorylation

sites, this program identifies all serine, threonine, and tyrosine residues in the protein that could potentially be phosphorylated (34). The program also provides for each residue a phosphorylation score ranging from 0 to 1.0 the value of which was proportional to the probability that the residue could, in fact, be phosphorylated *in vivo*. A score equal or larger to 0.5 was considered to predict a likely phosphorylation consensus site (35). The netphos 2.0 program identified four of the 17 tyrosine residues. The residues located at positions 184 and 361 of the human aromatase sequence have the highest consensus scores (0.992 and 0.976, respectively). The position at 361 corresponds to the residue present in the steroid-binding domain, an important functional domain of human aromatase, and notably this residue and its immedi-
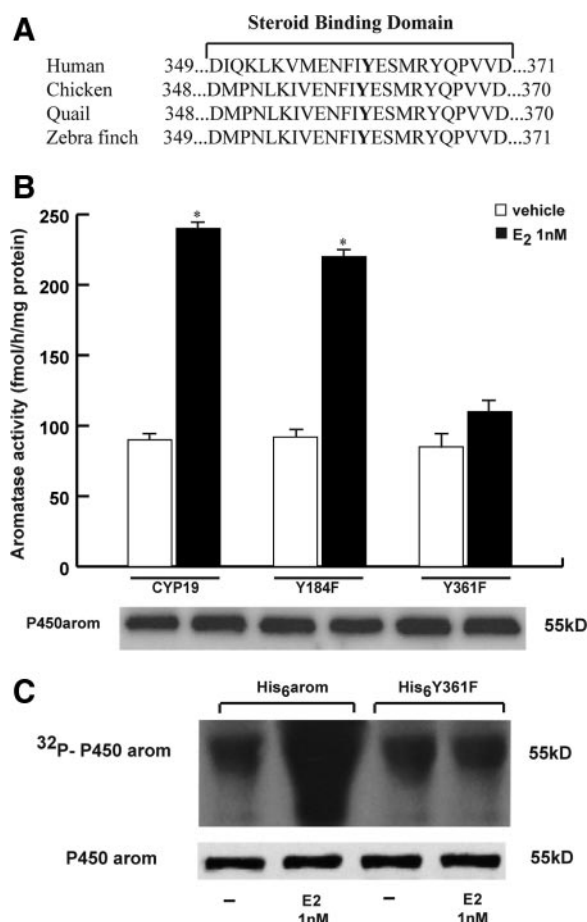
FIG. 3. Specific tyrosine residue involved in aromatase activation. A, Comparison of the amino acid sequences of steroid binding domain of aromatase in human, chicken, quail, and zebra finch. B, MCF-7 cells were transfected with CYP19 vector or Y184F or Y361F mutants, treated with vehicle or 1 nM E_2 for 10 min after which aromatase activity was performed. The values represent the means ± SE of three different experiments, each performed with triplicate samples. *, $P <$ 0.01 compared with vehicle. Western blotting shows the expression of DNA vectors used in the experiments. C, MCF-7 cells were transiently transfected with His$_6$-arom or His$_6$-Y361F, labeled with [^{32}P]orthophosphate, and then treated with vehicle (−) or 1 nM E_2 for 10 min. Aromatase was purified using Ni-NTA agarose beads after which the complexes were resolved in SDS-PAGE. The *top panel* shows autoradiography of the SDS-PAGE, and the *bottom panel* shows immunoblot analysis with antiaromatase antibody (P450$_{arom}$) as a control for expressed protein.

ate environment are well conserved across species (Fig. 3A). Thus, to address the location of the potential phosphorylation site within the aromatase protein, we mutated the conserved tyrosine (Y) at residue 361 as well as the one at 184 to phenylalanine (F) to create two different constructs, Y184F and Y361F. These plasmids were used in an aromatase enzymatic assay. We found that Y361F and Y184F mutation didn't affect the basal levels of aromatase activity. E_2 increased aromatase activity in cells transfected with Y184F mutant expression vector but had no effect in the presence of Y361F expression plasmid (Fig. 3B). To further confirm these results, we performed

an *in vivo* labeling experiment in MCF-7 cells transiently transfected with either His$_6$-arom or His$_6$-Y361F constructs. We found that the His$_6$-Y361F mutated construct was not efficiently phosphorylated *in vivo* upon E_2 treatment (Fig. 3C). These data directly prove that phosphorylation of the 361 tyrosine residue is crucial in the upregulation of aromatase activity and its phosphorylation under E_2 stimulation. This last result led us to question which specific cellular kinase might be responsible for phosphorylation of aromatase at the Y361 site. The research for consensus sequences corresponding to the protein kinases pointed to the Y361 site as potentially phosphorylated by c-Src tyrosine kinase (with a consensus score higher than the critical value of 0.5). Taking into account that estrogen stimulation of breast cancer cells led to an immediate tyrosine phosphorylation and activation of the c-Src kinase (26, 36), we sought to determine whether c-Src might be the tyrosine kinase involved in the E_2 activation of aromatase protein. Thus, we performed Western blot analysis using the c-Src inhibitor, 4-amino-5-(4-chlorophenyl)-7-(t.butyl)pyrazolo(3,4-d)pyramidine (PP2), and as shown in Fig. 4A, PP2 reduced the E_2-associated tyrosine phosphorylation of the purified aromatase. In addition, PP2 treatment was able to abrogate the E_2-induced increase on aromatase enzymatic activity (Fig. 4B).

To further support the crucial role of c-Src, we examined whether knockdown of the c-Src gene would similarly reduce tyrosine aromatase phosphorylation. Transfection with pool of two small interfering RNA (siRNA) duplex specifically direct against human c-Src, reduced the expression of this protein (Fig. 4C). As shown in Fig. 4D, silencing of c-Src significantly decreased tyrosine phosphorylation of the purified aromatase induced by E_2.

We next examined the physical association between c-Src kinase and either wt or Y361F mutant aromatase proteins. To test this possibility, we transiently transfected MCF-7 cells with either His$_6$-arom or His$_6$-Y361F constructs. Equal amounts of proteins were incubated with Ni-NTA agarose beads for isolation of recombinant P450$_{arom}$ proteins followed by immunoblot for c-Src and P450$_{arom}$. Results obtained showed that both wt and Y361F mutant aromatase proteins were able to bind c-Src tyrosine kinase (Fig. 4E). We confirmed the formation of this protein complex by immunoprecipitation of c-Src and then detection of aromatase on Western blotting (data not shown).

Using *in vitro* recombinant c-Src kinase, we found that wt aromatase was more efficiently phosphorylated than Y361F mutant aromatase protein (Fig. 4F), addressing the importance of this residue of aromatase protein as phosphorylation substrate of c-Src.
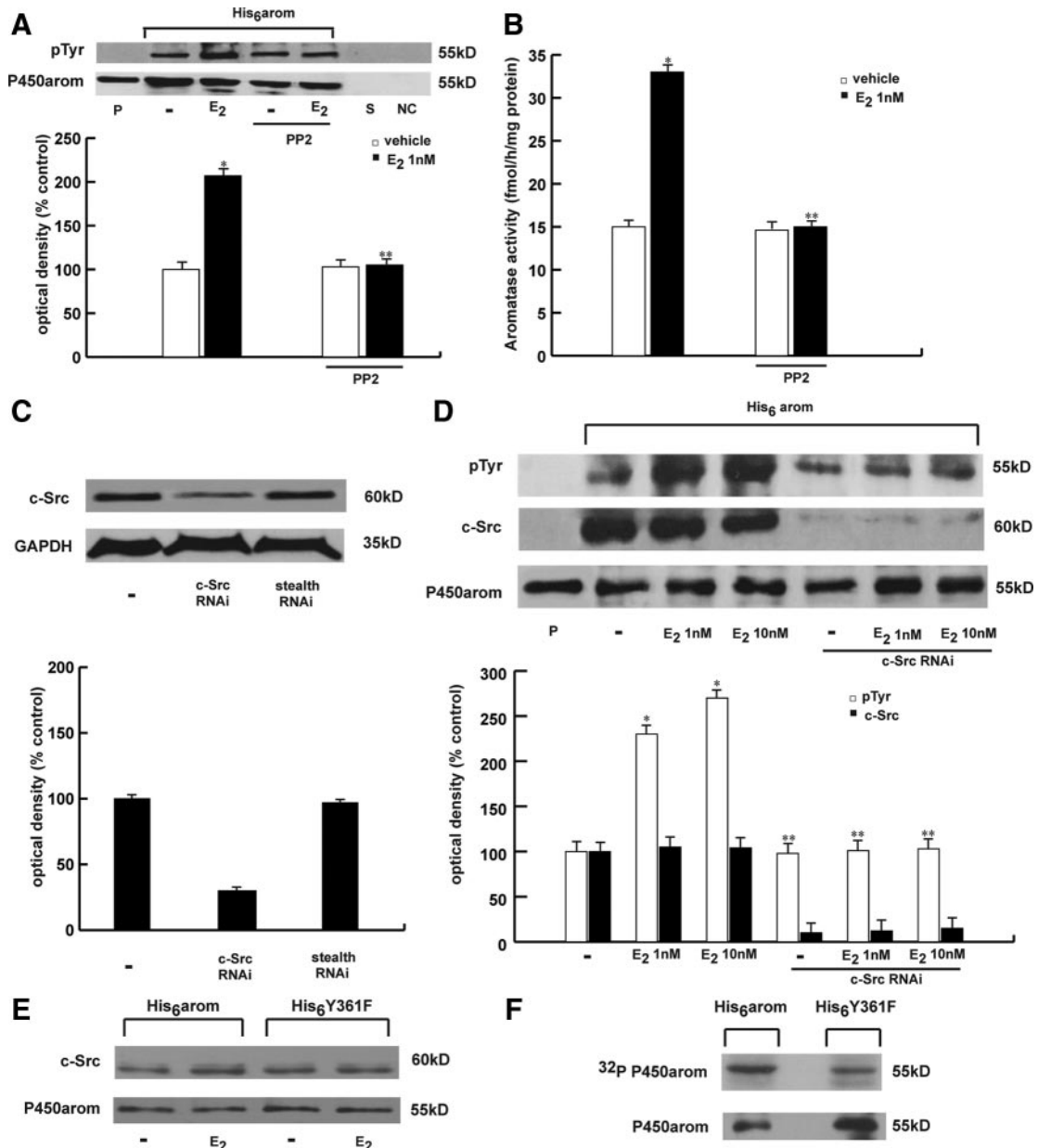
FIG. 4. c-Src signaling mediates E_2-induced aromatase activity. A, MCF-7 cells transiently transfected with His_6-arom were treated with vehicle (-) or 1 nM E_2 for 10 min, with or without pretreatment of PP2 (3 μM). The membrane was probed with antiphosphotyrosine (pTyr) antibody. To verify equal loading, the membrane was probed with antiaromatase antibody. Microsomal extracts from placenta (P) were used as positive control. As negative controls we used the supernatant removed after incubation with Ni-NTA agarose beads (S) and vector-transfected MCF-7 cell lysates incubated with Ni-NTA agarose beads (NC). The *histograms on the bottom* represent the means ± SE of three separate experiments in which band intensities were evaluated in terms of OD arbitrary units and expressed as percentages of the control, which was assumed to be 100%. *, $P < 0.01$ compared with vehicle; **, $P < 0.01$ compared with E_2-treated cells. B, MCF-7 cells were pretreated with or without PP2 (3 μM) before E_2 (1 nM) stimulation for 10 min, after which aromatase activity was performed. The values represent the means ± SE from triplicate assays. *, $P < 0.01$ compared with vehicle; **, $P < 0.01$ compared with E_2-treated samples. C, c-Src protein expression (evaluated by Western blotting) in MCF-7 cells not transfected (-) or transfected with RNAi targeted human c-Src mRNA or with a stealth RNAi control as reported in *Materials and Methods*. Glyceraldehyde-3-phosphate dehydrogenase was used as loading control. The *histograms* represent the means ± SE of three separate experiments in which band intensities were evaluated in terms of OD arbitrary units and expressed as percentages of the control, which was assumed to be 100%. D, MCF-7 cells were not transfected or transfected with c-Src RNAi, then transfected with His_6-arom, and exposed to 1 and 10 nM E_2 for 10 min. The membrane was probed with antiphosphotyrosine (pTyr) or anti-c-Src antibodies. To verify equal loading, the membrane was probed with antiaromatase antibody. Microsomal extracts from placenta (P) were used as positive control. The *histograms* represent the means ± SE of three separate experiments in which band intensities were evaluated in terms of OD arbitrary units and expressed as percentages of the control, which was assumed to be 100%. *, $P < 0.01$ compared with vehicle; **, $P < 0.01$ compared with E_2-treated cells. E, MCF-7 cells were transfected with His_6-arom or His_6-Y361F vectors and then treated with E_2. Aromatase protein was purified using Ni-NTA agarose beads after which the complexes were resolved in SDS-PAGE. Immunoblotting was performed using the anti-c-Src and anti-aromatase (P450$_{arom}$) antibodies. F, MCF-7 cells were transfected with His_6-arom or His_6-Y361F vectors. *In vitro* c-Src kinase assay was performed on aromatase protein purified using Ni-NTA agarose beads using recombinant full-length human Src kinase. Autoradiography is shown in the *upper panel*, and input aromatase is shown in the *bottom panel*. kD, Kilodaltons.

Evidence that ERα, growth factor receptors (GF-Rs), c-Src, and aromatase interact in a multiprotein complex

Studies in breast cancer culture have highlighted an intimate cross talk between the endogenous membrane ER and GF-Rs signaling pathways (37–39). This process may involve the sequential activation of the cellular tyrosine kinase c-Src (26, 36, 40, 41). We wondered whether the cross talk between ERα and GF-Rs, through c-Src, could be involved in the rapid modulation of E_2-induced aromatase activity in breast cancer cells. To evaluate a direct protein-protein interaction among ERα, epidermal growth factor (EGF) receptor (EGFR), and aromatase, we performed coimmunoprecipitation studies. Particularly, MCF-7 cells transiently transfected with His_6-arom were lysated after which protein extracts were incubated with Ni-NTA agarose beads for isolation of recombinant $P450_{arom}$ (Fig. 5A, *left panel*). Equal amounts of the lysates were immunoprecipitated with ERα-specific antibody (Fig. 5A, *right panel*). The membranes were probed with anti-ERα, anti-EGFR, anti-c-Src, or antiaromatase antibodies. The results showed that ERα, EGFR, c-Src, and aromatase were in a multiprotein complex. Notably, the presence of c-Src is required for this complex formation because silencing of c-Src reduced the interaction of aromatase with ERα and EGFR (Fig. 5A).

Next, to determine whether EGFR stimulation leads to an increased production of E_2 via an up-regulation of aromatase enzymatic activity, MCF-7 cells were treated with 100 ng/ml EGF for 10 min. Our data demonstrated that EGF is able to enhance aromatase enzymatic activity as well as the tyrosine-phosphorylated status in the His_6-tagged purified aromatase protein to the same extent as E_2. Pretreatment with AG1478, a specific EGFR inhibitor, or ICI completely abrogated these effects (Fig. 5, B and C). The same results were obtained under treatment with IGF and AG1024, a monoclonal antibody specific to IGF-1R (data not shown).

These data indicate that the induction of aromatase enzymatic activity may involve the cross talk between E_2/ER and GF-Rs signaling.

Discussion

In the present study we demonstrate that short exposure to E_2 induces an increase of aromatase enzymatic activity, through an enhanced tyrosine phosphorylation level of the enzyme, in estrogen-dependent MCF-7 breast cancer epithelial cells.

Our results showed that the rapid effect induced by E_2 in enhancing aromatase activity was specifically mediated by the interaction of E_2 with ERα, because it was abrogated in the presence of ER antagonists, such as TAM and ICI. Moreover, when in ER-negative CHO cells overexpressing aromatase, we transfected the membrane ERα construct yielding the ligand-binding domain of the receptor exclusively localized to the cytoplasmic face of the membrane, we also reproduced the stimulatory effects of E_2 on aromatase activity. This underlines the ability of membrane ERα, which is unable to generate genomic response, in modulating aromatase activity. We also reproduced similar results in ER-negative breast cancer cells SKBR3, ectopically expressing ERα, and in R2C rat Leydig tumor cells, which display high aromatase expression. These latter results suggest that the rapid changes in aromatase activity may represent a general mechanism not related to cell specificity even though it assumes more relevance in breast cancer in which growth and progression are strongly estrogen dependent. Our data appear opposite to previous findings demonstrating that E_2 treatment reduced aromatase activity in breast cancer cells (42, 43). However, it is worthwhile to point out that they come from a different experimental design performed after a long-term E_2 exposure of MCF-7 cells either cultured long term in estrogen-deprived medium or stably transfected with the aromatase gene (MCF-7aro). For instance, Pasqualini and Chetrite (42) observed the maximal inhibition in aromatase activity (evaluated by quantification of ^3H-estradiol from cell incubated with [^3H]testosterone) at the nonphysiological dose of 50 μM in MCF-7aro. Instead, in our study, we evaluated aromatase activity by tritiated water release assay using as substrate [1β-^3H] androst-4-ene-3,17-dione ($\Delta4$) in parental or transiently expressing aromatase MCF-7 cells. We demonstrated the maximal increase in aromatase enzymatic activity after a short time of exposure with low physiological doses of E_2.

The E_2 induced up-regulation of aromatase activity in MCF-7 cells was not correlated with any increase in the levels of aromatase mRNA and protein content, suggesting a posttranslational modulation of aromatase protein.

Posttranslational modification of enzymatic protein has been demonstrated for different members of the P450 enzyme family in vertebrates. For instance, cAMP-dependent protein kinase was essential for the activation of human and rat cholesterol 7α-hydroxylase (CYP7A) (44) as well as for phosphorylation of serine and threonine residues in human P450c17 (CYP17) (45, 46). Bovine P450scc (CYP11A1) has been identified as an active form phosphorylated by a protein kinase C (47), and similar activation of P450s through phosphorylation has been found in human liver enzymes such as CYP2E1 and CYP2B1 (48). Recently, phosphorylation of the cytochrome P450 aromatase has been proposed, even though
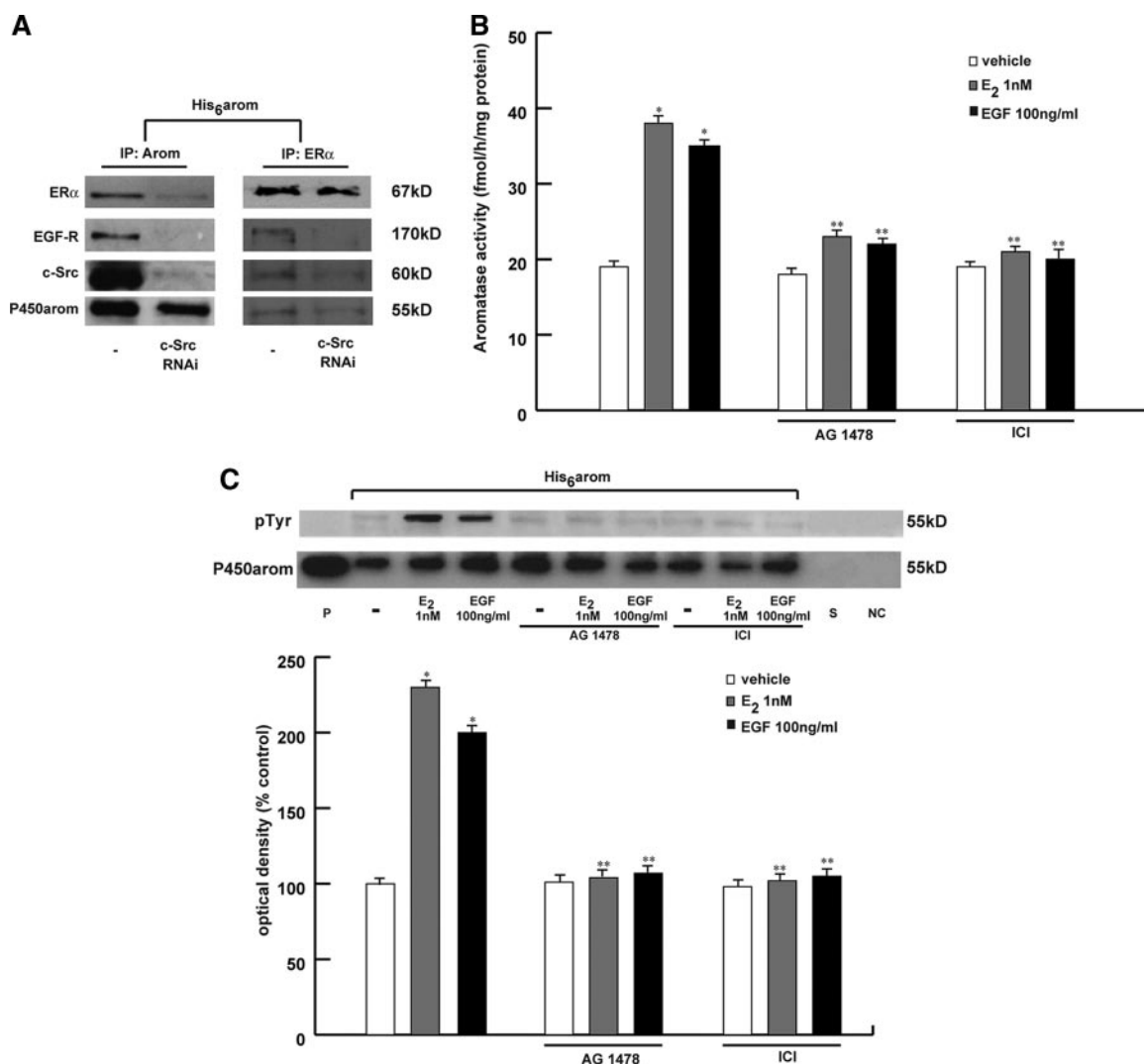
FIG. 5. Interaction between ERα and EGFR/c-Src in the aromatase activity induction. A, MCF-7 cells were not transfected (-) or transfected with c-Src RNAi and then transfected with His6-arom vector. Aromatase protein was purified using Ni-NTA agarose beads after which the complexes were resolved in SDS-PAGE. In another set of experiments the same amount of cell lysate was immunoprecipitated (IP) with ERα antibody. Immunoblotting was performed using the anti-ERα, anti-EGFR, anti-c-Src, and antiaromatase antibodies. B, MCF-7 were pretreated with 10 μM AG1478 or 1 μM ICI for 30 min and then exposed to 1 nM E$_2$ or 100 ng/ml EGF. After 10 min, aromatase activity was performed. The values represent the means ± SE from triplicate assays. *, $P < 0.01$ compared with vehicle; **, $P < 0.01$ compared with E$_2$- or EGF-treated samples. C, MCF-7 cells transiently transfected with His6-arom were pretreated with or without 10 μM AG1478 or 1 μM ICI for 30 min and then exposed to 1 nM E$_2$ or 100 ng/ml EGF for 10 min. The membrane was probed with antiphosphotyrosine (pTyr) antibody. To verify equal loading, the membrane was probed with antiaromatase antibody. P, Microsomal extracts from placenta. As negative controls we used the supernatant removed after incubation with Ni-NTA agarose beads (S) and vector-transfected MCF-7 cell lysates incubated with Ni-NTA agarose beads (NC). The *histograms* represent the means ± SE of three separate experiments in which band intensities were evaluated in terms of OD arbitrary units and expressed as percentages of the control, which was assumed to be 100%. *, $P < 0.01$ compared with vehicle; **, $P < 0.01$ compared with E$_2$- or EGF-treated samples. kD, Kilodaltons.

the specific kinases involved in this process are yet not well specified (16, 21). We demonstrated in MCF-7 cells that E$_2$ up-regulatory effects on aromatase activity resulted from a direct phosphorylation of enzymatic protein itself. Indeed, our *in vivo* labeling experiments showed, after E$_2$ treatment, a significant increase in phosphorylation of aromatase protein purified by Ni-NTA agarose beads. Particularly, we observed a specific enhancement of tyrosine phosphorylation levels of aromatase protein after E$_2$ exposure, whereas serine phosphorylation status

remains unchanged. This suggests that the rapid nongenomic effects of the hormone specifically target tyrosine residues.

Site-directed mutagenesis experiments revealed that phosphorylation of the specific tyrosine residue, located at position 361 in the steroid-binding domain of aromatase protein, is crucial in the up-regulation of enzymatic activity after E$_2$ treatment. The 361-tyrosine residue of aromatase sequence is well conserved across species and represents a potential consensus site of
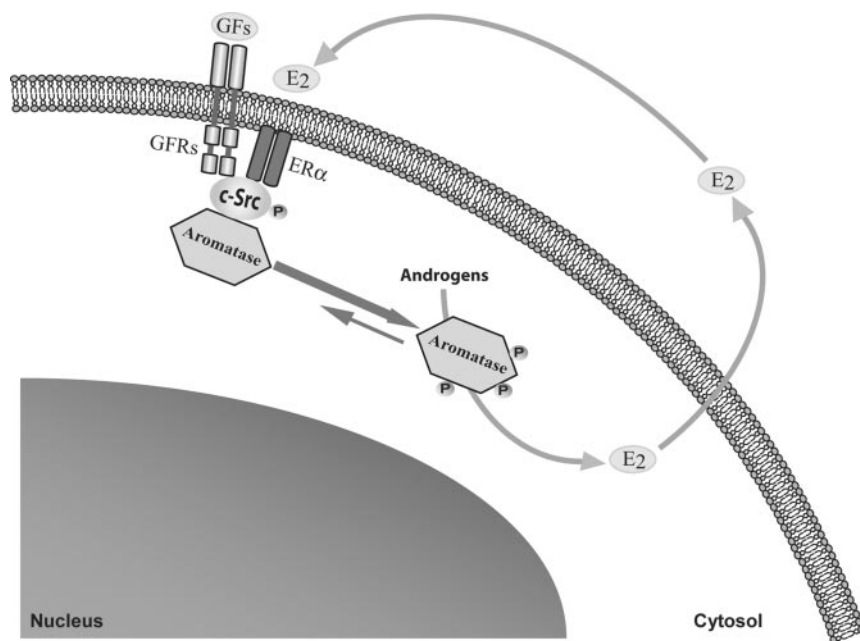
FIG. 6. Hypothetical model of the potential signaling transductional pathways through which E_2 and GFs (growth factors) may rapidly enhance aromatase activity in MCF-7 breast cancer cells. P, Placenta.

phosphorylation by an important nonreceptor tyrosine kinase c-Src (with a consensus score higher than the critical value of 0.5).

c-Src mediates signal transduction pathways implicated in proliferation, survival, cell adhesion, and migration (49). This kinase can be activated by many cell surface receptors and represents a crucial molecule downstream of ERα triggering estrogen rapid action (26, 36). In our study we demonstrated a direct interaction between c-Src and aromatase protein, and the involvement of this kinase in E_2-stimulated aromatase activity because blockade of c-Src activity completely reversed the E_2-induced increase of aromatase activity as well as reduced tyrosine phosphorylation of aromatase purified protein. Moreover, *in vitro* kinase activity assay using pure c-Src protein demonstrated that this kinase directly phosphorylates aromatase, and tyrosine located at the 361 site is involved in this event. Thus, we identified the phosphorylation of the critical residue 361 by c-Src kinase as a novel mechanism for regulating enzymatic activity and function of aromatase.

It is well known that ERα and GF-Rs utilize signaling pathways that intersect and directly interact at many levels. Estrogens have been shown to activate IGF-I receptor and EGFR (37–39), and it has been reported that E_2 up-regulates aromatase expression via cross talk between ER and GF in breast cancer cells (17). A number of proteins, such as c-Src, Fak (focal adhesion kinase) MNAR (50), are reported to form a complex with ERs and to be involved in extranuclear functions of ERα. Our study

shows the existence of a multipartite complex involving ERα, EGFR, c-Src, and aromatase. Silencing of c-Src by siRNA reduced the interaction of ERα and EGFR with aromatase, suggesting a key role of kinase active c-Src in the formation of this complex. In addition, treatment of MCF-7 cells with IGF-I and EGF increased, in a short time, aromatase activity as well as the tyrosine phosphorylation status of aromatase protein. Treatments with specific tyrosine kinase inhibitors of GF-Rs or with the antiestrogen ICI abrogated the GFs as well as the E_2 induction of aromatase activity. It has been largely demonstrated that c-Src is critical component of the bidirectional cross talk between ERα and GF-Rs (reviewed in Ref. 40). Thus, this may explain why, in the presence of antiestrogen or inhibitors of both GF-Rs tested, the up-regulatory effects on aromatase activity are not longer noticeable.

In summary, this study shows, for the first time, a new molecular mechanism by which E_2 rapidly increases aromatase activity through an enhanced tyrosine phosphorylation of the enzyme. We hypothesized that E_2, through an enhanced cross talk between GF-Rs, c-Src, and ER signaling, can phosphorylate and thus activate aromatase, resulting in a positive nongenomic autocrine loop between E_2 and aromatase in MCF-7 breast cancer cells (Fig. 6). All these data demonstrate that aromatase may be activated by different membrane cell signaling, which should be targeted in the novel therapeutic strategies for breast cancer treatment.

Materials and Methods

Cell cultures

MCF-7 and CHO cells were cultured in DMEM/F-12 medium containing 5% calf serum or 10% fetal bovine serum (FBS), respectively (Eurobio, Les Ullis Cedex, France). SKBR3 cells were cultured in RPMI 1640 medium supplemented with 10% FBS. R2C cells were cultured in Ham/F-10 supplemented with 15% HS, 2.5% FBS.

His₆-arom plasmid construction

His₆-arom plasmid construct was used to express the C-terminal 6×His-tagged form of human aromatase. The plasmid pUC19-arom containing the full-length of human aromatase gene (CYP19) was used as template. The 6×His epitope tag was inserted by two PCRs using the following primers: forward (5′-ATATAAGCT-TATGGTTTTGGAAATGCTGA-3′) and two reverse (5′-ATGAT-

TABLE 1. Oligonucleotide Primers Used for Mutagenesis Studies

Mutant	Template	Primers	Sequence
Y184F	CYP19	Forward	5′-CCAATGAATCGGGCtttGTGGACGTGTTGACCC-3′
		Reverse	5′-GGGTCAACACGTCCACaaaGCCCGATTCATTGG-3′
Y361F	CYP19	Forward	5′-GAAAACTTCATTtttGAGAGCATGCGGTACCAGCCTGTCG-3′
		Reverse	5′-CGACAGGCTGGTACCGCAGCTCTCaaaAATGAAGTTTTCC-3′
His$_6$-Y361F	His$_6$_arom	Forward	5′-GAAAACTTCATTtttGAGAGCATGCGGTACCAGCCTGTCG-3′
		Reverse	5′-CGACAGGCTGGTACCGCAGCTCTCaaaAATGAAGTTTTCC-3′

GATGGTGTTCCAGACACCT-3′), (5′-ATATTCTAGACTAAT-GATGATGATGATGATGGTGTTCCAGA-3′). PCR product was subcloned into *Hin*dIII/*Xba*I sites of pcDNA3.1, and Hys$_6$-arom sequence was confirmed by nucleotide sequence analysis. We proved that the enzymatic activity of polyhistidine-containing recombinant protein was well preserved by measuring aromatase activity in MCF-7 cells transiently transfected with Hys$_6$-arom vector.

Site-directed mutagenesis

This step was performed with the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to manufacturer's standard method. The templates and the specific oligonucleotides used are summarized in Table 1.

Transient transfection

Transient transfection was performed using the FuGENE 6 (Roche, Indianapolis, IN) reagent with the mixture containing 1 μg/well of CYP19 or Y184F, Y361F mutants. A set of experiments was performed cotransfecting 1 μg/well of CYP19 and 1 μg/well of the membrane ERα construct (mERα) or ERα wt.

Aromatase activity assay

The aromatase activity in subconfluent MCF-7, SKBR3, CHO, and R2C cells culture medium was measured by the tritiated water release assay using 0.5 μM [1β-^{3}H]androst-4-ene-3,17-dione as substrate (51). The incubations were performed at 37 C for 3 h under an air-CO$_2$ (5%) atmosphere. The results obtained were expressed as fentomoles per h (MCF-7, CHO, and SKBR3) or picomoles per h (R2C) and normalized to mg of protein.

RT-PCR assay

Total cellular RNA was extracted from MCF-7 cells using TRIzol (Invitrogen, Carlsbad, CA). Aromatase mRNA was analyzed by the RT-PCR method as previously described (52).

Immunoblotting and immunoprecipitation analysis

Whole-cell lysates were prepared in lysing buffer [50 mmol/liter HEPES (pH 7.5), 150 mmol/liter NaCl, 1.5 mmol/liter MgCl$_2$, 1 mmol/liter EGTA, 10% glycerol, 1% Triton X-100, protease inhibitors (Sigma, Italy)]. Equal amounts of total protein were resolved on 11% SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with antiserum against the human placental P450$_{arom}$ (Hauptman-Woodward Medical Research Institute, Buffalo, NY) or glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For immunoprecipitation studies, 300 μg of protein extracts was incubated with 1 μg of anti-ERα antibody (Santa Cruz) and 20 μl of protein A/G (Santa Cruz). The immunoprecipitated proteins were then subjected to Western blot analysis. Whole-cell lysates were used as input controls.

Detection of His$_6$-tagged aromatase protein by immunoblotting analysis

MCF7 cells were transiently transfected with His$_6$-arom or His$_6$-Y361F vectors and exposed to different treatments before lysis. Cellular proteins (300 μg) were incubated with Ni-NTA agarose beads (Invitrogen). Ni-NTA resin was used to isolate P450 aromatase tagged with six tandem histidine residues from cellular lysates. The beads containing bound proteins were washed in PBS buffer added with a mixture of protease inhibitors and analyzed by Western blot. Membrane was probed with a antibodies against human cytochrome P450$_{arom}$ (Serotec, Oxford, UK) or phosphotyrosine-containing proteins (pY99, Santa Cruz) or phosphoserine-containing proteins. For coimmunoprecipitation studies, membranes were probed with ERα, EGFR, and c-Src antibodies (Santa Cruz). Two set of controls were done in parallel: surnatant removed after the first centrifugation was added to one control and vector-transfected cell lysates plus Ni-NTA agarose beads was included in the other control. Microsomal extracts from placenta were used as positive control.

In vivo phosphorylation experiments

MCF-7 cells were transiently transfected with His$_6$-arom or His$_6$-Y361F construct, labeled for 2 h with [^{32}P]orthophosphate (PerkinElmer, Boston, MA) (0.5 mCi/ml in Krebs Ringer buffer, pH 7.4, containing 1% BSA), treated with E$_2$, washed with PBS, and immunoprecipitated with Ni-NTA agarose beads as described above. The supernatants were resolved onto 10% SDS-PAGE gel and transferred onto nitrocellulose membrane. Phosphorylated aromatase-purified protein was detected by autoradiography, and the aromatase protein level was determined by immunoblot of the same membrane with antiaromatase antibody.

In vitro c-Src kinase activity assay

C-Src kinase activity was measured by phosphorylation of aromatase protein specifically purified from lysates of MCF-7 cells transiently transfected with His$_6$-arom or His$_6$-Y361F constructs, as previously described. The washed Ni-NTA beads containing bound aromatase proteins were incubated with recombinant full-length human Src kinase (Cell Signaling Technology, Danvers, MA) in the presence of 10 μCi of [γ-^{32}P]ATP and 10 nmol/liter ATP in 40 μl kinase buffer at 30 C for 30 min. The reactions were stopped by the addition of sodium dodecyl sulfate loading buffer, and the samples were resolved by 10% SDS-PAGE. The phosphorylated aromatase protein bands were visualized by autoradiography.

c-Src knockdown by siRNA

MCF-7 cells were transfected with validated stealth RNA interference (RNAi) DuoPak (Invitrogen) targeted human c-Src or with a stealth RNAi control to a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. After 5 h the transfection medium was changed with serum free medium, transfected with His_6-arom vector, and then exposed to E_2. These transfected cells were used to immunoblotting analysis.

Statistical analysis

Each datum point represents the mean ± SE of three different experiments. Statistical analysis was performed using ANOVA followed by Newman-Keuls testing to determine differences in means. $P < 0.05$ was considered as statistically significant.

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Expression of the K303R Estrogen Receptor-α Breast Cancer Mutation Induces Resistance to an Aromatase Inhibitor via Addiction to the PI3K/Akt Kinase Pathway

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Abstract

Aromatase inhibitors (AI) are rapidly becoming the first choice for hormonal treatment of estrogen receptor-α (ERα)–positive breast cancer in postmenopausal women. However, de novo and acquired resistance frequently occurs. We have previously identified a lysine to arginine transition at residue 303 (K303R) in ERα in premalignant breast lesions and invasive breast cancers, which confers estrogen hypersensitivity and resistance to tamoxifen treatment. Thus, we questioned whether resistance to AIs could arise in breast cancer cells expressing the ERα mutation. As preclinical models to directly test this possibility, we generated K303R-overexpressing MCF-7 cells stably transfected with an aromatase expression vector. Cells were stimulated with the aromatase substrate, androstenedione, with or without the AI anastrozole (Ana). We found that Ana decreased androstenedione-stimulated growth of wild-type cells, whereas K303R-expressing cells were resistant to the inhibitory effect of Ana on growth. We propose that a mechanism of resistance involves an increased binding between the mutant receptor and the p85α regulatory subunit of phosphatidylinositol-3-OH kinase (PI3K), leading to increased PI3K activity and activation of protein kinase B/Akt survival pathways. Inhibition of the selective "addiction" to the PI3K/Akt pathway reversed AI resistance associated with expression of the mutant receptor. Our findings suggest that the K303R ERα mutation might be a new predictive marker of response to AIs in mutation-positive breast tumors, and that targeting the PI3K/Akt pathway may be a useful strategy for treating patients with tumors resistant to hormone therapy. [Cancer Res 2009;69(11):4724–32]

Introduction

Despite the clinical efficacy of the aromatase inhibitors (AI), many ERα-positive breast tumors initially fail to respond (de novo resistance), or resistance develops during treatment leading to disease progression (acquired resistance). To understand resistance mechanisms, several laboratories have developed in vitro cell line models to study the molecular changes associated with long-term estrogen deprivation (1–5). AI resistance has also been examined using aromatase-overexpressing MCF-7 breast cancer cell line models grown as xenografts in athymic nude mice (6), or breast cancer cells made resistant to AIs via long-term treatment with these drugs (7, 8). These models suggest the hypothesis that resistance to endocrine therapy may be through the acquisition of estrogen hypersensitivity, whereby low subphysiologic levels of estrogens remaining after estrogen deprivation are sufficient for maintenance of tumor growth. One unifying feature which has emerged is a retained mitogenic role for ERα (9). Recently, intracellular cross-talk between ERα and several signal transduction pathways have been shown to be associated with endocrine resistance (10, 11).

We have previously identified a frequent somatic mutation at nucleotide 908 of ERα (A908G) in premalignant breast lesions and invasive breast cancers (12, 13). This mutation results in a lysine to arginine transition at residue 303 (termed K303R), that confers hypersensitivity to estrogen (12, 14). We hypothesized that such a mutant could provide a continuous mitogenic stimulus to the breast even during phases of low circulating hormone, such as menopause, thus affording a proliferative advantage especially during treatment with AIs. Here, we present a new model of resistance to endocrine therapy, whereby the expression of the A908G ERα mutation conferred resistance to the AI anastrozole (Ana). We speculate that inhibition of the phosphatidylinositol-3-OH kinase (PI3K)/Akt pathways may represent a promising therapeutic strategy for hormone-resistant cancers that are "addicted" to these pathways due to mutation of the ERα target.

Materials and Methods

Reagents, hormones, and antibodies. 17β-Estradiol, 4-androstene-3,17-dione and heregulin were obtained from Sigma. Anastrozole and ICI182,780 were provided by AstraZeneca. PD98059, PI-103, Akt inhibitor VIII isozyme-selective (Akti1/2), and LY294002 were from Calbiochem. Exemestane (Exe) was obtained from Pfizer. Antibodies used for immunoblotting were ERα (clone 6F11) from Vector Laboratories and Rho GDIα from Santa Cruz Biotechnology. Total ERK1,2/MAPK, total Akt, phosphorylated p42/44 ERK1,2/MAPK (Thr^{202}/Tyr^{204}), Akt (Ser^{473}), ERα (Ser^{167}), and poly(ADP ribose)polymerase (PARP) were from Cell Signaling Technology; Bax and Bcl-2 were from Calbiochem; cytochrome P450 aromatase was from Serotec; and p85 was from Upstate Biotechnology. Secondary antibodies goat anti-mouse or goat anti-rabbit were obtained from Amersham Biosciences.

Plasmids. Full-length human aromatase cDNA was amplified from the pCMV6-Arom plasmid (OriGene Technologies) by PCR using the following primers: forward 5′-ACACTAGTATGGTTTTGGAAATGCTGAACCC-3′ and reverse 5′-ACGCGGCCGCCTAGTGTTCCAGACACCTGTCT. This PCR

product was subcloned into the *Spe* I/*Not* I sites of the pZeoSV2-vector (Invitrogen). The resulting pZeoSV2-*aromatase* expression vector (pZeo-Arom) sequence was confirmed by DNA sequencing. Generation of yellow fluorescent protein (YFP)–tagged expression constructs, YFP-WT and K303R-ERα, has been previously described (14).

Cell culture. MCF-7 parental breast cancer cells were cultured as described (14). MCF-7 wild-type (WT) and K303R ERα-expressing cells were generated as described (15). MCF-7 parental and YFP-K303R ERα clones were stably transfected with the pZeo-Arom expression vector using Fugene 6 reagent according to the manufacturer (Roche). Chinese hamster ovary (CHO) or MCF-7 Arom-expressing pools, stably transfected with YFP-WT ERα and YFP-K303R ERα expression vectors, were also used.

Aromatase activity assay. Aromatase activity was evaluated using a ^{3}H-water release assay using 0.5 μmol/L of [1β-^{3}H]-androst-4-ene-3,17-dione as substrate (16). The incubations were performed at 37°C for 1 h. The results were expressed as fmol-pmol/h/mg protein.

Tumor xenografts. All animal studies were carried out according to the guidelines and with the approval of the Baylor College of Medicine Animal Care and Use Committee. Female nude ovariectomized athymic mice were injected with MCF-7 YFP-WT and YFP-K303R ERα–expressing cells or MCF-7 Arom 1 and K303R Arom 1–expressing cells as described (17). Animals were supplemented with estrogen tubing releasing ~80 pg/mL, or supplemented daily with injections of the aromatase substrate androstenedione (AD; 100 μg).

Cell proliferation assays. Seven hundred cells were plated into 96-well plates in phenol red–free MEM containing 5% charcoal-stripped fetal bovine serum (FBS). After 24 h, cells were treated as indicated. Nine days later, proliferation was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent following the recommendations of the manufacturer. Soft agar anchorage-independent growth assays were performed as previously described; data shown are the mean colony numbers of three plates and is representative of two or three independent experiments (18).

Immunoprecipitation and immunoblot analyses. Cells were starved in serum-free MEM for 48 h and treated as indicated before lysis (15). For coimmunoprecipitation experiments, we used 500 μg of total cellular protein and anti-ERα (1:50) or anti-p85 (1 μg) antisera overnight and protein A/G with rotation at 4°C for 2 h. Protein extracts from tumor tissues were prepared by homogenizing the tissue in lysis buffer supplemented with 10% glycerol. Equal amounts of extracts were subjected to SDS-PAGE as described (14). Blots shown are representative of two or three individual experiments.

PI3K activity. PI3K was immunoprecipitated from cellular lysates with p85 antibody and the amount of phosphatidylinositol-(3,4,5)-trisphosphate (PIP$_3$) produced was assessed in a competitive ELISA (Echelon Biosciences).

ERα transactivation assays. Cells (50,000/well) were plated in phenol red–free MEM with 5% charcoal-stripped FBS in 24-well plates, and then cotransfected with 0.5 μg of an ERE$_2$-tk-luciferase reporter plasmid, plus 1 ng of the respective ERα expression plasmid and 50 ng of CMV-β-galactosidase plasmid as an internal control using Fugene 6 reagent. After 6 h, cells were treated as indicated for 18 to 24 h. Luciferase activities were determined as described (14).

Apoptosis assays. Cells were seeded into six-well plates (10^5/well) in phenol red–free MEM with 5% charcoal-stripped FBS for 48 h and then incubated with different treatments for 72 h. Apoptosis was measured using the Cell Death Detection ELISA Kit (Roche). Data are representative of two independent experiments performed in triplicate.

Statistical analyses. Data were analyzed by Student's *t* test using the GraphPad Prism5 software program.

Results

The K303R ERα mutation and estrogen hypersensitivity. We have previously described ERα-positive MCF-7 breast cancer cells that overexpress either WT ERα or the K303R ERα mutation (15). Cells expressing the K303R ERα mutant receptor expressed a 66 kDa

endogenous ERα protein, along with a 96 kDa receptor representing the exogenously added mutant tagged with YFP (Fig. 1*A*). We have shown that K303R ERα mutant overexpression conferred increased sensitivity to estrogen using *in vitro* growth assays (12). To confirm these findings, we examined the ability of increasing concentrations of estradiol (E$_2$) to induce anchorage-independent growth, which reflects *in vivo* three-dimensional tumor growth. Basal (E$_2$) mutant colony number was 2.9-fold higher (Fig. 1*B*); both cell lines exhibited estradiol-induced dose-response growth. Growth stimulation was not observed with low E$_2$ (10^{-12} mol/L) in parental cells, whereas low E$_2$ enhanced the growth of mutant cells.

To determine if low estrogen might favor the growth of mutant cells, we examined xenograft tumor models. Cells were injected into female mice supplemented with 80 pg/mL of estradiol, representing low premenopausal levels (Fig. 1*C*). K303R ERα tumors grew faster than the WT ERα tumors (*P* = 0.0466). Thus, expression of the mutant generated an estrogen hypersensitive phenotype *in vitro* and *in vivo*.

Expression of the mutation and AI resistance. Estrogen-deprived MCF-7 cell lines (1–3, 19), and breast cancer cells resistant to aromatase inhibitors (AI^R) have been generated (6, 7); one hypothesis emerging is that resistance may result from adaptive estrogen hypersensitivity or estrogen-independent ERα activation. This hypothesis led us to question if the K303R ERα mutant might play a role in AI^R due to its estrogen-hypersensitive phenotype.

To test for AI^R, cells were stably transfected with an aromatase cDNA expression vector. Figure 1*D* shows aromatase protein levels in a vector control clone (MCF-7 V), one clone stably expressing aromatase (MCF-7 Arom 1), and two clones coexpressing the YFP-K303R mutant ERα along with aromatase (K303R Arom 1 and Arom 2). These cells overexpressed aromatase protein and activity at ~1,000 times more than vector control cells (0.032 versus 29–65 pmol/h/mg protein).

We next evaluated proliferative responses in anchorage-independent assays (Fig. 1*E*). K303R Arom 1–expressing cells exhibited significantly enhanced control growth compared with WT ERα MCF-7 Arom 1–expressing cells, and growth was further increased by AD treatment. MCF-7 Arom 1 and K303R Arom 1 cells were also injected into mice to monitor xenograft growth; a significant increase in the growth of AD-treated mutant tumors was seen (Fig. 1*F*).

To investigate whether the mutant hypersensitive phenotype could cause AI^R, we examined the effects of the nonsteroidal aromatase inhibitor Ana on cell growth (Fig. 2*A*). Growth of MCF-7 vector (V) cells was significantly enhanced by E$_2$, but as expected, they did not respond to AD treatment. AD treatment enhanced growth to the same extent as E$_2$ in MCF-7 Arom 1 cells, as well as K303R Arom 1–expressing cells. Ana treatment decreased AD-stimulated growth at ~40% in MCF-7 Arom 1–expressing cells, but had no effect on the growth of mutant cells.

To extend the MTT data, we performed anchorage-independent soft agar growth assays (Fig. 2*B*). E$_2$ and AD treatments enhanced colony numbers, and treatment with Ana completely abrogated AD-stimulated growth of MCF-7 Arom 1 cells. In contrast, basal colony number was increased ~10-fold in K303R Arom 1 and Arom 2 clones, and Ana was unable to inhibit AD-induced colony growth.

Because it was possible that overexpression of exogenous ERα alone might stimulate cell growth and contribute to the AI^R phenotype, we also transfected MCF-7 Arom 1–expressing cells with an expression vector for YFP-WT ERα. Aromatase and exogenous YFP-ERα levels are shown (Fig. 2*C, top*). Pools
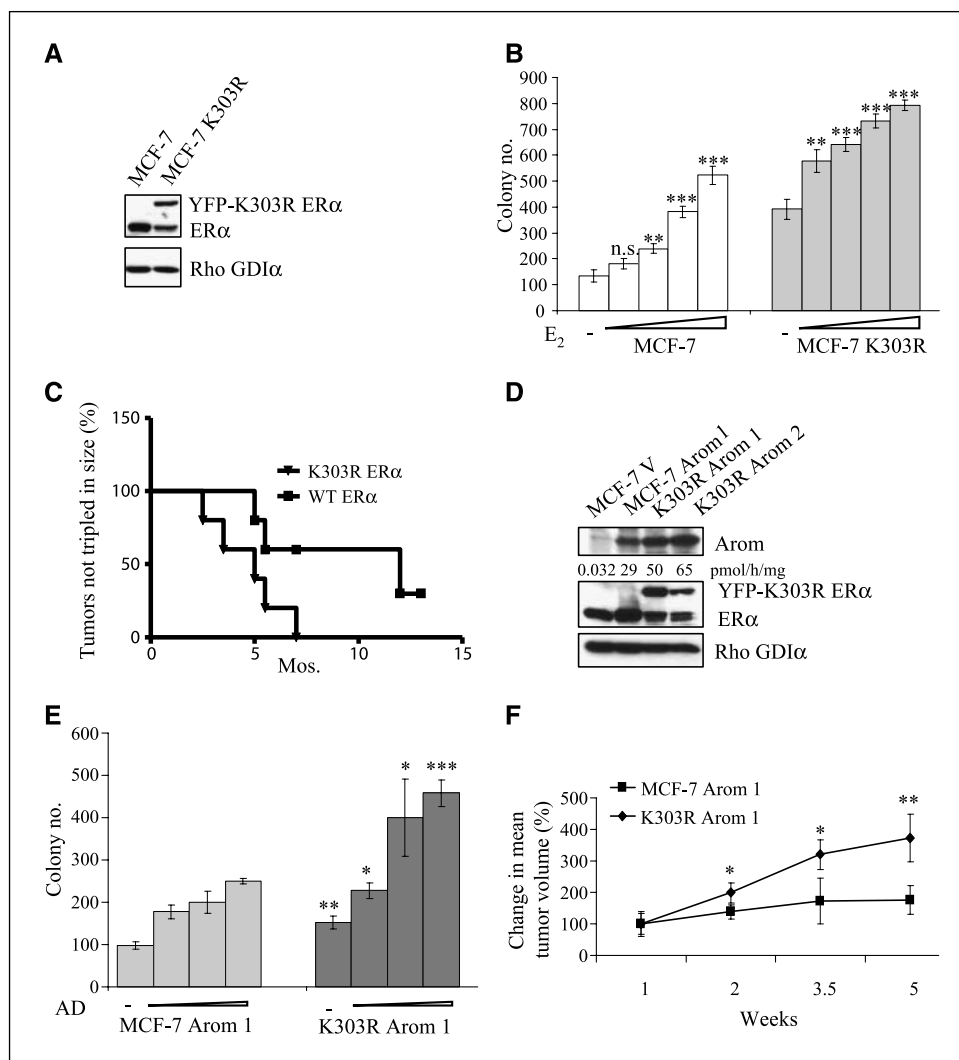
Figure 1. Characterization of K303R ERα-expressing cells. *A*, immunoblotting showing ERα and Rho GDIα (loading control) in MCF-7 parental (*MCF-7*) and YFP-K303R ERα-cells (*MCF-7 K303R*). *B*, anchorage-independent growth assay in cells treated with vehicle or 17β-estradiol (E_2, 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9} mol/L). *Bars*, SD; *n.s.*, nonsignificant (**, $P < 0.005$; ***, $P < 0.0005$ versus vehicle). *C*, WT ERα and K303R ERα cells were injected into mice ($n = 5$/group) supplemented with E_2. Tumor growth was plotted as the time for tumor tripling [months (*mos*)]. *D*, immunoblotting showing aromatase (*Arom*), ERα and Rho GDIα. Numbers below blots represent aromatase activity. *E*, anchorage-independent growth assay in cells treated with vehicle or 4-androstene-3,17-dione (*AD*, 1, 10, and 25 nmol/L). *Bars*, SD (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0005$). K303R Arom 1 group versus the respective treatment in MCF-7 Arom 1 group. *F*, MCF-7 Arom 1 and K303R Arom 1 cells were injected into mice, and supplemented with AD. Tumor growth was plotted as the percentage of change in mean tumor volume/animal ($n = 5$) compared with day 0 of treatment ± SD (*, $P < 0.05$; **, $P < 0.01$ K303R Arom 1 group versus MCF-7 Arom 1 group).

expressing exogenous WT or mutant receptor were evaluated in soft agar assays (Fig. 2*C, bottom*). The basal growth of K303R ERα−Arom pools (P) was significantly higher than WT pools. Inhibition of aromatase activity by Ana caused a significant reduction in AD-stimulated growth in WT ERα Arom cells, but Ana was unable to reduce AD-stimulated mutant cell growth, confirming our stably transfected clones results. In the mutant pool AD + Ana stimulated growth compared with AD treatment alone, which may be a variable clonal effect. We also generated stable pools of YFP-tagged ERα in ER-negative, aromatase-positive CHO cells (Fig. 2*D*). Ana did not reverse AD-stimulated growth in CHO mutant cells, confirming that the AI^R phenotype was associated with expression of the mutant in a number of different cellular backgrounds. We also examined whether there was cross-resistance to the nonsteroidal AI Exe (Supplementary Fig. S1*A*). Exe treatment decreased AD-stimulated growth of mutant cells, although Exe was unable to reduce their high basal nonstimulated growth. Aromatase activity was effectively reduced by both Ana and Exe treatment (Supplementary Fig. S1*B*). We are currently examining the mechanism(s) associated with the partial response to Exe in mutant-expressing cells.

K303R ERα aromatase cells exhibit enhanced PI3K/Akt signaling. Previous studies have revealed the importance of

increased MAPK activity and/or PI3K signaling in the adaptation of ERα-positive cells to long-term estrogen deprivation or AI treatment (1–4, 6). To explore the mechanisms underlying the mutant AI^R, we focused on short-term signaling events. Cells were treated with increasing AD concentrations (Fig. 3*A*) for different times (Fig. 3*B*). MCF-7 Arom 1 cells showed a low basal level of pAkt that was increased in a dose-dependent and time-dependent manner by AD treatment. In contrast, mutant cells showed elevated constitutive pAkt that was not further increased by AD; no changes in phosphorylated p42/44 ERK1,2/MAPK levels were seen (data not shown). pAkt levels were also increased ∼4-fold in mutant tumor xenografts compared with MCF-7 Arom 1−over-expressing tumors (Fig. 3*C*).

We next investigated the expression and activation of Akt in cells treated with Ana (Fig. 3*D*). MCF-7 Arom-1 cells showed slightly increased levels of pAkt following AD treatment that was reduced with Ana cotreatment, whereas K303R Arom 1 and Arom 2– expressing cells showed elevated constitutive pAkt that was unaffected by Ana. It has been shown that treatment with the antiestrogen ICI182,780 leads to a rapid down-regulation in ERα levels (20); there were no changes in pAkt with short-term treatment (Fig. 3*E*). Treatment with ICI182,780 (1–48 h) reduced ERα levels in MCF-7 Arom 1, but not basal pAkt; in contrast,

ICI treatment resulted in a decrease in pAkt levels in mutant cells, concomitant with a reduction in YFP-ERα levels (Fig. 3F). This suggests that activation of pAkt may be related to nongenomic activities of the mutant receptor, as short-term treatments did not block Akt phosphorylation, but are known to block ERα's genomic activities (21).

ERα can bind to the p85α regulatory subunit of PI3K and activate PI3K/Akt signaling in cells (22). We have previously shown that the mutant ERα receptor exhibits altered binding with several nuclear receptor coregulatory proteins, such as the TIF-2 coactivator, and for the ERBB2 (HER2) oncogene when compared with WT ERα (12, 18). We next examined whether the mutation might alter binding with the PI3K subunit p85, and alter PI3K activity. ER-negative CHO cells were transiently transfected with YFP-tagged receptors, and coimmunoprecipitation studies were performed. Enhanced binding of the p85 subunit to mutant receptor in the absence of ligand was observed (Fig. 4A). Equal amounts of lysates of CHO transfected cells (CHO + WT or K303R) or stable pools (CHO WT or K303R P; Fig. 4B) were immunoprecipitated with an anti-p85 antibody, and kinase activity was determined. Mutant ERα either transiently expressed or in stable pools significantly induced PI3K activity. We also performed an *in vitro* PI3K assay in our stably transfected MCF-7 clones, and found constitutively increased PI3K activity in the mutant cells, which was abrogated by PI3K inhibitor

LY294002 treatment (Fig. 4C). We also detected elevated constitutive pAkt in CHO cells transiently or stably expressing the mutant receptor (Fig. 4D). These data support a mechanism whereby enhanced binding of the regulatory subunit of PI3K results in enhanced downstream Akt signaling in the mutant cells.

Phosphorylation of ERα by a number of kinases is an important mechanism by which ERα activity can be regulated (23–25). Akt kinase phosphorylates ERα at serine (S) 167. Increased levels of pS167 YFP-ERα were found in K303R mutant cells (Fig. 4D), and the mutant increased ERE-transcriptional activity $\sim$ 8-fold (Fig. 4E). ERE activity was inhibited by treatment with either the PI3K inhibitor LY294002, or the antiestrogen ICI182,780. Thus, Akt may serve as a functional link between nongenomic and genomic ERα activities, engaging in a bidirectional cross-talk that may set up a vicious cycle between these two growth-regulatory pathways and augment signaling between them.

K303R ERα-aromatase cells exhibited altered apoptotic responses. Akt/protein kinase B signaling is involved in the control of survival and apoptosis (26, 27), and AIs induce cell death by apoptosis (28). This suggestion led us to question if the K303R ERα mutation could protect cells from apoptosis induced by Ana, providing these cells with a potential survival advantage. We first evaluated PARP proteolysis, and found a reduction in the basal levels of the proteolytic form of PARP (85 kDa) in mutant cells

Figure 2. K303R ERα mutation confers resistance to anastrozole. *A,* MTT growth assays in cells treated with vehicle, E$_2$ (1 nmol/L), AD (10 nmol/L), and/or anastrozole (Ana 100 nmol/L, 1 μmol/L, 10 μmol/L). Cell proliferation is expressed as fold change relative to vehicle-treated cells. Data are representative of three independent experiments, performed in quadruplicate. *Columns,* mean; *bars,* SD (**, *P* < 0.005; *n.s.,* nonsignificant AD versus Ana + AD). *B,* anchorage-independent growth assay in cells treated with vehicle, E$_2$ (1 nmol/L), AD (10 nmol/L) $\pm$ Ana (1 μmol/L). *Bars,* SD (*, *P* = 0.01; *n.s.,* nonsignificant AD versus Ana + AD). *C* and *D,* anchorage-independent growth assay in MCF-7 Arom P (*C*) and CHO P (*D*) pools treated with vehicle, AD (10 nmol/L) $\pm$ Ana (1 μmol/L). *Bars,* SD (**, *P* < 0.01; *n.s.,* nonsignificant AD versus Ana + AD). *C* and *D,* ERα and Rho GDIα (*top*). Numbers below blots represent aromatase activity.
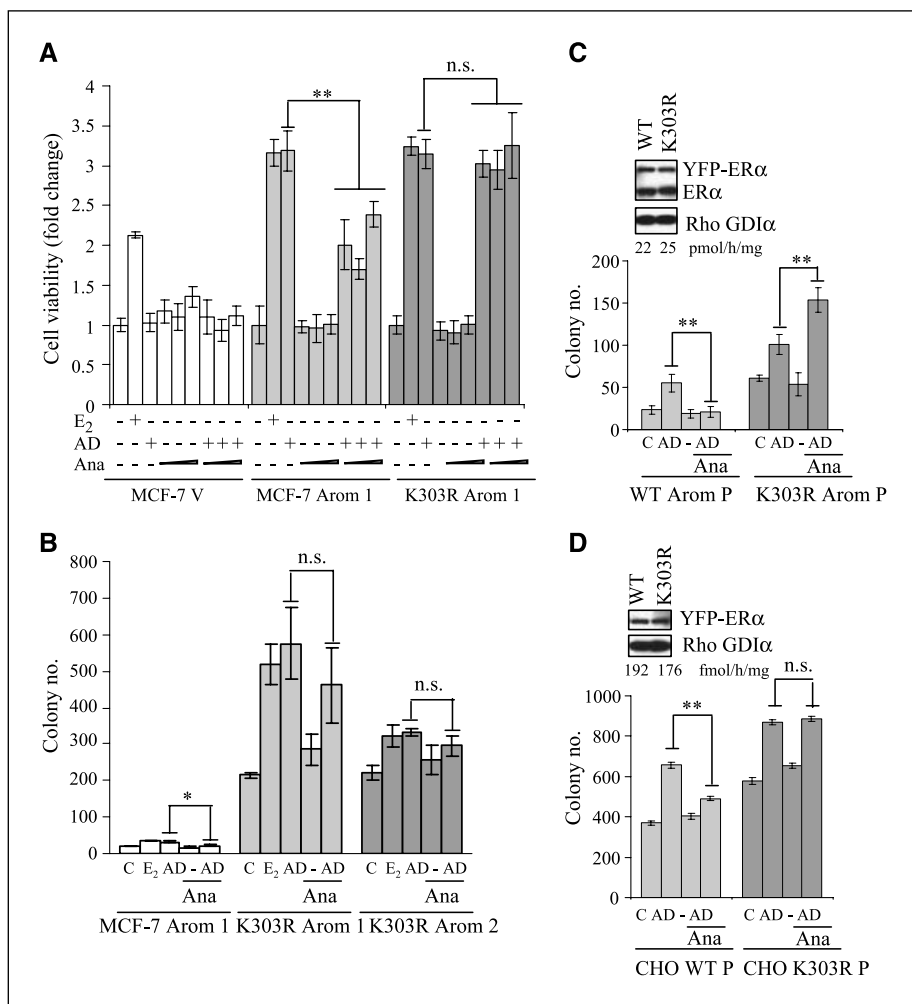
under control conditions (Fig. 5*A*). AD treatment reduced its levels in a time-dependent fashion in WT and mutant cells, but this reduction was less pronounced in WT cells. E$_2$ was able to reduce PARP-cleavage levels only in mutant cells, suggesting that reduced apoptosis may underlie their hypersensitivity. Ana treatment of MCF-7 Arom 1–expressing cells increased proteolysis compared with AD treatment; cleavage was unchanged in mutant cells (Fig. 5*B*). In addition, we found an increase in the Bcl-2/Bax ratio in K303R Arom 1–expressing cells, which was further increased with AD and Ana treatments (Fig. 5*C* and *D*).

To determine the levels of cellular apoptosis, we also used ELISA cell death detection assays (Fig. 5*E*), and found that AD-stimulated mutant cells exhibited a lower apoptosis compared with MCF-7 Arom 1–expressing cells, suggesting that the proliferative advantage provided by the mutation may be achieved by a decreased apoptotic response of these cells. In addition, Ana treatment induced an increase in cell apoptosis only in MCF-7 Arom 1 cells. We hypothesize that mutant-expressing cells are resistant to AI-induced cellular apoptosis.

The AI^R phenotype is dependent on the PI3K/Akt pathway. We next addressed whether activated PI3K/Akt signaling may be a functional mechanism of resistance to AI therapies using PI3K/Akt

inhibitors. To define the effective dose, mutant cells were treated with different doses of these agents and analyzed for pAkt. LY (10 μmol/L), PI-103 (1 μmol/L), Akti1/2 (1 μmol/L) effectively blocked basal Akt phosphorylation (Fig. 6*A*). We then performed soft agar growth assays. LY completely inhibited mutant proliferation, whereas it induced a slight reduction in the growth of MCF-7 Arom 1 cells (Fig. 6*B*). Similar results were obtained using the PI-103 inhibitor in another mutant clone (K303R Arom 2; data not shown). Because PI3K inhibitors affect not just all three Akt isozymes, but also other PH domain-containing molecules (29), we tested a specific Akt inhibitor, Akti1/2 (Fig. 6*C*), and found that basal, AD-stimulated and Ana-stimulated growth was inhibited by ∼30% in MCF-7 Arom 1–expressing cells, whereas growth was inhibited by >90% in mutant cells. These results suggest that Akt signaling is essential for the growth of mutant cells, but may not play an important role in the growth of WT cells.

We also used a MEK1 inhibitor (PD98059) in soft agar assays (Fig. 6*D*); antiproliferative effects were not observed and PD treatment was unable to reverse the AI^R mutant phenotype. These results confirm that the p44/42 Erk1,2/MAPK pathway was not involved in the AI^R mutant phenotype. We also used ICI182,780, and it suppressed the colony growth of both cell lines, indicating
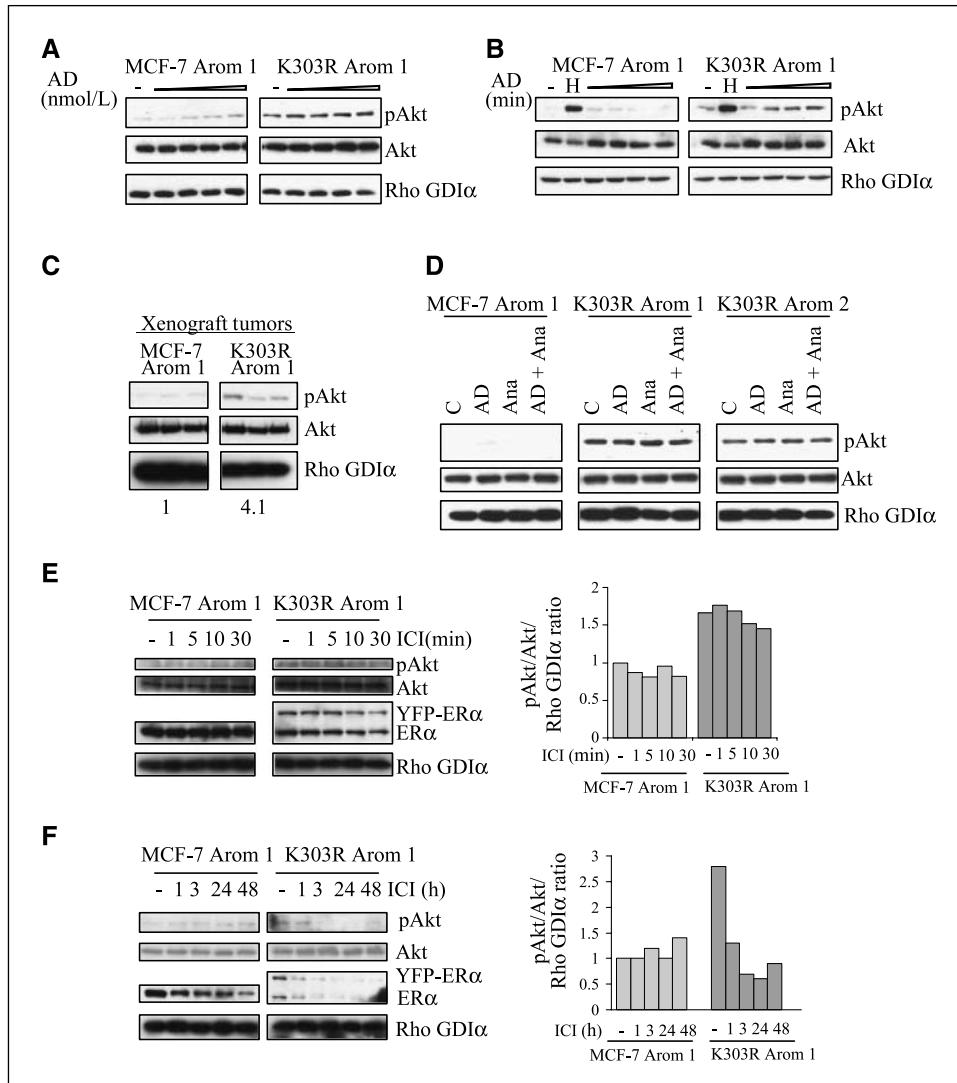

Figure 3. Mutant cells exhibited constitutive pAkt. *A* to *F,* immunoblotting showing phosphorylated Akt (*pAkt*), Akt, and Rho GDIα in cells treated with vehicle or AD 1, 10, 25, and 50 nmol/L (1 h, *A*), or AD 10, 30, 60, and 120 min (10 nmol/L, *B*), or heregulin 2 ng/mL (H 5 min, *B*); in xenograft tumors extracts (*C,* numbers below blots represent the average fold change in pAkt levels of K303R Arom 1 samples versus MCF-7 Arom 1 samples); in cells treated with vehicle, AD 10 nmol/L ± Ana 1 μmol/L (1 h, *D*); in cells treated with vehicle and ICI 1 μmol/L for 1, 5, 10, and 30 min (*E*) or 1, 3, 24, and 48 h (*F*). Quantitative analysis is the fold difference in pAkt/Akt/Rho GDIα ratio relative to vehicle-treated MCF-7 Arom 1 cells.
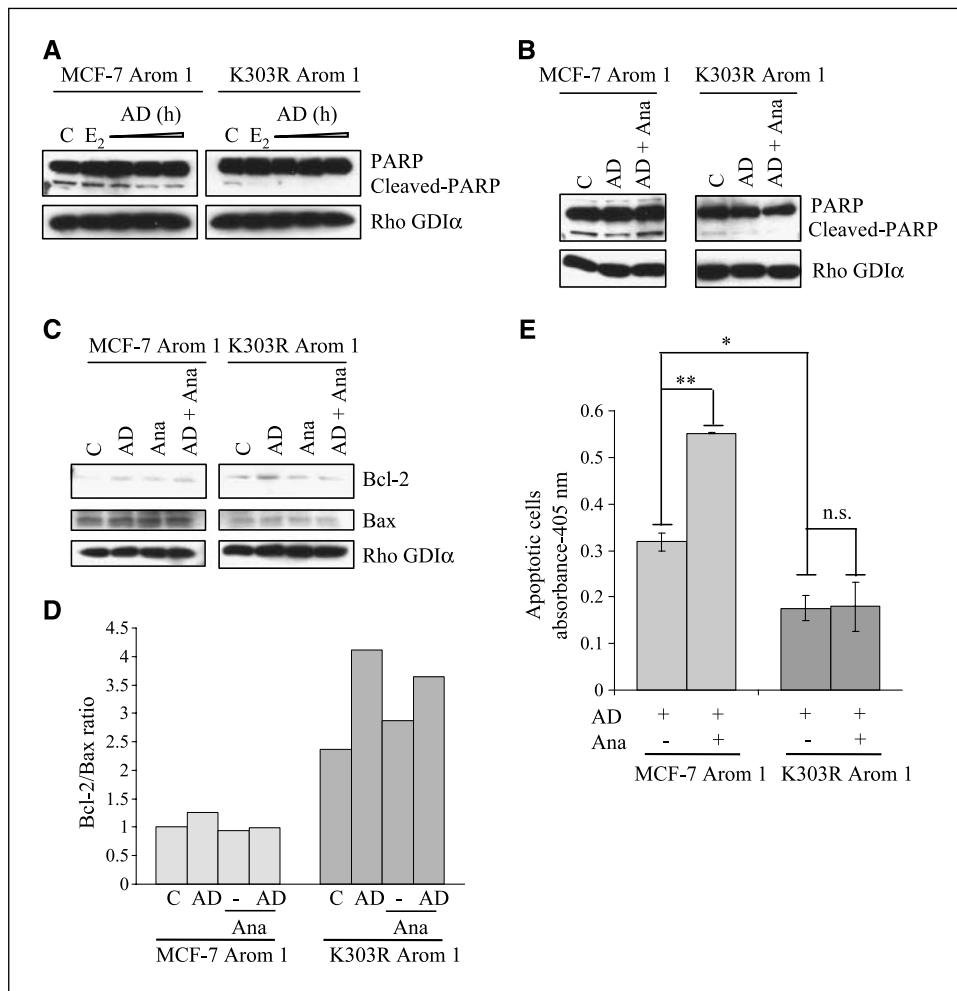
Figure 5. Altered apoptotic response in mutant cells. *A* to *C,* immunoblotting showing PARP and Rho GDIα in cells treated with vehicle, E_2 1 nmol/L (24 h), AD 10 nmol/L (12, 24, and 48 h, *A*), or AD 10 nmol/L and AD + Ana 1 μmol/L (24 h, *B*); Bcl-2, Bax, and Rho GDIα in cells treated with vehicle, AD 10 nmol/L ± Ana 1 μmol/L (24 h, *C*). *D,* quantitative analysis is the fold difference in Bcl-2/Bax ratio relative to vehicle-treated MCF-7 Arom 1 cells. *E,* ELISA cell detection assay in cells treated with AD 10 nmol/L ± Ana 1 μmol/L. *Columns,* mean; *bars,* SD (*, $P < 0.05$, **, $P < 0.005$, *n.s.,* nonsignificant AD versus Ana + AD).

detect increased HER2 levels in our aromatase-overexpressing mutant cells (data not shown), highlighting the uniqueness of our resistance model. K303R ERα-expressing cells exhibited constitutive activation of the PI3K/Akt signaling pathway, and increased binding to the p85α regulatory subunit of PI3K that led to increased PI3K activity and downstream Akt phosphorylation. The increased levels of phosphorylated Akt in mutant-expressing cells were associated with a specific increase in ERα pS167, a known Akt phosphorylation site. In agreement with these data, we found that the mutant had enhanced transcriptional activation, which was inhibited by treatment with either the PI3K inhibitor LY294002, or the antiestrogen ICI182,780. This suggests that Akt may serve as a functional link between both cytoplasmic and nuclear ERα functions, resulting in an integrated bidirectional signaling that promoted and sustained the proliferation of mutant cells. Despite the extensive disruption in the genome of cancer cells (34, 35), there are examples whereby the reversal of only one or a few of these abnormalities can profoundly inhibit the cancer growth (36). PI3K/Akt inhibition drastically inhibited the growth of mutant cells suggesting an exquisite "dependency" of the mutant cells on the PI3K/Akt pathway for maintenance of AI^R.

It has been reported that estrogen deprivation, antiestrogen, or AI treatment induced apoptotic cell death (28, 37). We found a lower frequency of apoptosis in AD-stimulated K303R ERα-expressing cells, suggesting an altered apoptotic response in these cells. In addition, Ana treatment induced a significant increase in cell apoptosis in WT cells, but no changes were observed in the apoptotic response of mutant-expressing cells. It has been proposed that Bcl-2/Bax protein ratios may determine whether a cell will undergo apoptosis (38). We found an increased Bcl-2/Bax ratio in the mutant cells that was further increased after AD and Ana treatments, confirming a potential survival advantage for the mutant receptor cells.

There are relatively few mutations which have been reported in the ERα gene, which is surprising because many clinical resistance mechanisms involve mutation of the target. The K303R ERα mutation was associated with poor outcomes in univariate analyses, and its presence was correlated with older age, larger tumor size, and lymph node–positive disease, all factors associated with worse outcomes (13). Four other studies did not detect the mutation in invasive cancers (39–42), but our studies suggest that the detection method used might be insensitive. However, the K303R mutation has been reported, but at low frequency in invasive breast tumors by Conway and colleagues (43). Molecular analyses have shown that the mutated arginine at the 303 position allows ERα to be more highly phosphorylated by protein kinase A (14) and Akt kinase signaling.[6] We have

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[6] Our unpublished data.

Figure 4. Mutant cells showed increased PI3K/Akt activation. *A,* lysates from CHO cells transiently transfected with WT or K303R ERα were immunoprecipitated (*IP*) with anti-p85 or anti-ERα and immunoblotted for ERα or p85. *Input,* whole-cell lysates. *B and C, in vitro* PI3K activity of p85 immunoprecipitates from CHO cells transiently transfected with YFP-WT or YFP-K303R ERα, and CHO WT P and CHO K303R P pools (*B*) or MCF-7 Arom 1 and K303R Arom 1 cells treated with vehicle or LY294002 (LY 10 μmol/L) for 45 min (*C*). The enzyme activity was expressed as amounts of PIP$_3$ (in picomoles) produced by cells. *Columns,* means from triplicate readings in two experiments; *bars,* SD (*, $P < 0.05$; **, $P = 0.001$). *B and C,* p85 expression in immunoprecipitates (*top*). *D,* immunoblotting of pAkt, Akt, phosphorylated Ser167-ERα (*pS167*), YFP-ERα, and Rho GDIα in CHO cells transiently transfected with YFP-WT or YFP-K303R ERα, or CHO WT P and CHO K303R P pools. *E,* CHO cells were transiently transfected with YFP-WT or YFP-K303R ERα plus an ERE-luciferase reporter, and treated with vehicle, LY (10 μmol/L), or ICI (1 μmol/L). Luciferase activities were normalized to β-galactosidase for relative luciferase units. Data are representative of three independent experiments, performed in triplicate. *Columns,* mean; *bars,* SD (***, $P = 0.0008$ versus vehicle). ERα and Rho GDIα expression was determined by immunoblotting (*right*).
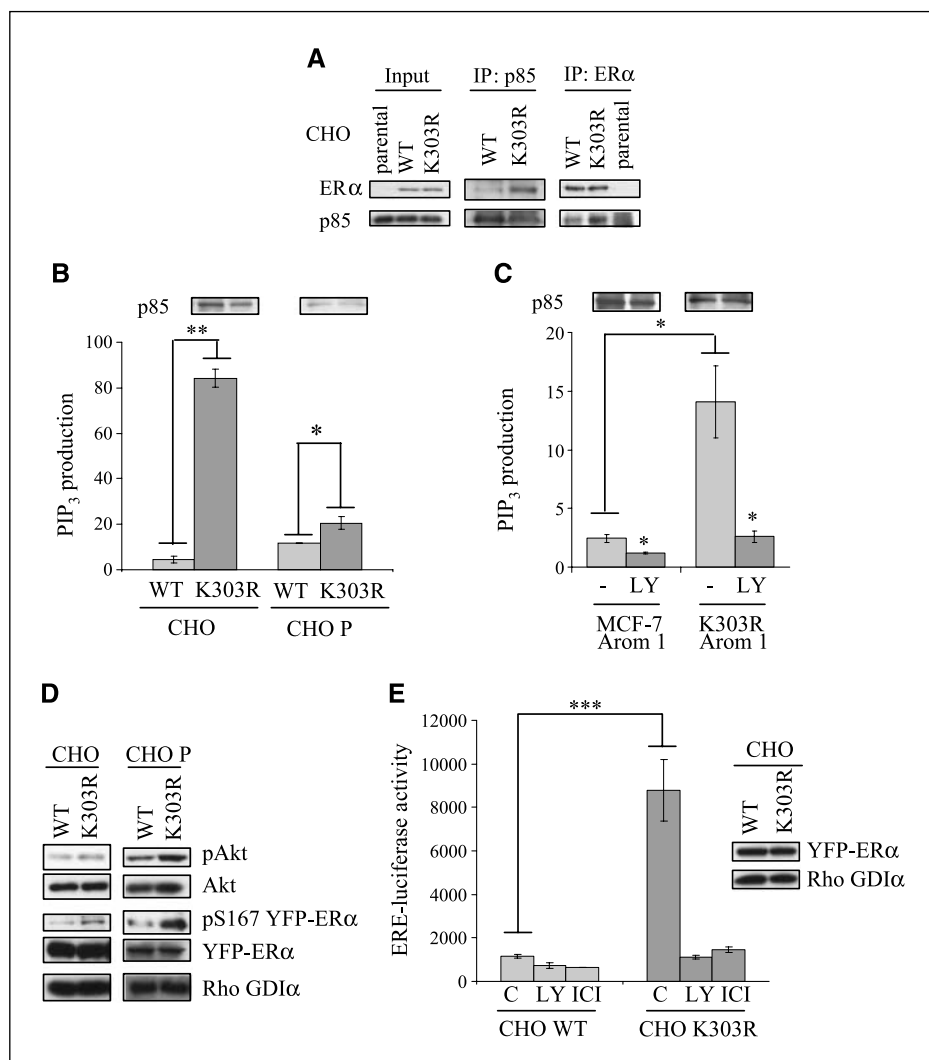
that ERα expression remained important in their growth regulation (Fig. 6*D*).

We next evaluated the effects of the PI3K inhibitor LY294002 on apoptosis using an ELISA cell death detection assay (Fig. 6*E*), and found that LY treatment in MCF-7 Arom 1 cells showed similar apoptosis induction as Ana-treated cells (1.7-fold increase), whereas inhibitor treatment of mutant cells was able to induce a 3.6-fold increase in the apoptotic rate compared with AD, and most significantly, after Ana treatment. We conclude that the mutation provides a selective dependency on the PI3K/Akt survival pathway for sustained proliferation and/or survival. We suggest that inhibition of this potential pathway addiction may provide an effective means to reverse AI^R associated with the expression of K303R ERα mutation.

Discussion

Despite significant advances in the treatment of breast cancer following the introduction of AIs (30, 31), *de novo* and acquired resistance remains a major clinical concern. Here, we report that expression of the K303R ERα mutant conferred resistance to the AI anastrozole, and show that resistance occurs through a constitutive

activation of the PI3K/Akt prosurvival signaling pathway to which the mutant cells have become addicted for maintenance of growth.

Breast cancer cells can adapt in response to the selective pressures exerted by exposure to estrogen deprivation therapy by developing enhanced sensitivity to the proliferative effects of low estrogen levels (32). A number of laboratories have developed *in vitro* models with long-term estrogen deprivation (1–5), and they propose a dynamic interplay between growth factor signaling and ERα action. Letrozole resistance involves cross-talk between the HER2 growth factor receptor pathway and ERα, leading to activation of Erk1,2/MAPK and ERα phosphorylation (6). Herein, we show that a naturally occurring mutation of ERα, termed K303R, conferred hypersensitivity to estradiol, and show that the mutant ERα in aromatase-expressing cells exhibited enhanced growth in basal conditions, as well as with AD treatment. We found that the hypersensitive phenotype associated with mutant expression resulted in the acquisition of resistance to the AI anastrozole. The growth of mutant cells was suppressed by fulvestrant treatment, indicating that ERα remained essential for growth.

There is extensive evidence to support the role of HER2 as a mediator of resistance to endocrine therapy (33), but we did not

Figure 6. Inhibition of PI3K/Akt pathway reversed AI^R. *A,* immunoblotting showing phosphorylated Akt and Akt in cells treated with vehicle, LY 10 μmol/L or PI-103 (1, 5, and 10 μmol/L) or Akti1/2 (1, 5, and 10 μmol/L) for 30 min. *B to D,* anchorage-independent growth assay in cells treated with vehicle, AD 10 nmol/L, Ana 1 μmol/L ± LY 10 μmol/L (*B*) or Akti1/2 1 μmol/L (*C*) or PD98059 (PD 10 μmol/L, *D*) or ICI 1 μmol/L (*D*). *Bars,* SD (***, *P* < 0.0005 LY, Akti1/2 and ICI-treated cells versus control cells and cells treated with AD or Ana + AD). *E,* cell death detection assay in cells treated with AD 10 nmol/L ± Ana 1 μmol/L ± LY 10 μmol/L. *Columns,* mean; *bars,* SD (*, *P* < 0.05 versus AD in MCF-7 Arom 1 and AD and Ana + AD in K303R Arom 1).
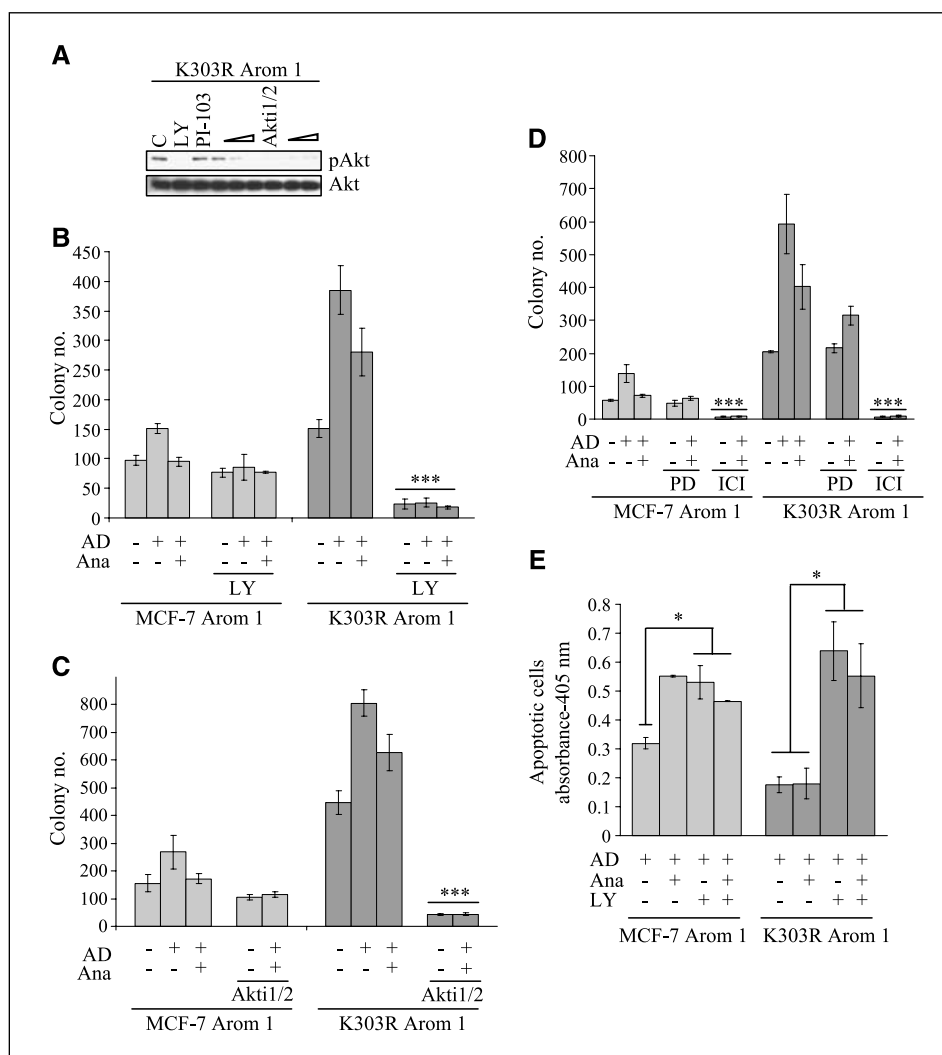
previously shown that overexpression of the K303R ERα mutation in ERα-positive MCF-7 breast cancer cells conferred estrogen hypersensitivity *in vitro* (12, 14), and decreased sensitivity to tamoxifen treatment when engaged in cross-talk with growth factor receptor signaling (18). Here, we provide a unique model of endocrine resistance, whereby expression of the K303R ERα mutant receptor conferred resistance to the AI anastrozole. The potential mechanisms of resistance include cellular strategies to evade apoptosis, and increased proliferation through an enhanced cross-talk between the mutant receptor and the PI3K/Akt signaling pathway.

There are two major clinical implications of our study. First, because K303R mutant cells may escape from growth inhibition when treated with AIs, genetic assays for the mutation might offer a new predictive marker for hormonal response. Second, these data

support the development of treatment strategies utilizing signal transduction inhibitors, which may be used to extend the duration of sensitivity to estrogen deprivation, or to reverse resistance at its time of emergence.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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PRECLINICAL STUDY

Growth factor-induced resistance to tamoxifen is associated with a mutation of estrogen receptor α and its phosphorylation at serine 305

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Abstract Estrogens play a crucial role in breast tumor growth, which is the rationale for the use of antiestrogens, such as tamoxifen, in women with estrogen receptor (ER)-α-positive breast cancer. However, hormone resistance is a major clinical problem. Altered growth factor signaling to the ERα pathway has been shown to be associated with the development of clinical resistance. We previously have identified a mutation that replaces arginine for lysine at residue 303 (K303R) of ERα, which confers hypersensitive growth in low levels of estrogen. To determine if the K303R mutation could participate in the evolution of hormone resistance, we generated MCF-7 breast cancer cells stably transfected with either wild-type (WT) or K303R ERα. We found that the mutation confers decreased sensitivity to tamoxifen in the presence of the growth factor heregulin, using anchorage-independent growth assays. K303R ERα-expressing cells were hypersensitive to growth factor signals. Our data suggest that phosphorylation of serine 305 within the hinge domain of ERα might play a key role in increasing ligand-independent activity of the mutant receptor. We hypothesize that the mutation adapts the receptor for enhanced bidirectional cross-talk with the HER2 growth factor receptor pathway, which then impacts on responsiveness to tamoxifen.

Keywords Breast cancer · Estrogen receptor · K303R mutant ERα · HER2

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Introduction

Estrogens play a crucial role in regulating the growth and differentiation of normal breast epithelium and also of breast cancers, with approximately two-thirds of all breast cancers dependent for their growth on a functional estrogen receptor α (ERα). ERα is a member of the nuclear hormone receptor superfamily that regulates transcription of ER target genes by binding with specific estrogen response elements [1]. However, ERα also regulates the expression of many genes without direct binding to DNA. This occurs via protein–protein interactions with other transcription factors, such as activator protein-1, and with extranuclear signaling complexes that, in turn, modulate downstream gene expression [2, 3]. Therapeutic strategies directed at inhibiting the action of ERα using antiestrogens, such as tamoxifen (Tam), or reducing estrogen levels using aromatase inhibitors, are the standard therapies offered to women with ERα-positive cancer. However, not all patients who have ER positive tumors respond to endocrine therapies (termed de novo resistance), and a large number of patients who do respond will eventually develop disease

progression or recurrence while on therapy (acquired resistance).

In previous work we identified an A to G somatic mutation at ERα nucleotide 908 (A908G) in early premalignant breast lesions [4]. This transition introduces a lysine to arginine substitution at residue 303 (K303R ERα) within exon 4, at the border between the hinge and the hormone-binding domain of the receptor; the mutation confers increased sensitivity to subphysiological levels of estrogen [4]. Others have reported that the K303R ERα mutation was not present in invasive tumors [5, 6], but we have demonstrated that the detection method used by these other investigators, standard dye-terminator sequencing, was not sensitive for detection of this specific mutation [7]. In addition, Conway et al. [8] have identified this mutation in 6% of breast cancers using a different detection method. Therefore, the exact frequency of this mutation in breast tumors remains to be established. The mutation resides at major post-translational modifications sites (acetylation, ubiquitination, methylation) adjacent to a protein kinase A (PKA) phosphorylation site at serine residue 305 (S305). Cui et al. [9] have demonstrated that this naturally occurring mutation is a more efficient substrate for phosphorylation by PKA, and is hypoacetylated which subsequently alters estrogen sensitivity. Michalides et al. [10] have suggested that phosphorylation of ERα S305 by PKA induces a switch from antagonistic to agonistic effects of Tam, which induces resistance to this antiestrogen. It has also been shown that the ERα S305 site can be an in vivo substrate for p21-activated kinase 1 (PAK 1)-mediated phosphorylation, and that activation of ERα S305 might confer a conformational change which allows for a better interaction with ligands such as Tam [11, 12].

We know that estrogen regulation of breast cancer cell growth can also be modulated by complex interactions with a variety of peptide growth factors. A large body of evidence supports the idea that rapid membrane effects of ERα may activate various components of growth factor tyrosine kinase signaling, such as that from insulin-like growth factor-IR (IGF-IR), epidermal growth factor receptor (EGFR), and c-erbB2/HER2 [13–15]. Furthermore, the kinase cascade signaling initiated by growth factor receptors can activate ERα (termed ligand-independent effects) and its coregulatory proteins, causing an interdependent loop of cross-talk that leads to enhanced tumor cell survival and proliferation [16–19]. Moreover, several preclinical and clinical studies suggest that overexpression of EGFR or HER2, and/or high levels of phosphorylated Akt and extracellular signal-regulated kinases (ERKs) in breast cancers contribute to Tam resistance [20–25]. We therefore hypothesized that the K303R mutation adapts ERα for enhanced reception of intracellular signal transduction, which leads to antiestrogen resistance. To test this hypothesis we used as an experimental model of MCF-7 breast cancer cells stably transfected with either wild-type (WT) or the K303R mutant ERα. We found that cells expressing the estrogen hypersensitive K303R ERα mutant showed elevated levels of growth factor signaling, and enhanced cross-talk between the mutant and the HER2 growth factor receptor. These results suggest that the presence of the A908G ERα somatic mutation may be useful as a predictive marker of hormonal response in patients whose tumors exploit ERα and/or growth factor cross-talk to evade treatment.

Materials and methods

Reagents, hormones and antibodies

17β-Estradiol (E$_2$), 4-Hydroxytamoxifen (4-OH), Epidermal growth factor (EGF), Insulin like growth factor-1 (IGF-1), and Heregulin (H) were from Sigma (St. Louis, MO). Herceptin was form Genentech (San Francisco, CA) Antibodies used for immunoblotting were: ERα (6F11) from (Novocastra, Newcastle, United Kingdom), progesterone receptor (PR) from DAKO (Carpinteria, CA), Rho GDIα from Santa Cruz Biotechnology (Santa Cruz, CA), total MAPK, total Akt, total c-Src, phosphorylated p42/44 MAPK (Thr^{202}/Tyr^{204}), Akt (Ser^{437}), c-Src (Tyr^{416}) from Cell Signaling Technology (Beverly, MA), total HER2 from NeoMarker (Fremont, CA), phosphorylated HER2 (Tyr^{1248}), phosphor-ER-S305 from UPSTATE (Temecula, CA), and Living colors^{TM} Full Length polyclonal antibody (Clontech, Mountain View, CA).

Cell culture

MCF-7 breast cancer cells, originally obtained from Dr. Benita Katzenellenbogen (University of Illinois, Urbana, IL), were maintained on plastic in minimal essential medium (MEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Summit Biotechnology, Fort Collins, CO), 0.1 nmol/l nonessential amino acid, 2 mmol/l L-glutamine, 50 units/ml penicillin/streptomycin, at 37°C with 5% CO$_2$/95% air. HeLa cells were obtained form American Type Culture Collection (Manassas, VA), and were maintained in the same media. Generation of the yellow-fluorescent protein (YFP)-tagged expression constructs, YFP-WT ERα and YFP-K303R ERα, has been previously described [9]. MCF-7 cells were stably transfected using Fugene 6 according to the manufacturer's instructions (Roche, Indianapolis, IN), and individual clones were isolated and expanded with G418 selection. In some experiments we used a pool of stably transfected cells selected for 1 week with G418 antibiotic. Stably transfected clones were screened for expression of exogenous and endogenous ERα using immunoblot analysis.

Quantitative image analyses by high throughput microscopy

PRL-Hela is a cell line specifically engineered for the single cell study of ER function [26]. PRL-Hela cells contain multiple genomic integrations of a replicated prolactin (PRL) promoter/enhancer. The multiple integrations (PRL array) are spatially confined and are visualized by the accumulation of fluorescently tagged ERα. PRL-Hela cells transiently expressing YFP-WT ERα or YFP-K303R ERα, pretreated with forskolin (10 μM) for 15 min, and then treated with increasing concentrations of E_2 or Tam for 30 min, were fixed and DAPI-stained as previously reported [26, 27]. The cells were imaged using the Cell Lab IC 100 Image Cytometer (Beckman Coulter, Fullerton, CA) with a Nikon 40X Plan S flour 0.90 NA objective. Two channels were imaged: channel 0 (DAPI stain) was used to find the focus and nuclei, when channel 1 was used to image YFP ERα. A proprietary algorithm (GPRC) developed at Beckman Coulter was used to identify and quantify the YFP-ERα-targeted PRL array. The parameters for the GPRC algorithm were: object scale = 30 and minimum peak height = 10. Foci identified by the GPRC algorithm were masked. The area of the mask in pixels was the measure of PRL array size. Channel 1 was offset 2 μm from DAPI focused for cells in all treatment conditions. After image acquisition and application of the GPRC algorithm, the total cell populations for each treatment were progressively filtered (gated) using the same criteria. Nuclei clusters, and mitotic cells were filtered from the total cell population using an intersection of DNA content and DNA clusters gates. In addition, low YFP ERα expression and low aggregate number gates were generated and applied to produce the final cell population to be analyzed. From the final population of cells, the array size was determined using the GPRC mask [28]. The images and masks were visually inspected for accuracy. Unpaired student's t-test assuming equal variance was performed to determine statistical significance (two-tailed, $P < 0.05$). Standard deviations are shown.

Immunoprecipitation and immunoblot analysis

Cells were starved in phenol red free MEM with 5% charcoal-stripped FBS for 48 h and treated as indicated before lysis [50 mmol/l Tris–HCl (pH 7.4), 150 mmol/l NaCl, 2% NP40, 0,25% deoxycholic acid, 1 mmol/l EDTA, 1 mmol/l Na_3VO_4, and 1:100 protease inhibitors cocktail; Calbiochem]. For coimmunoprecipitation experiments, we used 1 mg of total cellular protein and a 1:200 dilution of anti-Living colors Full Length polyclonal antisera (Clontech) that recognizes native and denatured forms of recombinant YFP fusion proteins expressed in mammalian cells, and 2 μg of HER2 polyclonal antisera

overnight, followed by protein A/G precipitation with rotation at 4°C for 2 h. Immunoprecipitated proteins were washed thrice with lysis buffer. Equal amounts of cell extract and immunoprecipitated proteins were resolved under denaturing conditions by electrophoresis in 8 to 10% polyacrylamide gels containing SDS (SDS–PAGE), and transferred to nitrocellulose membranes (Schleicher & Schuell, Keen, NH) by electroblotting. After blocking the transferred nitrocellulose membranes were incubated with primary antibodies overnight at 4°C, with secondary antibodies goat anti-mouse or goat anti-rabbit antisera (1:3000; Amersham Biosciences; Piscataway, NJ) for 1 h at room temperature and developed with enhanced chemi luminescence reagents (Alpha Innotech, San Leandro, CA).

Anchorage-independent soft agar growth assays

Cells (5,000/well) were plated in 4 ml of 0.35% agarose with 5% charcoal-stripped FBS in phenol red-free MEM, in a 0.7% agarose base in 6-well plates. Two days after plating, media containing control vehicle or hormonal treatments (E_2 1 nM, heregulin 2 ng/ml, EGF 10 ng/ml, IGF-1 10 ng/ml with or without Tam 100 nM) was added to the top layer, and the appropriate media was replaced every 2 days. In some experiments a pool of stably transfected cells were treated with heregulin with or without herceptin (10 μg/ml). After 14 days, 150 μl of MTT was added to each well and allowed to incubate at 37°C for 4 h. Plates were then placed in 4°C overnight and colonies >50 μm diameter from triplicate assays were counted. Data are the mean colony number of three plates and representative of two independent experiments analyzed for statistical significance ($P < 0.05$) using a two-tailed student's Test, performed by Graph Pad Prism 5 (GraphPad Software, Inc., San Diego, CA). Standard deviations are shown.

Blocking peptide delivery

A blocking peptide of 13 residues (IKRSKKN**S**LALSC) from the sequence (residues 298–310) surrounding the S305 residue (in bold) of the human ERα was transferred into cells using a cationic amphiphile molecule, PULSin™ delivery reagent (Polyplus transfection, Illkirch, France), as suggested by the manufacturer. Briefly, cells were plated in a 6-well plate with regular growth media, and then starved for 48 h in a phenol red free MEM with 5% charcoal stripped FBS. After starvation cells were washed with PBS to remove all traces of serum, and fresh phenol-red free media without serum was added. The mixture containing the S305 blocking peptide (4 μg/well) diluted in 200 μl of 20 nM Hepes, and 16 μl of PULSin™ was incubated for 15 min at room temperature, and then added to the cells.

The media was changed after 4 h of incubation, cells were treated as indicated, and cellular extracts were prepared. Delivery of R-phycoerythrin was used as a positive control and live cells were observed by fluorescence microscopy after 4 h.

Results

Ligand-independent signaling to the K303R ERα mutant reduces Tam sensitivity

We have previously shown that K303R ERα mutant-overexpressing cells display enhanced ligand-independent activity when stimulated with cyclic AMP, in part because the mutation generates a more efficient substrate for PKA-mediated signaling [9]. It is well known that ligand-independent signaling can influence cellular responsiveness to Tam [29]. For instance, PKA-mediated phosphorylation of ERα at S305 allows the antagonist Tam to behave as an agonist, which then results in ERα-dependent transactivation [10]. Here we have utilized the stable cell line PRL-Hela containing a multi-copy integrated prolactin enhancer/promoter DNA array that is responsive to ER such that when the receptor is expressed as a YFP-fusion protein, the integration site can be easily visualized (PRL-array) [26]. High-throughput microscopy has been used to identify ligand-independent changes in the size of the PRL-array which is an indicator of the chromatin condensation status at the promoter [28]. Arrays rapidly decondense after E_2 treatment or condense after antiestrogen treatment (both within minutes). Thus, in PRL-Hela cells, the array size is an indicator of receptor-mediated transcription function in response to different treatments and allows direct and live observation of ER-dependent chromatin remodelling. Therefore, using a mammalian-based, stably transfected prolactin promoter array system, we analyzed live cell dynamics on an estrogen-responsive promoter, to visualize chromatin remodelling induced by E_2 and Tam. We transiently transfected PRL-Hela cells with YFP tagged-ERα WT or the YFP-ERα K303R mutant receptor expression vector, and examined E_2 (Fig. 1a) and Tam's (Fig. 1b) effect on chromatin remodelling in the presence of forskolin, an activator of PKA signaling. Using high through-put microscopy, the amount of chromatin remodelling was quantified [28]. Twenty four hours after transfection cells were pretreated with forskolin (FSK), and then treated with E_2 or Tam at different doses, as indicated, for 30 min. Over 60 nuclei in two separate experiments were analyzed to determine the size of the PRL-reporter array. Vehicle control showed similar values for the array size in the presence of the WT receptor and the mutant (Fig. 1b). In
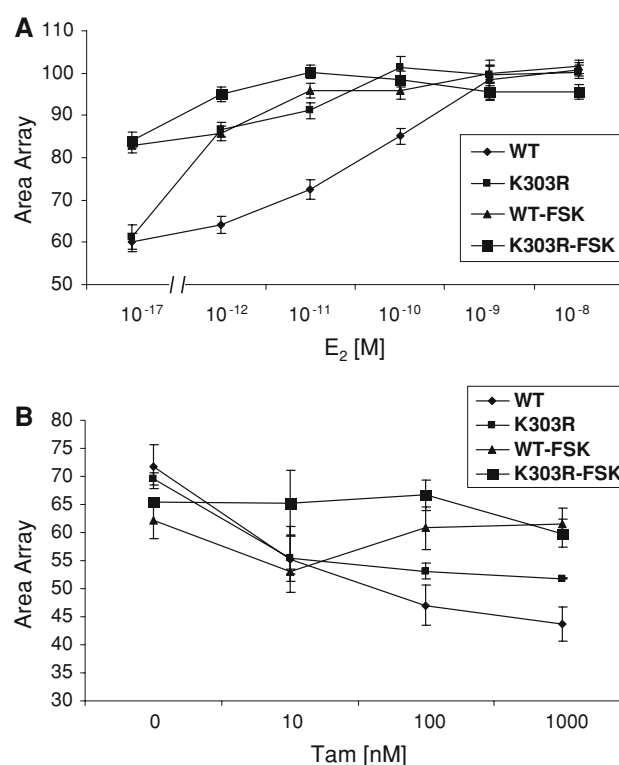

Fig. 1 E_2 and Tam effects on large-scale chromatin structure in PRL-Hela cells. Cells transiently expressing YFP-ERα WT (WT) or the YFP-K303R ERα (K303R) mutation were pretreated with forskolin (FSK) for 15′ min and then were treated with E_2 (a) or Tam (b) at different doses for 30 min. After fixing and counter-staining with DAPI, cells were imaged and array size was quantified using high throughput microscopy (HTM) as described in "Materials and methods". Results are expressed as array size normalized to control cells obtained from three independent experiments

PRL-Hela cells expressing the WT receptor, estrogen treatment increased array size in a dose-dependent manner in estrogen concentrations between 10^{-12} and 10^{-9} M. Mutant-expressing cells showed a linear dose-response in the range of 10^{-17} M through 10^{-11} M of estrogen. Indeed, mutant ERα expressing cells exhibited a much lower EC50 (3×10^{-13} M) compared with WT ERα-expressing cells (5.1×10^{-11} M). These data demonstrate that K303R mutant-expressing cells are hypersensitive to estrogen and form larger arrays in the presence of low estrogen concentrations. With forskolin treatment, WT and mutant ERα cells stimulated with estrogen both displayed significantly increased array sizes. WT ERα-expressing cells demonstrated a one-log reduction in the EC50 (4.2×10^{-12} M ± 0.0038 compared to 5.1×10^{-11} M ± 0.0132 in the absence of forskolin) and a significantly increased average array size (Fig. 1a). Mutant-expressing cells also displayed a significantly increased average size, but without a significant change in the EC50 (8×10^{-13} M ± 612.5 compared to 3×10^{-13} M ± 0.0004 in the absence of forskolin). Thus, forskolin increased average array size,

and reduced the EC50 of WT ERα cells thus enhancing estrogen sensitivity, whereas forskolin treatment of mutant-expressing cells only increased the array size, suggesting that these cells displayed an inherent hypersensitivity to estrogen. As shown in Fig. 1b, Tam treatment decreased the array size in WT ERα-expressing cells, however, the mutant receptor was significantly less responsive to Tam ($P < 0.0001$). Forskolin treatment blocked Tam-induced promoter condensation (reduced size of the array), and the mutant receptor array size was not affected by physiological levels of Tam (100 nM) in the presence of forskolin. All together these results suggest that hormone-independent kinase signaling to the mutant receptor K303R ERα may confer resistance to antiestrogen treatment in breast cancer cells.

MCF-7 K303R-ERα mutant expressing cells exhibit altered growth factor signaling

To further explore ligand-independent activation of signaling in mutant-expressing breast cancer cells, we developed MCF-7 ERα-positive human breast cancer cell lines overexpressing either WT or the K303R ERα mutant. We chose to introduce the mutant receptor into parental WT ERα-expressing cells to simulate the situation in invasive human breast tumors, where WT receptor is most frequently co-expressed along with the mutant [7]. To differentiate the exogenously expressed receptor, we tagged the vector with YFP. Stably transfected clones were screened for expression of ERα using immunoblot analysis (Fig. 2a). Parental MCF-7 cells are shown along with one clone stably expressing YFP-WT, and three clones expressing YFP-K303R ERα (MCF-7 K303R-1-3).

It is well accepted that the response to endocrine therapies in human breast cancer patients correlates with ERα and progesterone receptor (PR) levels. Several studies have shown that patients with ERα/PR-positive breast cancers derive greater benefit from adjuvant hormonal therapy than those patients whose tumors lack PR [30–32], however, it must be noted that other studies have not always found this result [33]. It has been hypothesized that high growth factor signaling activity in breast cancers may be associated with decreased PR levels (for a review see [34]), and elegant studies have shown that up-regulation of pMAPK Erk1/2 leads to a loss of PR via degradation by the 26S proteasome [35]. In clinical samples we have previously reported a borderline significant inverse correlation between the presence of the K303R ERα mutation and the PR-B isoform [7], and we have shown that a reduction in PR-B levels was associated with a poorer response to endocrine therapy [36]. Therefore, we first evaluated the levels of PR-A and B in our parental MCF-7 cells, and two stably transfected clones (Fig. 2b). Parental MCF-7 cells

expressed both PR isoforms, with PR-A as the predominant form in these cells. PR is a known estrogen-induced gene, and as expected we saw higher levels of both PR-A and B in MCF-7 cells after estrogen (E_2) treatment. In comparison, MCF-7 WT-overexpressing cells exhibited slightly less PR induction with E_2. In contrast, under control, basal conditions, K303R ERα mutant-overexpressing cells demonstrated much lower levels of both PR isoforms, and less were induced with E_2 treatment. Lower levels of PR mRNA levels were also detected in estrogen-stimulated mutant cells using Real-Time polymerase chain reaction (qPCR) ($P = 0.0008$, data not shown). These results suggest that growth factor signals might be altered in the mutant-overexpressing cells.

To test for altered intracellular signaling, we next examined the effects of short-term treatments with E_2, heregulin (H), EGF (E), and IGF-1 (I) on phosphorylation levels of downstream growth factors signaling components, such as HER2, Akt, and MAPK using immunoblot analysis (Fig. 2c) which is quantitatively represented in Fig. 2d. Cells were maintained under estrogen-depleted conditions for 2 days, and then treated for 10 min with E_2 or different growth factors as indicated, and cellular extracts were prepared. MCF-7 K303R mutant cells showed constitutively higher levels of phosphorylated HER2 as well as total HER2, compared to MCF-7 WT ERα-overexpressing cells (Fig. 2c). To begin to understand the mechanism associated with higher levels of total HER2 in the mutant cells we analyzed the expression level of HER2 mRNA in different clones of mutant ERα expressing cells, by qPCR; but we did not find differences in HER2 mRNA levels between WT and K303R ERα expressing cells (data not shown). This result suggests that post-translational modification and increase in protein stability could be involved in the HER2 up-regulation that we found in mutant ERα-expressing cells, and we are currently exploring this possibility. Treatment with E_2, heregulin, EGF, and IGF-1 induced higher levels of pHER2 in mutant-overexpressing cells, but only a small induction was seen in MCF-7 WT cells which contain low endogenous levels of HER2. Treatment with heregulin or EGF led to increased phosphorylation of the downstream signaling molecules Akt and MAPK in mutant-overexpressing cells compared with MCF-7 WT cells. IGF-1 treatment also induced enhanced phosphorylation of Akt, but increased levels of phosphorylated MAPK were only detected in K303R ERα mutant-overexpressing cells. In both cell lines, no enhancement of Akt and MAPK phosphorylation was seen with estrogen treatment.

To investigate if the kinetics of growth factor signaling might be altered in mutant-expressing cells, we performed a time-course study with heregulin treatment in WT ERα and K303R ERα mutant cells (Fig. 2e). After 2 days of
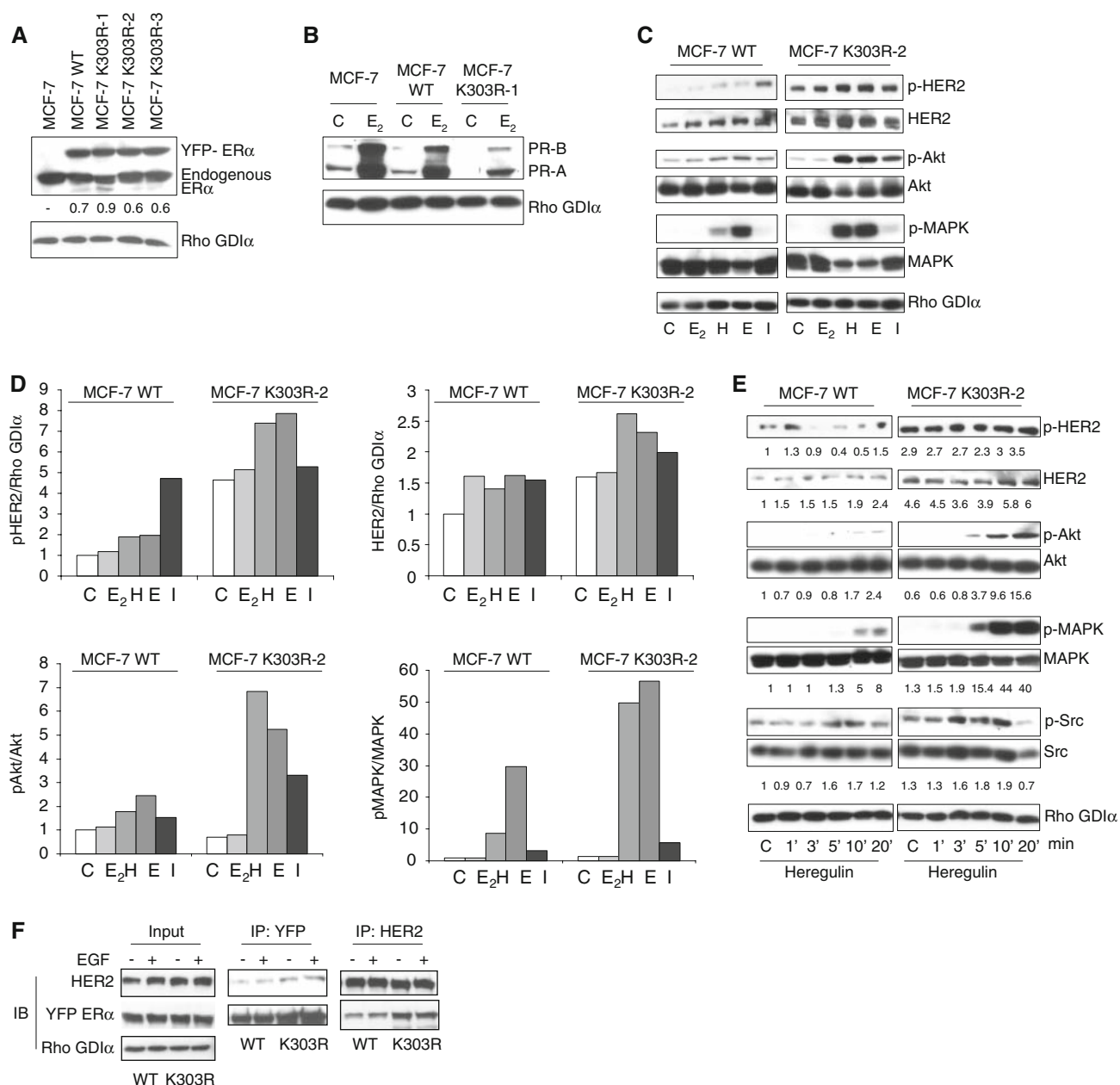
Fig. 2 Growth factor signaling in MCF-7 WT-ERα and MCF-7 K303R ERα-expressing cells. **a** MCF-7 cells stably transfected with a yellow-fluorescent protein (YFP)-tagged expression construct YFP-WT ERα (MCF-7 WT) and different clones transfected with the mutant YFP-K303R ERα construct (MCF-7 K303R-1, -2, -3) were screened for expression of exogenous (96 kDa) and endogenous ERα (66 kDa) by immunoblot analysis using an antibody against human ERα. Parental cells (MCF-7) were used as a control for endogenous ERα expression. Rho GDIα was used as a control for equal loading and transfer. *Numbers below the blot* represent the ratio between YFP-ERα and endogenous ERα protein expression. **b** MCF-7 parental, MCF-7 WT, and MCF-7 K303R-1 cells were serum-starved for 48 h, and then treated with or without 1 nM E_2 for 24 h before lysis. Equal amounts of total cellular extract were analyzed for progesterone receptor (PR-A and PR-B) levels by Western blotting. **c** MCF-7 WT and MCF-7 K303R-2 cells were treated with vehicle ethanol (C), 1 nM E_2, 2 ng/ml heregulin (H), 10 ng/ml EGF (E) and 10 ng/ml IGF-1 (I) for 10 min before lysis. Levels of phosphorylated (p) HER2 (Tyr[1248]), Akt (Ser[473]), and MAPK (Thr[202]/Tyr[204]), at the indicated residues, and total non-phosphorylated protein were measured in cellular extracts by immunoblot analysis. **d** Quantitative analysis of the blots shown above. **e** MCF-7 WT and MCF-7 K303R-2 cells were treated with heregulin (H) at 2 ng/ml for different times before lysis. Cellular extracts were analyzed as in *panel c*, and for p-Src (Tyr[416]) at the indicated residues. *Numbers below the blot* represent fold change in protein expression of MCF-7 K303R-2 cells compared to MCF-7 WT cells. **f** MCF-7 WT (WT) and MCF-7 K303R-3 (K303R) cells after 48 h of starvation were treated with or without EGF 100 ng/ml for 5 min before lysis. YFP-WT ERα and YFP-K303R ERα proteins were immunoprecipitated using an anti-YFP polyclonal antibody (IP:YFP), or an anti-HER2 polyclonal antibody (IP:HER2), and immunoblotted (IB) with HER2 and anti-ERα antibodies, respectively. Whole-cell lysates (Input) were used as input controls. Rho GDIα was used as a control for equal loading and transfer. Immunoblots are representative of three separate experiments

starvation cells were treated for 1, 3, 5, 10, or 20 min with heregulin, and then lysed as described in Materials and methods. As shown in Fig. 2e a rapid response to heregulin was seen within 1' in MCF-7 WT cells with activation of phospho-HER2, and phospho-AKT/phosphoMAPK within 10–20' (numerical quantitation relative to control WT is shown beneath each immunoblot). In contrast, mutant-expressing cells exhibited enhanced activation of pHER2, pAKT, and pMAPK. Compared with WT-expressing cells, these molecules were all stimulated at earlier time points in mutant cells; heregulin induced phosphorylation of MAPK and Akt by 5 min, but levels of these phosphorylated kinases were not detectable at this time-point in WT ERα cells. We saw that the non receptor tyrosine kinase c-Src also exhibited an earlier time-point of activation (by 3 min), and higher phosphorylation levels at the activating c-Src tyrosine residue 416 (Tyr^{416}) was seen in K303R ERα cells. These collective data demonstrate that the mutant-overexpressing cells are hypersensitive to growth factor signal transduction, and suggest that the mutant receptor could impact on ligand-independent signaling pathways commonly known to be employed to evade anti-hormonal therapeutic strategies in breast cancer.

Previous reports have shown that ERα and growth factor pathways can interact at different levels, through a direct association or complex formation of ERα with key signaling molecules such as c-Src, Shc, and the p85α regulatory subunit of PI3K [37–40]. C-Src family tyrosine kinases are involved in signaling of a number of different growth factor receptors, including EGFR/HER2, in breast cancer cells. In previous work we have reported that MCF-7 cells stably expressing the K303R ERα mutant receptor exhibited increased c-Src kinase activity and c-Src tyrosine phosphorylation, when compared with WT ERα-expressing cells [41]. Therefore, we next addressed whether the mutation might alter the ability of the receptor to bind with the HER2 tyrosine kinase receptor, which is expressed at higher levels in mutant cells (Fig. 2c). To evaluate potential protein–protein interactions between WT and mutant receptors with HER2 in our model system, we treated MCF-7 cells overexpressing WT or the mutant for 5' with EGF 100 ng/ml and then lysates were prepared (Fig. 2f). Equal amounts of protein were immunoprecipitated with either anti-YFP antisera or anti-HER2 antisera followed by immunoblot for HER2 and YFP-ERα. As shown in Fig. 2f, similar amounts of input from the whole-cell lysates were used. In the absence of treatment, both WT ERα and HER2 resided in a protein complex; and EGF treatment slightly increased the amount of the protein in the complex. The levels of mutant receptor bound to HER2 under basal conditions were higher, and EGF treatment did not further increase the amount of receptor in the complex. The bottom panel shows that equal amounts of YFP-ERα and

HER2 were immunoprecipitated under all conditions tested. These results suggest that the K303R ERα mutant may be constitutively associated with HER2, which could be involved in the enhanced bidirectional crosstalk that we report between ERα and growth factor receptor signaling pathways.

Tamoxifen fails to inhibit anchorage-independent growth induced by heregulin in MCF-7 K303R ERα-overexpressing cells

It is already appreciated that altered crosstalk between several receptor tyrosine kinases and ERα may contribute to endocrine resistance [18], and it has been shown that elevated levels of HER2 contribute to resistance in a tamoxifen-resistant subline of MCF-7 cells compared to unselected parental cells [42]. Since the K303R ERα mutation conferred enhanced heregulin-mediated signaling, we next addressed whether this alteration might confer a decrease in response to antiestrogen therapy. To test this question we next performed anchorage-independent growth assays using our two ERα-overexpressing models. Cells were plated in soft agar and then treated with estrogen (E$_2$, 1 nM) or heregulin (H, 2 ng/ml), EGF (10 ng/ml, E), IGF-1 (10 ng/ml, I) in the presence or absence of tamoxifen (Tam, 100 nM). After 14 days of growth colonies >50 μM in diameter were counted (Fig. 3a). Control (C) basal growth of mutant-expressing cells was higher compared to WT-expressing cells ($P = 0.001$); as expected treatment with estrogen as well as heregulin increased the number of colonies in both cell lines, while EGF and IGF-1 slightly increased proliferation but only in MCF-7 K303R ERα-overexpressing cells. Tam treatment induced a significant reduction in the number of colonies in MCF-7 WT cells under control conditions, and was able to inhibit growth of cells stimulated with estrogen or heregulin (reduction in colonies 78 and 96%, respectively). In contrast Tam treatment was less efficient at inhibiting estrogen or heregulin-stimulated proliferation in mutant-overexpressing cells (reductions were only 54 and 21%, respectively), but effectively reduced EGF and IGF-1 stimulated growth in these cells. Thus, response to Tam was greatly affected by the enhanced growth factor signaling by heregulin in mutant cells; this enhanced sensitivity prevented the major antagonistic activity of tamoxifen on the proliferation of mutant-expressing cells.

Next, we examined whether the HER2 pathway found to be up-regulated in the mutant-expressing cells, was responsible for the higher growth of mutant cells compared to WT ERα cells. We therefore evaluated anchorage-independent growth of either mutant or WT ERα cells after herceptin treatment. Herceptin is a humanized monoclonal antibody directed against the extracellular domain of
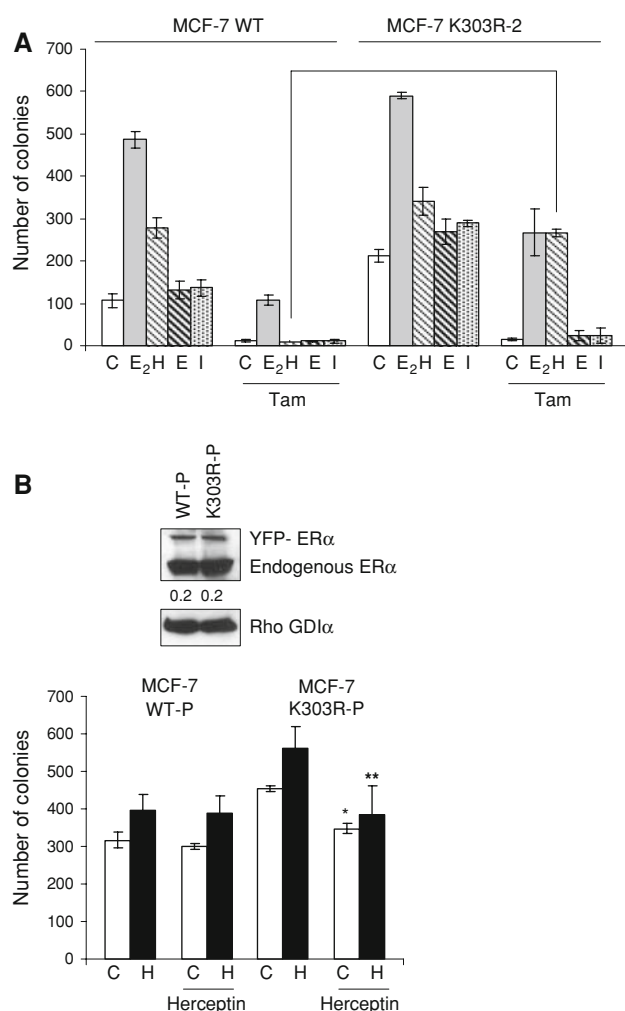
Fig. 3 Heregulin treatment reduced the ability of tamoxifen to inhibit anchorage-independent growth of MCF-7 K303R cells. **a** MCF-7 WT and MCF-7 K303R-2 stably transfected cells were seeded (5,000/well) in 0.35% agarose and then treated with vehicle (C), E_2 (1 nM), heregulin (2 ng/ml, H), EGF (10 ng/ml, E), IGF-1 (10 ng/ml, I) with or without Tam (100 nM). Cells were allowed to grow for 14 days and the number of colonies >50 μm were quantified and the results were graphed. *$P = 0.0001$ versus control (C) of MCF-7 WT cells. **b** Immunoblot analysis showing YFP-ERα and endogenous ERα protein expression (*upper panel*) in the pool of stable transfectants. *Numbers below the blot* represent fold change in protein expression of MCF-7 K303R-2 cells compared to MCF-7 WT cells. MCF-7 WT-P and MCF-7 K303R-P pool of transfected and overexpressing cells were plated in soft agar and then untreated or treated with heregulin 2 ng/ml (H) in the presence or absence of Herceptin (10 μg/ml). *$P = 0.0002$ versus control (C); **$P = 0.03$ versus heregulin (H) treated cells (*bottom panel*); standard deviations are shown

HER2, and was developed as an agent to inhibit the growth of HER2-overexpressing tumor cells [43, 44]. Cells were plated in soft agar and then treated with heregulin in the presence or absence of herceptin (10 μg/ml). After 14 days of growth, colonies were counted (Fig. 3b). As expected since HER2 levels are low in WT cells, herceptin had no effect on their growth either under basal conditions or with

heregulin treatment. In contrast, herceptin significantly inhibited anchorage-independent growth of K303R mutant cells in control untreated conditions, and with heregulin treatment (*$P = 0.0002$ vs. control; **$P = 0.03$ vs. heregulin treated cells). These data confirm that the HER2 pathway may be responsible for the higher constitutive growth of mutant-overexpressing cells rendering these cells more sensitive to the inhibitory effect of this selective HER2-targeted agent.

Phosphorylation at serine residue 305 (S305) of ERα K303R mutant is involved in growth factor signaling up-regulation

ERα is a known target of several post-translational modifications, such as phosphorylation, sumoylation, and acetylation [9, 45–47]. For instance, receptor phosphorylation, which regulates receptor affinity, coregulator protein binding, and transcriptional activity, can be induced in the absence of ligand via cross-talk with various signal transduction pathways [48]. We previously have reported that the K303R ERα mutant is a more efficient substrate for phosphorylation by PKA at S305 which enhanced hormone sensitivity and stimulated cellular growth [9]. We hypothesize that the phosphorylation status of S305 in the mutant receptor may control receptor activity, and be a conduit for enhanced downstream cross-talk with growth factor signaling networks. To explore this possibility, we first evaluated the phosphorylation status of the S305 residue in either WT ERα or K303R mutant cells after estrogen treatments between 5 min and 2 h. Cellular extracts were subjected to immunoblot assay using a specific anti-phospho-S305 ERα antibody (Fig. 4a). Estrogen treatment enhanced the phosphorylated levels of S305 within 15' in YFP-WT-expressing cells, and these levels remained elevated at 2 h. In contrast, K303R ERα mutant-expressing cells exhibited elevated levels of pS305 under basal control conditions, and this elevated phosphorylation remained constant with longer estrogen treatments. Since it is well established that ERα can be activated in a ligand-independent manner by MAPK [49] at serine 118 (S118), we also evaluated the levels of phospho-S118 in WT ERα or K303R ERα-overexpressing cells under the same conditions described above. We did not detect significant changes in S118 phosphorylation patterns between the two cell lines (data not show). These results suggest that constitutively higher phosphorylation of S305 in the mutant receptor might play a role in the ligand-independent activation of the receptor itself. It is possible that this enhanced S305 phosphorylation within the mutant might play a key role in the observed up-regulation of growth factor signaling cascades seen in these cells as well.
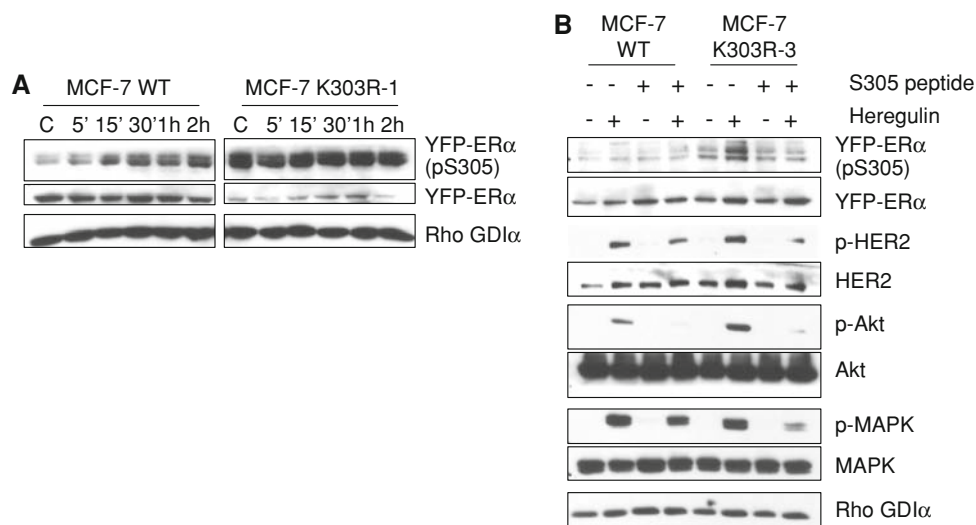
Fig. 4 Serine residue 305 (S305) in the K303R ERα mutant is involved in growth factor signal up-regulation. **a** MCF-7 WT and MCF-7 K303R-1 cells were treated for different times with E_2 (1 nM) before lysis. Cellular extracts were analyzed for phosphorylation levels of S305 YFP ERα (pS305) and total non-phosphorylated YFP-ERα. **b** Cells were incubated with the S305 peptide (4 μg/well) for 4 h in serum-free media and then treated with or without heregulin (2 ng/ml) for 10 min before lysis. Levels of phosphorylated (p) S305 YFP-ERα, HER2 (Tyr^{1248}), Akt (Ser^{473}), and MAPK (Thr^{202}/Tyr^{204}), and total non-phosphorylated proteins were measured in cellular extracts by immunoblot analysis. *Blots* are representative of three separate experiments. Rho GDIα was used as a control for equal loading and transfer

To test this hypothesis we performed immunoblot analysis to evaluate the phosphorylation levels of a number of growth factor signaling components after incubation with an S305 blocking peptide. To block phosphorylation at S305 we delivered a peptide (S305 peptide, residues 298 to 310) to the cells. After peptide delivery, the cells were subjected to short term treatments (10 min) with heregulin and then growth factor signaling molecules were analyzed by immunoblot analysis (Fig. 4b). Heregulin enhanced S305 phosphorylation in K303R mutant-overexpressing cells, but had no effect on WT receptor. Phosphorylation of the mutant receptor was abrogated by the S305 peptide. Addition of the S305 blocking peptide also inhibited heregulin-induced phosphorylation of HER2, Akt, and MAPK in both cell lines. Interestingly, reduction in phospho-HER2 and MAPK levels were more pronounced in mutant-expressing cells compared with WT, suggesting that the mutant cells were more sensitive to the inhibitory effect of S305 blocking peptide. These data indicate that phosphorylation of the S305 residue may be crucial in mediating enhanced cross-talk between HER2 and mutant ERα, and suggest that phosphorylation blockade might be a potential therapeutic strategy to block mutant function.

Discussion

Despite the improvements in the efficacy of hormonal therapies for the treatment of breast cancer patients with ER-positive tumors, de novo and acquired resistance remain major clinical problems that limit the efficacy of these therapies. In most cases, ERα remains essential to the problem of resistance due to its intimate cross-talk with growth factor signaling pathways [50, 51]. In this study, we show that expression of the K303R ERα mutant in ERα-positive MCF-7 breast cancer cells confers a decreased sensitivity to tamoxifen treatment in the presence of growth factor stimulation. Furthermore, this naturally occurring mutant is constitutively phosphorylated at S305, and shows an enhanced bidirectional cross-talk with the HER2 signaling pathway.

The A to G somatic mutation of ERα at nucleotide 908 (A908G) was previously identified in about 30% of pre-malignant breast lesions, but at a higher frequency (50%) in invasive breast tumors [4, 7]. The mutation was found to be associated with biologic measures of poor outcome, including elevated HER2 protein, larger tumor size and axillary lymph node positivity. To date no other somatic ERα mutation has been identified in more than a few invasive breast cancers [52], making this mutation novel. Recently, in a study using a population-based, case-control study design, the A908G mutation was detected, but at a low frequency (7%), in invasive breast tumors [53], confirming our identification of the mutant in cancer. We previously demonstrated that the exogenous expression of the K303R ERα mutant in MCF-7 breast cancer cells conferred a hypersensitive growth in very low physiological levels of estrogen (10^{-12} to 10^{-11} M) [4]. In the

present study we focused on the K303R ERα mutation and its potential role in modulating hormonal response in breast cancer cells, and on the molecular pathways that could be involved in its hormone action.

Our studies showed that growth factor signaling pathways were up-regulated in K303R ERα-expressing cells. In particular, MCF-7 mutant ERα expressing cells showed constitutively higher levels of total and phosphorylated HER2, the tyrosine kinase receptor that belongs to the epidermal growth factor receptors family. Preliminary quantitative RT-PCR analysis demonstrated that HER2 mRNA levels were not increased in mutant expressing cells compared to WT ERα-expressing cells. Thus, transcriptional regulation may not be the major mechanism for the observed increased levels of HER2 in mutant cells. In the present study we did not investigate the possible post-transcriptional mechanisms associated with higher levels of HER2, but instead focused on the effects that HER2 overexpression induced on downstream cell signaling and hormone responsiveness in mutant-expressing cells. It is well known that HER2 catalytic activity can amplify the signal of other c-erbB family receptors by the formation of HER2-containing heterodimers, which increases ligand binding affinity and receptor stability [54, 55]. Moreover, it has been shown that c-neu, the mouse homolog of HER2, is able to multimerize, be phosphorylated, and thus activated when present at high density on the cell surfaces [56]. Both mechanisms result in the amplified activation of downstream signaling cascade, such as Akt and MAPK, which are involved in cell survival and proliferation.

We found that the peptide growth factors heregulin and EGF strongly enhanced phosphorylation of the two major downstream signaling cascades Akt and MAPK, in mutant-expressing cells compared to WT ERα-expressing cells. Furthermore, analysis of rapid kinetics showed that many of these downstream molecules, as well as the c-Src non receptor tyrosine kinase, were stimulated at earlier time points in mutant-expressing cells. The rapid responses of these downstream kinase cascades to heregulin suggest that the presence of K303R ERα mutation could modify the responsiveness of the cells to the growth factor signaling possibly through enhanced non-genomic activity of the mutant receptor.

Recent research into the mechanisms associated with Tam resistance suggest that some of the same growth factor receptor pathways implicated in adaptive hypersensitivity, such as Akt and MAPK, or specific oncogenes involved in intracellular signal transduction, become activated and are used to bypass normal hormonal responsiveness. Several reports indicate that the up-regulation of HER2 tyrosine kinase signaling in breast cancer plays an important role in the development of endocrine resistance [57]. Preclinical studies have demonstrated that HER2 overexpression in ERα positive MCF-7 human breast cancer xenografts rendered them resistant to Tam [58], and markedly increased levels of EGFR and HER2 were found in some sublines of MCF-7 cells with acquired Tam resistance [59, 60]. Tam resistance in these cells was reversed by EGFR/HER2 tyrosine kinase inhibitors, and combined treatment with these inhibitors and Tam was effective in reversing resistance in xenograft models [61], thereby strongly implicating this signaling network in resistance. Although HER2 overexpression occurs only in a minority of ERα-positive patients [62], clinical studies confirm that HER2-overexpressing tumors are less responsive to Tam treatment [63, 64]. In a previous retrospective study we found an association between the K303R ERα mutation and elevated HER2 levels in invasive breast cancer [7]. Here we observed increased HER2 levels in K303R mutant-expressing cells that was concomitant with an altered response to Tam with growth factor stimulation.

We also demonstrated that mutant-expressing cells exhibited a higher level of growth under all conditions tested. Importantly, Tam sensitivity was significantly affected with estrogen and heregulin treatments. Our data indicate that the Tam-resistant phenotype associated with the mutant was most pronounced in the presence of growth factor activation; in the presence of heregulin Tam inhibited soft agar growth only 21% compared with a 96% inhibition in WT ERα-expressing cells. We speculate that bidirectional cross-talk between the HER2 and mutant receptors could play a role in conferring a selective advantage in terms of growth to those patients that express the K303R ERα mutant that are treated with Tam. We are currently examining for the presence of the mutant in a retrospective cohort of patients treated with Tam who have long-term clinical follow-up.

Different therapeutic agents targeting the activity of the c-erbB family of receptors have been recently developed and tested in patients. For instance, herceptin (Trastuzumab[TM]), a monoclonal antibody against HER2, was approved for therapeutic use in patients with HER2-overexpressing breast cancer [65, 66]. By binding to the juxtamembrane domain of HER2 [67], this agent blocks HER2 homo- and heterodimerization with the other members of the c-erbB family, and thereby interrupts the activation of downstream proliferative signaling. Here we show that herceptin elicited its antiproliferative effects either under basal conditions or with hergulin treatment in K303R ERα mutant cells, but did not affect growth of the WT cells. These findings confirm our hypothesis that the HER2 pathway, which appears to be elevated in mutant cells, could be involved in the regulation of cell growth in breast tumor cells bearing the mutation, probably through increased crosstalk between HER2 and ERα pathways.

The existence of bidirectional cross-talk between ERα and growth factor receptor pathways, and its involvement

in the development of endocrine resistance has been well documented [16, 19]. Several studies have demonstrated direct or indirect activation of growth factor signaling via ERα. For example, ligand-independent activation of serine 118 ERα by EGFR/MAPK-mediated phosphorylation regulates growth of tamoxifen-resistant MCF-7 breast cancer cells [68]. Chung et al. [69] have also demonstrated that HER2 and ERα can directly interact at the cell membrane, and this interaction protected breast cancer cells from Tam-induced apoptosis. Moreover, membrane or cytoplasmic ERα can induce phosphorylation of EGFR through activation of G-proteins, c-Src, and matrix metalloproteinases [70], and can directly interact with adaptor proteins such as c-Src, Shc and the p85α regulatory subunit of PI3K [37–40]. These processes activate downstream kinases that in turn activate ERα and its coregulatory proteins, thus also enhancing genomic activities of the receptor [71, 72]. All together these effects amplify the bidirectional crosstalk which multiplies signals between and downstream of the growth factor receptors and ERα, thus sustaining survival and proliferative signals in breast cancer cells.

The present findings suggest an enhanced hormone-independent physical association/complex between the mutant receptor and HER2 compared to WT receptor. This suggests that the mutation, present in the hinge region of the receptor, may increase the ability of ERα to interact with HER2 or other components of the complex. As yet, the interaction surface between HER and ERα has not been defined. Altered interactions as we describe may imply that Tam may not antagonize the mutant receptor because the HER2 pathway may be dominant and non-genomic action predominates. This is consistent because we have already demonstrated that the mutation can alter coregulator protein binding. The mutation demonstrates enhanced binding ability to bind to the TIF-2 coactivator [4], and the AIB coactivator at very low and physiological levels of estradiol, but decreased binding to the corepressor NCoR1. Mutant receptor binding to BRCA-1 has also been shown to be enhanced. These collective data suggest that altered affinity for ER coregulators, and possibly signaling molecules such as HER2, could be one mechanism by which the K303R mutation confers hypersensitivity to low levels of estrogen, and reduced sensitivity to Tam. The mutant receptor appears to exhibit increased ligand-independent activity which bypasses antiestrogen treatment.

To explore why the K303R ERα mutation has increased cross-talk with the HER2 pathway, we focused on the differences in post-translational modifications between WT and mutant receptor. It is known that ERα activity can be modulated by several post-translational modifications, such as protein phosphorylation [45], acetylation [46], ubiquitination [73], and sumoylation [47]. The majority of studies in this field have focused their attention on the phosphorylation

status of the receptor, and its effect on receptor activity. For instance, receptor phosphorylation by different kinases such as c-Src, PKA, MAPK, and Akt can all regulate receptor affinity, coregulator protein binding, and transcriptional activity [45]. We have previously shown that the K303R ERα mutation renders the receptor a more efficient substrate for PKA-induced phosphorylation at residue S305 which has distinct biological results—enhanced hormone sensitivity for growth [9]. Phosphorylation at the S305 residue can also be mediated by both protein kinase A (PKA) and p21-activated kinase-1 (PAK-1) signaling network [9, 12].

Several reports have identified the serine residue at 305 as a physiologically important site that modifies response to Tam. In particular it has been demonstrated, using fluorescence resonance energy transfer (FRET) analysis, that PKA signaling to ERα S305 causes a conformational arrest in the ERα and switches Tam from an antagonist to an agonist [10]. Michalides et al. [74] have also demonstrated that PKA phosphorylation at S305 ERα induces Tam resistance through an altered orientation of ERα towards the co-activator SRC-1. In addition, S305 ERα phosphorylation by PAK-1 up-regulates cyclin D1 expression in breast cancer cells [75]. Here we show that K303R mutant cells have elevated phosphorylation levels of S305 compared with WT-expressing cells suggesting that the mutant has constitutive ligand-independent activity. The contribution of the S305 site in enhanced cross-talk with HER2 was established using a blocking peptide. We show that heregulin-stimulation enhanced S305 phosphorylation in mutant expressing cells, but it did not significantly influence the phosphorylation status of WT receptor. Interestingly, we found that the S305 peptide affected heregulin-induced ERα phosphorylation, and prevented downstream phosphorylation events, such as activation of MAPK and Akt. These effects were even more prominent in K303R mutant cells possibly due to the prominent role of the S305 site in ligand-independent activity of the mutant receptor. A similar experimental approach was used by Varricchio et al. [76]. They demonstrated that a six amino acid peptide surrounding the phosphotyrosine residue 537 was able to block ER/c-Src interactions, cyclin D1 expression, and growth of MCF-7 and LNCaP cells.

We have previously shown that transcriptional activity of the mutant receptor was induced at very low concentrations of estradiol (10^{-12} M), and only the specific lysine to arginine substitution at 303 residue resulted in a receptor with enhanced sensitivity to estrogen. We have also demonstrated that cAMP-dependent signaling can enhance the receptor's intrinsic sensitivity to hormone, and that blocking PKA activity reversed the hypersensitive proliferative phenotype in mutant-expressing cells [9]. The data obtained herein using live cell dynamics agrees with these earlier results. The live cell high through-put analyses

allowed us to study mutant receptor/promoter interaction and chromatin remodelling [26, 28]. Our results confirm that the K303R mutant is inherently hypersensitivity to estrogen, and only WT receptor showed increased sensitivity to estrogen after forskolin treatment. We used the same experimental approach to test for altered Tam activity in the presence of forskolin. Tam treatment inhibited transcriptional responses in WT, but at statistically significant lower levels in mutant cells. Forskolin treatment blocked Tam-induced promoter condensation, suggesting that ligand-independent kinase signaling to the mutant receptor decreased tamoxifen sensitivity.

Clinical studies have reported that breast tumors with HER2 amplification show reduced levels of PR [77], and the absence of PR is a marker of a more aggressive phenotype [78]. Patients whose tumors lack PR derive less benefit from adjuvant hormonal therapy [30–32]. In vitro studies suggest that amplified growth factor signaling may underlie the reduction of PR levels in breast cancer cells. [79]. In this report we found that PR protein expression was almost undetectable in K303R cells under basal conditions, and estrogen induced only a small increase in PR content, compared to the induction elicited in WT-expressing cells. The lack of PR expression observed in K303R mutant-expressing cells may be the consequence of altered growth factor signaling that contributed to the Tam-resistant phenotype observed in our model system.

We conclude that the K303R ERα hypersensitive phenotype involves an integration of post-translational modification events, such as phosphorylation at S305, with enhanced bidirectional cross-talk between the mutant and growth factor receptors such as HER2, and that genomic and nongenomic mechanisms probably contribute to Tam resistance. Because our molecular and biological data demonstrate that the ERα mutation may be resistant to Tam, we suggest that the mutation is a potential novel predictive marker of hormonal response in breast cancer tumors. In addition, our molecular studies suggest that use of a specific blocking peptide to prevent S305 phosphorylation of the mutant may reduce ligand-independent activity, and be a new therapeutic approach to treat patients with mutation-positive tumors which are resistant to Tam therapy.

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Evidence That Leptin Through STAT and CREB Signaling Enhances Cyclin D1 Expression and Promotes Human Endometrial Cancer Proliferation

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Obesity is a risk factor for endometrial cancer in pre- and post-menopausal women. Leptin, an adipocyte-derived hormone, in addition to the control weight homeostasis, is implicated in multiple biological actions. A recent study demonstrated that leptin promotes endometrial cancer growth and invasiveness through STAT/MAPK and Akt pathways, but the molecular mechanism involved in such processes still needs to be elucidated. In an attempt to understand the role of leptin in regulating endometrial cancer cells proliferation, we have demonstrated that leptin treatment reduced the numbers of cells in G0/G1-phase while increased cell population in S-phase. This effect is associated with an up-regulation of cyclin D1 together with a down-regulation of cyclin-dependent kinase inhibitor p21[WAF1/Cip1]. Mutagenesis studies, eletrophoretic mobility shift, and chromatin immunoprecipitation analysis revealed that signal transducers and activators of transcription 3 (STAT3) and cyclic AMP-responsive element (CRE) binding protein motifs, within cyclin D1 promoter, were required for leptin-induced cyclin D1 expression in Ishikawa endometrial cancer cells. Silencing of STAT3 and CREB gene expression by RNA interference reversed the up-regulatory effect of leptin on cyclin D1 expression and cells proliferation. These results support the hypothesis that STAT3 and CREB play an important role in leptin signaling pathway that leads to the proliferation of Ishikawa cells, thus establishing a direct association between obesity and endometrial tumorogenesis.

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Leptin, the product of the *ob* gene, mainly secreted by adipocytes, is involved in the control of body weight and its plasma levels strongly correlate to the body fat mass (Zhang et al., 1994; Ahima and Flier, 2000).

In addition to its regulatory role in energy metabolism, leptin is also implicated in the modulation of many other processes such as reproduction, lactation, hematopoiesis, immune responses, and cell proliferation (Ahima and Flier, 2000; Huang and Li, 2000; Brann et al., 2002). More interestingly, recent studies have demonstrated that leptin stimulates growth, migration, invasion, and angiogenesis in tumor cell models, suggesting that it is able to promote an aggressive cancer phenotype (Bouloumie et al., 1998; Somasundar et al., 2004b; Garofalo and Surmacz, 2006).

The activities of leptin are mediated through the transmembrane leptin receptor (ObR) present in a variety of tissues (Tartaglia, 1997), by activation of the Janus-activated kinase/signal transducers and activators of transcription (JAK/STAT) (Bahrenberg et al., 2002; Ahima and Osei, 2004) as well as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase/(PI-3K)/Akt pathways (Sweeney, 2002; Zabeau et al., 2003). Besides, induction of ObR can activate several genes involved in cell proliferation, such as c-fos, c-jun, jun B, and egr-1 (Sweeney, 2002; Zabeau et al., 2003).

Epidemiological studies have suggested a positive correlation between obesity and an increased risk of different cancers, including breast, prostate, colon, and endometrial (Calle and Kaaks, 2004; Garofalo and Surmacz, 2006; Somasundar et al., 2004a). There is convincing and consistent evidence from both case-control and cohort studies that obesity is tightly related to endometrial cancer in both pre- and post-menopausal women (Calle and Thun, 2004).

Endometrial cancer is the most common gynecological malignancy and the fourth most common malignancy in women in the developed world after breast, colorectal and lung cancer. The incidence is estimated at 15–20 per 100,000 women per year (Ryan et al., 2005).

Several studies demonstrated that serum–leptin levels among cases with endometrial cancer were significantly higher compared to controls (Petridou et al., 2002; Yuan et al., 2004). Expression of leptin and its functional receptor, short (ObRs) and long (ObRl) isoform has been shown in both cancer and non-cancer endometrium (Gonzalez et al., 2000; Kitawaki et al., 2000; Koda et al., 2007). The levels of ObRl were similar in cancer and normal tissue, but the short isoform was significantly decreased in malignant cells. Moreover, induction of the expression of this receptor resulted in inhibited proliferation of cancer cells due to delayed start of the mitotic S-phase, suggesting that loss of ObRs in the endometrial cancer might contribute to malignant progression (Yuan et al., 2004).

A recent report demonstrated that leptin promotes endometrial cancer growth and invasiveness through STAT/MAPK and Akt pathways. Particularly, treatment with leptin resulted in increased proliferation and induces invasion of ECC1 and Ishikawa cells (Sharma et al., 2006). However, the molecular mechanism by which leptin-induced endometrial cancer cell proliferation still needs to be elucidated.

In the last years, a large body of evidence has shown that disruption of cell cycle control mechanism is a common pathway in human cancer and overexpression of cyclin D1 is one of the most commonly observed alterations (Cordon-Cardo, 1995; Fu et al., 2004; Knudsen et al., 2006). Cyclin D1, an important cell cycle regulator is required for completion of the G1/S transition in normal mammalian cells (Fu et al., 2004). Moreover, its expression increases from normal endometrium to hyperplasia and carcinoma, suggesting that it may play a role in endometrial carcinogenesis (Ruhul-Quddus et al., 2002).

The aim of the present study was to explore the molecular mechanism eliciting the proliferative effect of leptin in endometrial cancer cell. We demonstrated that leptin enhances cyclin D1 expression through regulation of STAT binding site (GAS) and cyclic AMP-response element (CRE) located within its promoter.

Our findings have provided evidence for better understanding the association between obesity and endometrial cancer.

Materials and Methods
Materials

Dulbecco's modified Eagle's medium (DMEM), L-glutamine, Eagle's non-essential amino acids, penicillin, streptomycin, fetal bovine serum (FBS), bovine serum albumin (BSA), phosphate-buffered saline were purchased from Eurobio (Les Ullis Cedex, France). TRIzol and Lipofectamine 2,000 reagent by Invitrogen (Carlsbad, CA), FuGENE 6 by Roche Applied Science (Indianapolis, IN). TaqDNA polymerase, RETROscript kit, 100-bp DNA ladder, Dual Luciferase kit, and TK Renilla luciferase plasmid were provided by Promega (Madison, WI). Aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, U0126, AG490, H89, and recombinant human leptin were purchased by Sigma (Milan, Italy). Antibodies against phospho p44/42 MAPK (Thr 202/Tyr 204) (#9101S), p44/42 MAPKinase (#9102), pCREB (Ser133), and CREB (48H2) were provided by Cell Signaling. Antibodies against cyclin D1 (M-20), GAPDH (FL-335), p21$^{WAF1/Cip1}$ (H164), pSTAT3 (B-7), STAT3 (F-2), and polymerase II (N20) by Santa Cruz Biotechnology (Santa Cruz, CA). An ECL system and Sephadex G-50 spin columns were purchased from Amersham Biosciences (Buckinghamshire, UK). [λ^{32}P]ATP and [^{3}H]thymidine from PerkinElmer Life Sciences (Wellesley, MA). RNase A (Calbiochem, La Jolla, CA).

Plasmids

The plasmids containing the human cyclin D1 promoter or its deletions (p−2966/+142, p−944/+142, p−848/+142, p−136/

+142) were kindly provided by Prof A. Weisz (University of Naples, Italy). These fragments were inserted into the luciferase vector pXP2.

Site-directed mutagenesis

The cyclin D1 promoter plasmids bearing both STAT3 binding recognition (GAS) and cyclic AMP-responsive element (CRE) mutated sites (pGAS/CRE mut) were created by site-directed mutagenesis using QuickChange kit (Stratagene, La Jolla, CA). Briefly, this was based on a PCR reaction with two complementary oligonucleotide primers containing the mutation. The PCR was performed with the Pfu DNA polymerase during 16 cycles (30 sec at 95°C, 30 sec at 55°C, and 8 min at 68°C), using as template the human cyclin D1 promoter p−136/+142 and the following mutagenic primers (mutations are shown as lowercase letters): 5′-CGGACTACAGGGGAGTagcGTTGAAGTTGCAAAGTCC-TGGAG-3′ and 5′-CTCCAGGACTTTGCAACTTCAACgctAC-TCCCCTGTAGTCCG-3′ (GAS MUT); 5′-GATCTTTGCTTAA-CAACAGTAACtctACACGGACTACAGGGGAG -3′ and 5′-CTCCCCTGTAGTCCGTGTagaGTTACTGTTGTTAAG CAAAGATC-3′ (CRE MUT). The PCR products were then incubated with DpnI which only digests the parental methylated cDNA and the constructed mutated expression vectors were confirmed by DNA sequencing.

Cell culture

Ishikawa human endometrial cancer cells were obtained from D. Picard (University of Geneva, Geneva, Switzerland). Ishikawa cells were maintained in DMEM without phenol red supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin. Cells were switched to medium without serum 48 h before each experiment.

DNA flow cytometry

Ishikawa cells were harvested, fixed, and stained with Propidium iodide (100 μg/ml) after treatment with RNase A (20 μg/ml). Stained cells were analyzed for DNA content by Flow Cytometry using FAC-Scan (Becton Dickinson and Co., Franklin Lakes, NJ).

Total RNA extraction and reverse transcription-PCR assay

Total cellular RNA was extracted from Ishikawa cells using Triazol reagent as suggested by the manufacturer. The evaluation of genes expression was performed by the reverse transcription-PCR method using a RETROscript kit as suggested by the manufacturer. The cDNAs obtained were amplified by PCR using the following primers: 5′-TCTAAGATGAAGGAGACCATC-3′ and 5′-GCGGTAGTAGGACAGGAAGTTGTT-3′ (cyclin D1); 5′-GCTTCATGCCAGCTACTTCC-3′ and 5′-CTGTGCTCACTTCAGGGTCA -3′ (P21$^{WAF1/Cip1}$); 5′-CTCAACATCTCCCCCTTCTC-3′ and 5′-CAAATCCCATATCCTCGTCC-3′ (36B4). The PCR was performed for 30 cycles for cyclin D1 (94°C for 1 min, 60°C for 1 min, and 72°C for 2 min), 30 cycles for p21 (94°C for 1 min, 58°C for 1 min, and 72°C for 2 min) and 15 cycles (94°C for 1 min, 58°C for 1 min, and 72°C for 2 min) to amplify 36B4 in the presence of 1 μl of first strand cDNA, 1 μM each of the primers mentioned above, 0.5 mM dNTP, Taq DNA polymerase (2 units/tube), and 2.2 mM magnesium chloride in a final volume of 25 μl. To check for the presence of DNA contamination, a reverse transcription-PCR was performed on 1 μg of total RNA without Moloney murine leukemia virus reverse transcriptase (the negative control). DNA quantity in each lane was analyzed by scanning densitometry.

Immunoblotting

Ishikawa cells were grown in 10 cm dishes to 50–60% confluence and lysed in 500 μl of 50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM EdTA, 0.1% SDS, and a mixture of protease inhibitors (aprotinin, PMSF,

and sodium ortho-vanadate). Equal amounts of total proteins were resolved on an 11% SDS–polyacrylamide gel, transferred to a nitrocellulose membrane and probed with the appropriated antibody. As internal control, all membranes were subsequently stripped (glycine 0.2 M, PH 2.6 for 30 min at room temperature) of the first antibody and reprobed with anti GADPH Ab. The antigen–antibody complex was detected by incubation of the membranes for 1 h at room temperature with peroxidase-coupled goat anti-mouse or anti-rabbit IgG and revealed using the ECL System. The blots were then exposed to film, and the bands of interest were quantified by Scion Image laser densitometry scanning program. The results obtained as optical density arbitrary values were transformed to percentages of the control (percent control) taking the samples from cells not treated as 100%.

Transient transfection assay

Ishikawa cells were starved with serum free medium for 24 h and then transfected using the FuGENE 6 reagent with the mixture containing 0.25 µg of human cyclin D1 promoter constructs. Twenty-four hours after transfection, the cells were untreated or treated with 1,000 ng/ml leptin for 6, 12, and 24 h. TK Renilla luciferase plasmid (10 ng per each 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 (EMSA)

Nuclear extracts from Ishikawa cells were prepared as previously described (Catalano et al., 2003). The probe was generated by annealing single-stranded oligonucleotides, labeled with $[\lambda^{32}P]$ ATP and T4 polynucleotide kinase, and purified using Sephadex G50 spin columns. The DNA sequences used as probe or as cold competitors are the following (the nucleotide motifs of interest are underlined, and mutations are shown as lowercase letters): 5′-TTAACAACAGTAACGTCACACGGACTA-3′ and 5′-TAGTCCGTGTGACGTTACTGTTGTTAA-3′ (CRE); 5′-AGGGGAGTTTTGTT GAAGTTGCAAA-3′ and 5′-TTTGCAACTTCAACAAAACTCCCCT-3′ (GAS); 5′-C TTAACAACAGTAAttgCACACGGACTA-3′ and 5′-TAGTCCGTGTGcaaTT ACTGTTGTTAAG-3′ (CRE MUT); 5′-AGGGGAGTagcGTTGAAGTTGCAAA-3′ and 5′-TTTGCAAC TTCAACgctACTCCCCT-3′ (GAS MUT). In vitro transcribed and translated CREB protein was synthesized using the T7 polymerase in the rabbit reticulocyte lysate system. 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 Ishikawa nuclear protein or an appropriate amount of CREB protein, 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. For experiments involving STAT3 and CREB antibodies, the reaction mixture was incubated with these antibodies at 4°C for 12 h before addition of labeled probe. 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 (ChIP)

ChIP was performed as previously described (Morelli et al., 2004). Ishikawa cells were untreated or treated with 1,000 ng/ml leptin for 1 h. The cells were then cross-linked with 1% formaldehyde and sonicated. Supernatants were immunocleared with salmon sperm DNA/protein A and immunoprecipitated with specific anti-STAT3, anti-CREB and anti polymerase II antibodies or a normal mouse serum IgG as negative control. Pellets were washed as reported, eluted with elution buffer (1% SDS, 0.1 M NaHCO₃) and digested with proteinase K. DNA was obtained by phenol/chloroform/ isoamyl alcohol extractions and precipitated with ethanol; 3 µl of

each sample were used for PCR amplification with the following primers flanking GAS/CRE sequence present in the cyclin D1 promoter region: 5′-TGCGCCCGCCCCCGCCCCCCTC-3′ and 5′-TGTTCCATGGCTGGGGCTCTT-3′. The PCR conditions were 1 min at 94°C, 1 min at 65°C, and 2 min at 72°C. The amplification products obtained in 35 cycles were analyzed in a 2% agarose gel and visualized by Ethidium bromide staining.

RNA interference (RNAi)

Cells were plated in 6-well dishes with regular growth medium the day before transfection to 60–70% confluence. On the second day the medium was changed with SFM without P/S and cells were transfected with RNA duplex of stealth RNAi targeted human STAT3 mRNA sequence 5′-GCC UCA AGA UUG ACC UAG Att-3′ (Ambion, Austin, TX), with RNA duplex of validate RNAi targeted human CREB mRNA sequence 5′- GGC UAA CAA UGG UAC CGA Utt -3′ or with a stealth RNAi control (Ambion) to a final concentration of 50 nM using Lipofectamine 2000 as recommended by the manufacturer. After 5 h the transfection medium was changed with SFM in order to avoid Lipofectamine 2000 toxicity and cells were exposed to Leptin 1,000 ng/ml for 24 or 48 h. These transfected cells were used to examine the effects of silencing STAT3 and CREB gene expression on: (1) cyclin D1 protein content, (2) cellular proliferation by [³H]thymidine incorporation.

[³H]thymidine incorporation

Ishikawa cells were untreated or treated with leptin 1,000 ng/ml for 24 or 48 h. For the last 6 h, [³H]thymidine (1 µCi/ml) was added to the culture medium. After rinsing with phosphate-buffered saline, the cells were washed once with 10% and three times with 5% trichloroacetic acid. The cells were lysed by adding 0.1 N NaOH and then incubated for 30 min at 37 °C. Thymidine incorporation was determined by scintillation counting.

Statistical analysis

Each datum point represents the mean ± SE of three different experiments. Statistical analysis was performed using ANOVA followed by Newman–Keuls testing to determine differences in means. $P < 0.05$ was considered as statistically significant.

Results
Leptin modulates cell cycle progression in endometrial cancer cells

Flow Cytometric analysis was used to investigate the role of leptin on Ishikawa cell cycle progression. Cells were synchronized by 48 h serum starvation and then induced to re-enter the cell cycle by treatment with hormone. Twenty-four hours leptin treatment reduced the percentage of cells in the G0/G1-phase of the cell cycle and increased the fraction of the cells in S-phase, compared with control group (Fig. 1).

Leptin enhances cyclin D1 and down-regulates p21[WAF1/Cip1] expression in Ishikawa cells

To characterize the molecular mechanism associated with the proliferative effects induced by leptin, we examined changes in the expression of genes involved in these processes. Since cyclin D1 is a critical modulator in the cell cycle G1/S transition and its overexpression is one of the most commonly observed alterations in human endometrial cancers (Lukas et al., 1994; Nikaido et al., 1996; Fu et al., 2004), we aimed to evaluate the potential ability of leptin to modulate cyclin D1 mRNA and protein content in Ishikawa human endometrial cancer cells. RT-PCR showed an increased mRNA expression of the cyclin D1 gene after treatment with leptin 1,000 ng/ml for 6, 12, and 24 h. Cyclin D1 mRNA expression was normalized using the human housekeeping gene 36B4 (Fig. 2A). The leptin-induced
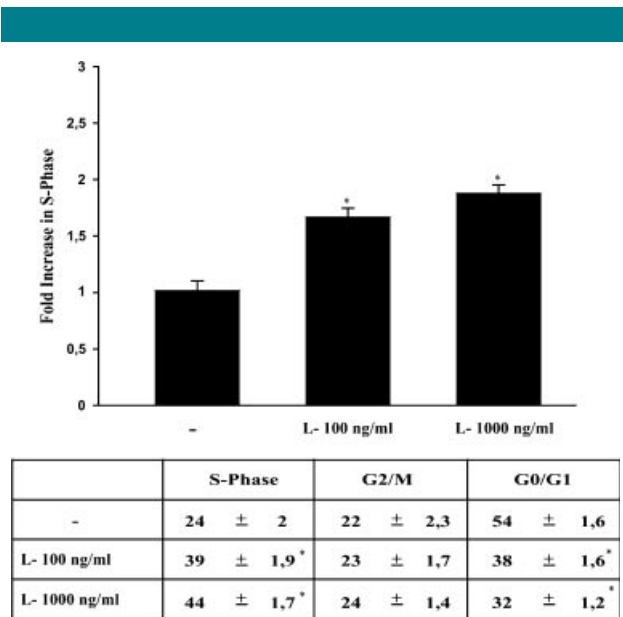
Fig. 1. Leptin increases the fraction of Ishikawa cells in S-phase of the cell cycle. Ishikawa cells were synchronized in serum-free media for 48 h and then exposed to 100 ng/ml and 1,000 ng/ml leptin (L) for 24 h or left untreated (–). The distribution of Ishikawa cells in the cycle was determined by Flow Cytometry using Propidium-iodide stained nuclei. The results indicate the fold-increase of Ishikawa cells in S-phase after serum starvation or leptin treatment. The histograms represent the mean ± SE of three separate experiments done in triplicate. The table shows the distribution of Ishikawa cells in the various phases of cell cycle. $*P < 0.01$ compared with untreated cells.

expression of cyclin D1 was evidenced at protein level, at all investigated times, by Western blotting analysis (Fig. 2B).

To study more properly the involvement of leptin in cell cycle G1/S transition phase, the expression of $p21^{WAF1/Cip1}$, the major cyclin-dependent-kinase inhibitor, was analyzed. As shown in Figure 2A and B $p21^{WAF1/Cip1}$ mRNA and protein levels decreased in leptin treated cells.

These observations indicate that leptin may promote G1/S-phase progression by stimulating expression of cyclin D1 and inhibiting the expression of $p21^{WAF1/Cip1}$.

Leptin-induced cyclin D1 expression is STAT, ERK, and PKA dependent in Ishikawa cells

Leptin exerts its biological functions through binding to its receptors that mediate a downstream signal by activating multiple signaling pathways (Sweeney, 2002; Ahima and Osei, 2004). To gain insight into the mechanism underlying the modulatory role of leptin on cyclin D1 expression in endometrial cancer cells, we examined the changes in signal transductional pathways involved in mediating leptin action. Cellular proteins were extracted from Ishikawa cells treated with 1,000 ng/ml of leptin at different times, and by Western blotting analysis we determined the status of STAT3 and ERK1/2 phosphorylation. As shown in Figure 3A, leptin significantly induced phosphorylation of STAT3 within 15 min of treatment while an increased phosphorylation of ERK was observed after 5 min of leptin stimulation (Fig. 3B). Besides, we also examined the effect of leptin on phosphorylated CREB/ATF-1, a downstream substrate of MAPK, but also an effector of PKA (Delghandi et al., 2005). The CREB Ab produces two bands and recognizes both CREB (upper band) and ATF-1 (lower band). Notably, leptin exposure for 15 min significantly induced phosphorylation of CREB/ATF1 (Fig. 3C), which was inhibited by pretreatment of these cells with PKA and ERK1/2 inhibitors, H89 and U0126 respectively (Fig. 3D).
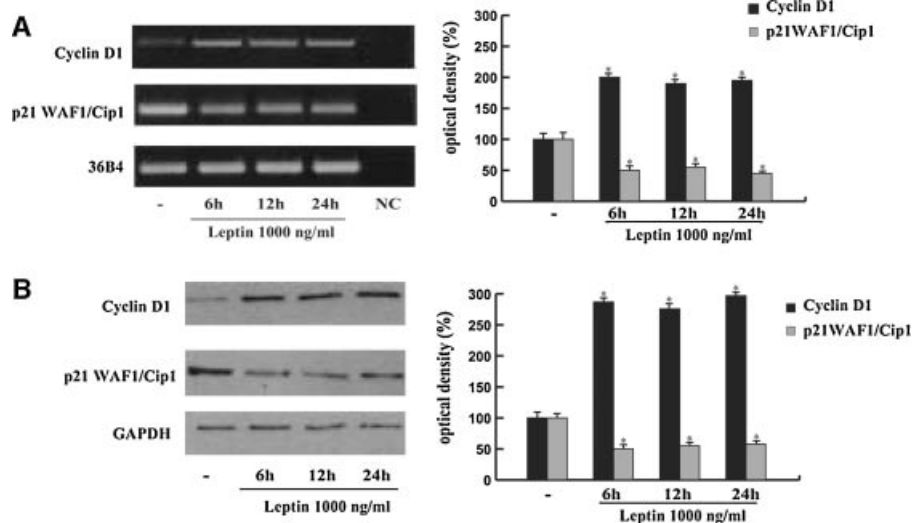

Fig. 2. Effects of Leptin on cyclin D1 and p 21$^{WAF1/Cip1}$ expression in Ishikawa cells. Ishikawa cells were serum-starved for 48 h followed by treatment with 1,000 ng/ml leptin for 6, 12, and 24 h or left untreated (–). A: Total RNA was isolated from Ishikawa cells and reverse transcribed. cDNA was subjected to PCR using specific primers for cyclin D1, p21$^{WAF1/Cip1}$ or 36B4. NC: negative control, RNA sample without the addition of reverse transcriptase. 36B4 mRNA levels were determined as control. B: Protein extracts obtained from Ishikawa cells were resolved by SDS–PAGE and subjected to immunoblot analysis with rabbit antiserum against human cyclin D1 and p21$^{WAF1/Cip1}$. GAPDH served as loading control. The histograms represent the mean ± SE of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed as 100%. $*P < 0.01$ compared to untreated cells.
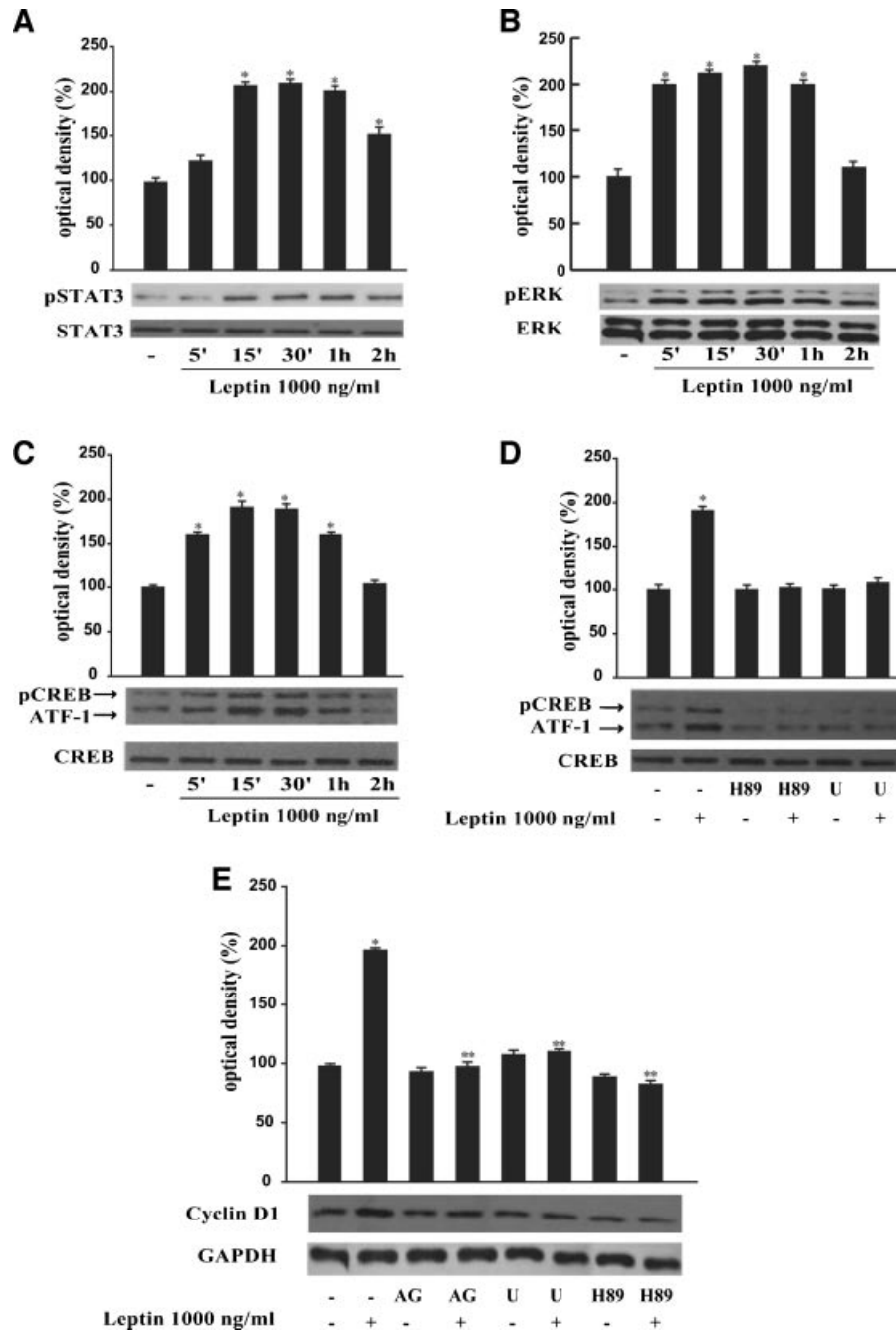
Fig. 3. Activation of leptin signalling in the up-regulation of cyclin D1 expression. Ishikawa cells were serum-starved for 48 h and then treated with 1,000 ng/ml leptin for various time intervals or left untreated (–). Protein extracts obtained from Ishikawa cells were resolved by SDS–PAGE and subjected to immunoblot analysis with specific antibodies against total or phosphorylated (p) forms of STAT3 (A), ERK (B), and CREB/ATF-1 (C). For combined treatment, cells were pretreated with H89 (10 μM) and U0126 (U) (10 μM) for 30 min followed by leptin treatment for 15 min. Equal amounts of protein were resolved by SDS–PAGE and subjected to immunoblot analysis and immunoblotted with specific antibodies against total or phosphorylated CREB/ATF1 (D). E: Ishikawa cells were serum-starved for 48 h and treated with 1,000 ng/ml leptin for 24 h or left untreated (–). For combined treatment, cells were pretreated with AG490 (AG) (20 μM), U0126 (U) (10 μM), and H89 (10 μM) for 30 min followed by leptin treatment. Specific antibodies against cyclin D1 and GAPDH were used. The histograms represent the mean ± SE of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed as 100%. *$P < 0.01$ compared to untreated cells; **$P < 0.01$ compared to leptin treatment alone.

Leptin had no effect on total STAT3, ERK and CREB protein expression levels.

Next, to investigate the signal transduction pathways involved in leptin-induced cyclin D1 expression, chemical inhibitor of JAK/STAT (AG490), ERK1/2 (U0126), and PKA (H89) were added to serum-starved Ishikawa cells before treatment with 1,000 ng/ml of leptin for 24 h. Our results revealed that AG490, U0126, and H89 effectively prevent leptin induction of cyclin D1 expression (Fig. 3E).

Leptin transactivates cyclin D1 promoter in Ishikawa cells

The aforementioned observations prompted us to evaluate whether leptin signalling could affect cyclin D1 transcriptional activity. Thus, we transiently transfected Ishikawa cells with a luciferase reporter construct containing the upstream region of the cyclin D1 gene spanning from -2.966 to $+142$. As shown in Figure 4B, a significant increase in promoter activity was observed in the transfected cells exposed to leptin 1,000 ng/ml for 6, 12, and 24 h.

Cyclin D1 promoter contains multiple regulatory elements, including binding sites for AP-1, STATs, NF-kb, Oct-1, Sp1, CRE, and TCF/LEF (Allan et al., 2001; Natsume et al., 2003; Bartusel et al., 2005; Brockman and Schuler, 2005; Saxena et al., 2007b).

To delimit the cis-element involved in cyclin D1 transcriptional activation by leptin, we transiently transfected Ishikawa cells with plasmids containing a series of $5'$ deleted segments of human cyclin D1 promoter. Schematic representation of constructs is shown in Figure 4A.

In transfection experiments performed using $p-944/+142$, $p-848/+142$, and $p-136/+142$ the responsiveness to leptin was still observed at all investigated time (Fig. 4B), suggesting that the region from -136 to $+142$ was required for the transactivation of cyclin D1 by leptin.

The nucleotide sequence analysis of this region evidenced a CRE and STAT3 binding motif (GAS) located at position -52 and -27 respectively, putative effectors of leptin signalling as previously demonstrated in other systems (Mauro et al., 2007; Saxena et al., 2007b). Thus, mutation analysis of both CRE and GAS sites on cyclin D1 promoter was carried out.

As shown in Figure 4D mutation of both GAS and CRE completely abolished leptin responsiveness of cyclin D1 promoter in Ishikawa cells demonstrating their involvement in the up-regulation of cyclin D1 induced by leptin.

Leptin increases STAT3-DNA and CREB-DNA binding activity to cyclin D1 promoter

To further investigate the specific role of GAS and CRE motifs in the transcriptional activation of cyclin D1 by leptin, we performed EMSA experiments.

Using synthetic oligodeoxyribonucleotides corresponding to the GAS and CRE motifs, we observed the formation of a complex in nuclear extract from Ishikawa cells (Fig. 5A and B, lane 1), which was abrogated by 100-fold molar excess of unlabeled probe (Fig. 5A and B, lane 2) demonstrating the specificity of the DNA binding complex. This inhibition was not longer observed when mutated oligodeoxyribonucleotides were used as competitor (Fig. 5A and B, lane 3). Leptin-induced both GAS and CRE activation compared with control samples at the same time-point (Fig. 5A and B, lane 4, 5, and 6). Incubation of anti-STAT3 with the nuclear extracts resulted in a greatly reduced band, indicating the presence of STAT3 protein in the complex (Fig. 5A, lane 7). Similarly, incubation of anti-CREB with the nuclear extracts resulted in reduced and supershifted band (Fig. 5B, lane 7). IgG did not affect either GAS or CRE complex formation (Fig. 5A and B, lane 8). Using transcribed and translated in vitro CREB protein, we obtained a complex migrating at the same level as that of Ishikawa nuclear extracts (Fig. 5B, lane 9).

Leptin enhances recruitment of STAT3 and CREB to the promoter region of cyclin D1

Although our findings clearly demonstrated the role of STAT3 and CREB in leptin mediated regulation of cyclin D1 promoter, we further sought to determine that STAT3 and CREB directly participate in leptin mediated cyclin D1 gene regulation using ChIP assay. Using specific antibody against STAT3, formaldehyde cross-linked protein-chromatin complexes were immunoprecipitated from Ishikawa cells cultured with or without leptin 1,000 ng/ml. The resulting precipitated genomic DNA was then analyzed by PCR using primers spanning the STAT3 binding element in the promoter region of cyclin D1. As shown in Figure 5C, ChIP analysis with anti-STAT3 antibody revealed that treatment with leptin for 1 h increased STAT3 recruitment to cyclin D1 promoter. Interestingly, we also observed upon leptin stimulation a significant increase in CREB recruitment to the cyclin D1 promoter as evidenced by ChIP analysis using anti-CREB antibody. In addition, the enhanced recruitment of STAT3 and CREB was correlated with greater association of polymerase II to the cyclin D1 regulatory region.

Our data suggest that cyclin D1 may be a target for leptin mediated growth stimulation of Ishikawa cells and the molecular mechanism may involve recruitment of STAT3 and CREB transcription factors.

STAT3 and CREB siRNAs prevent leptin-induced cyclin D1 expression and cell proliferation

We studied the effect of silencing STAT3 and CREB gene expression by siRNA on leptin-induced cyclin D1 expression and cellular proliferation in Ishikawa cells to better define the contribution of these two transcription factors. In preliminary experiment we evaluated, after 24 and 48 h of siRNA transfection, that STAT3 and CREB protein expression was effectively silenced as revealed by Western Blotting (Fig. 6A). As shown in Figure 6B, silencing of the STAT3 and CREB genes significantly decreased cyclin D1 activation induced by leptin, while no changes was observed after transfection of cells with scrambled dsRNA upon identical experimental conditions. We next determined the effect of STAT3 and CREB siRNA on growth stimulation by measuring changes in the rate of DNA synthesis (Thymidine incorporation). We confirmed, as previously demonstrated (Sharma et al., 2006), that treatment with leptin 1,000 ng/ml for 24 and 48 h induces proliferation of Ishikawa cells. The growth stimulatory effect of leptin was severely affected in Ishikawa cells with silenced STAT3 or CREB expression (Fig. 6C).

These results further support that STAT3 and CREB play an important role in leptin signaling pathway that leads to the proliferation of Ishikawa cells.

Discussion

Increasing epidemiologic data in humans as well as many in vitro investigative reports have linked obesity with various disease states and suggested a strong link between leptin and tumor progression (Garofalo and Surmacz, 2006). Indeed, several studies have described a mitogenic effect of leptin on gastric (Pai et al., 2005), breast (Hu et al., 2002; Catalano et al., 2003; Mauro et al., 2007), ovarian (Choi et al., 2004), and prostate cancer cells (Somasundar et al., 2004a). Recently, growth stimulatory effect of leptin in human endometrial cancer cells was proposed (Sharma et al., 2006), even though the molecular mechanism underlying this effect remains to be fully elucidated.

As first attempt, to provide new insight into the stimulatory action exerted by leptin on cell growth, we analyzed leptin signaling on cell cycle profile in Ishikawa endometrial cancer cells. Our results showed that leptin treatment was able to speed up cell cycle progression by reducing G0/G1 arrest with an increase of cell population in S-phase.

The cell cycle is regulated by the coordinate action of cyclin-dependent kinases (cdk), specific cyclin proteins and cdk inhibitors. Alterations of the mechanisms controlling cell cycle progression play a relevant role in the pathogenesis of different human neoplasia. Among the molecules involved in cell cycle regulation cyclin D1 abnormalities may contribute to such malignant transformation (Cordon-Cardo, 1995; Fu et al., 2004;
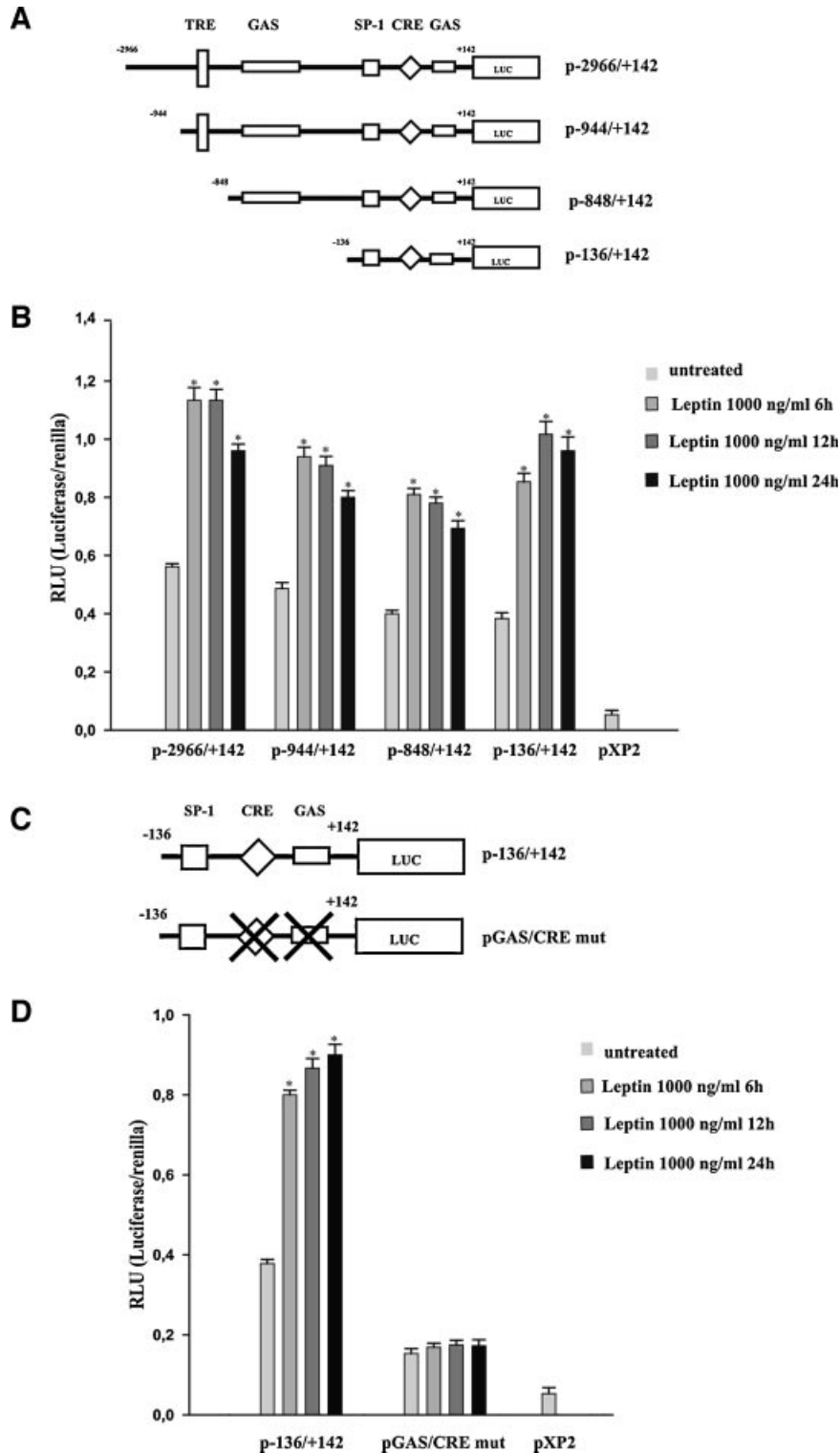
Fig. 4. Leptin transactivates cyclin D1 gene promoter through GAS and CRE motifs. A: Schematic representation of human cyclin D1 promoter fragments used in this study. All of the promoter constructs contain the same 3′ boundary (+142). The 5′ boundaries of the promoter fragments varied from −2 966 to −136. Each fragment was subcloned into the pXP2 vector. B: Transcriptional activity of Ishikawa cells with promoter constructs is shown. Ishikawa cells were serum-starved for 48 h, transfected for 24 h and left untreated or treated with 1,000 ng/ml leptin for 6, 12, and 24 h. C: Schematic representation of the mutated plasmid used in this study. D: Transcriptional activity of Ishikawa cells with promoter constructs is shown. Ishikawa cells were serum-starved for 24 h, transfected for 48 h and left untreated or treated with 1,000 ng/ml leptin for 6, 12, and 24 h. The values represent the mean ± SE of three separate experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. pXP2: basal activity measured in cells transfected with pXP2 basal vector *P < 0.01 compared to untreated cells.
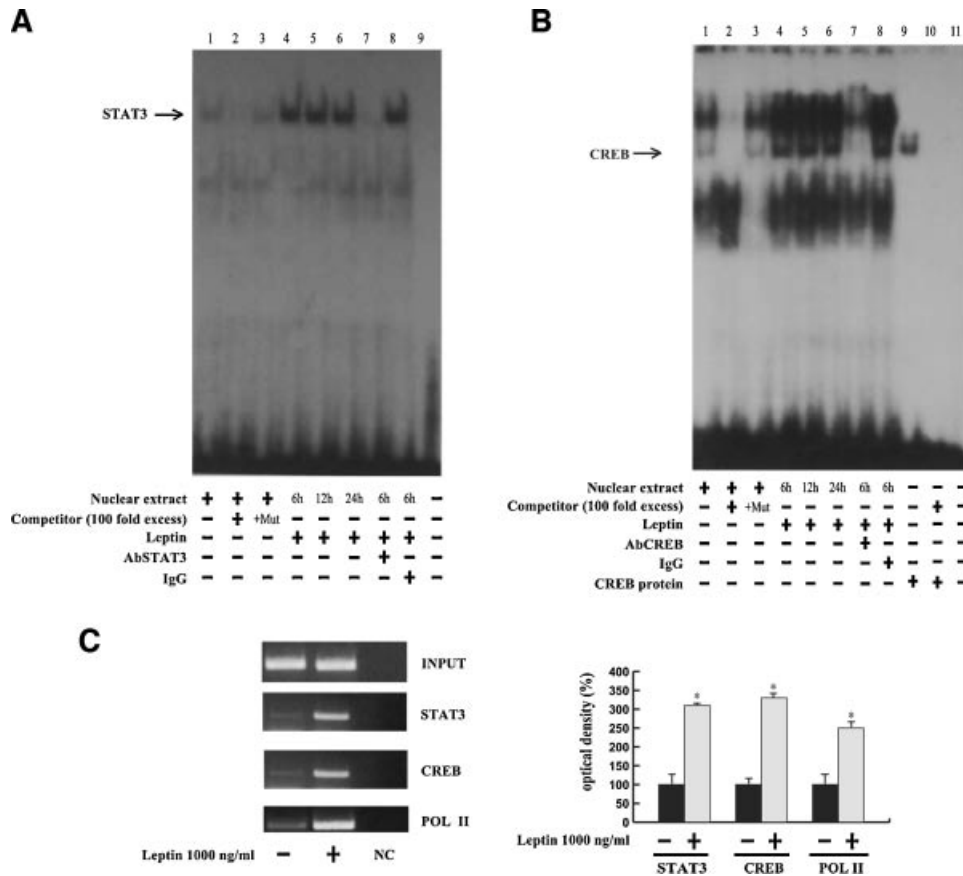
Fig. 5. Leptin induces activation of STAT3-DNA and CREB-DNA binding activity in Ishikawa cells. Nuclear extracts from Ishikawa cells were incubated with a double-stranded STAT3-specific (A) and CREB-specific (B) consensus sequence probe labeled with [λ32P] ATP and subjected to electrophoresis in a 6% polyacrylamide gel (lane 1). A: Competition experiments were done by adding as competitor a 100-fold molar excess of unlabeled probe (lane 2) or a 100-fold molar excess of unlabeled oligonucleotide containing a mutated GAS (lane 3). Ishikawa nuclear extracts treated with 1,000 ng/ml leptin for 6, 12, and 24 h incubated with probe (lane 4, 5, and 6). The specificity of the binding was tested by adding to the reaction mixture a STAT3 antibody (lane 7). IgG did not affect GAS complex formation (lane 8). Lane 9 contains probe alone. B: Competition experiments were done by adding as competitor a 100-fold molar excess of unlabeled probe (lane 2 and 10) or a 100-fold molar excess of unlabeled oligonucleotide containing a mutated CRE (lane 3). Ishikawa nuclear extracts treated with 1,000 ng/ml leptin for 6, 12, and 24 h incubated with probe (lane 4, 5, and 6). The specificity of the binding was tested by adding to the reaction mixture a CREB antibody (lane 7). IgG did not affect CRE complex formation (lane 8). We used as positive control a transcribed and translated in vitro CREB protein (lane 9). Lane 11 contains probe alone. (C) The cells were serum-starved for 48 h and left untreated (–) or treated with 1,000 ng/ml leptin for 1 h. The preacleared chromatin was immunoprecipitated with specific antibody anti-STAT3, anti-CREB, anti polymerase II antibodies, and with a normal mouse serum (NC) as negative control. Cyclin D1 promoter sequences containing GAS and CRE sites were detected by PCR with specific primers, as detailed in Materials and Methods Section. To determine input DNA, the cyclin D1 promoter fragment was amplified from 30 μl, purified soluble chromatin before immunoprecipitation. The histograms represent the mean ± SE of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control, which was assumed to be 100%. *P < 0.01 compared to untreated cells.

Knudsen et al., 2006). Altered expression of cyclin D1 may result from rearrangement (Arnold et al., 1989), translocation (Withers et al., 1991), amplification and/or overexpression in head and neck, breast, squamous cell carcinomas, non-small cell lung cancer, colon and urinary bladder cancer (Hall and Peters, 1996). In addition, it has been reported that cyclin D1 overexpression in endometrial glands increases progressively in intensity and extent from normal endometrium to complex hyperplasia and carcinoma (Ruhul-Quddus et al., 2002).

Of interest, we found that leptin exposure up-regulates both cyclin D1 mRNA and protein levels at all the investigated times with a concomitant decrease of p21$^{WAF1/Cip1}$ expression. Our results are consistent with previous studies showing similar induction of cyclin D1 expression in human breast cancer cells (Okumura et al., 2002; Chen et al., 2006), in colon cancer HT-29

cell line (Rouet-Benzineb et al., 2004) as well as in human hepatocarcinoma cells (Chen et al., 2007; Saxena et al., 2007a).

Moreover, in our study, we demonstrated that leptin-stimulated cyclin D1 expression requires JAK/STAT, MAPK, and PKA activation, as it emerges by the observation that the chemical inhibitors of the above mentioned pathways completely reversed the increase of cyclin D1 protein levels.

It is worth noting that our findings recall previous reports indicating the involvement of JAK/STAT and MAPK signalling pathways in leptin mediated cell growth in diverse cellular contexts (Dieudonne et al., 2002; Choi et al., 2004; Sharma et al., 2006; Chen et al., 2007; Saxena et al., 2007a). For instance, recently, in Ishikawa endometrial cancer cells, leptin through ERK1/2 has been linked to cell proliferation (Sharma et al., 2006;), whereas in MCF-7 breast cancer cells,
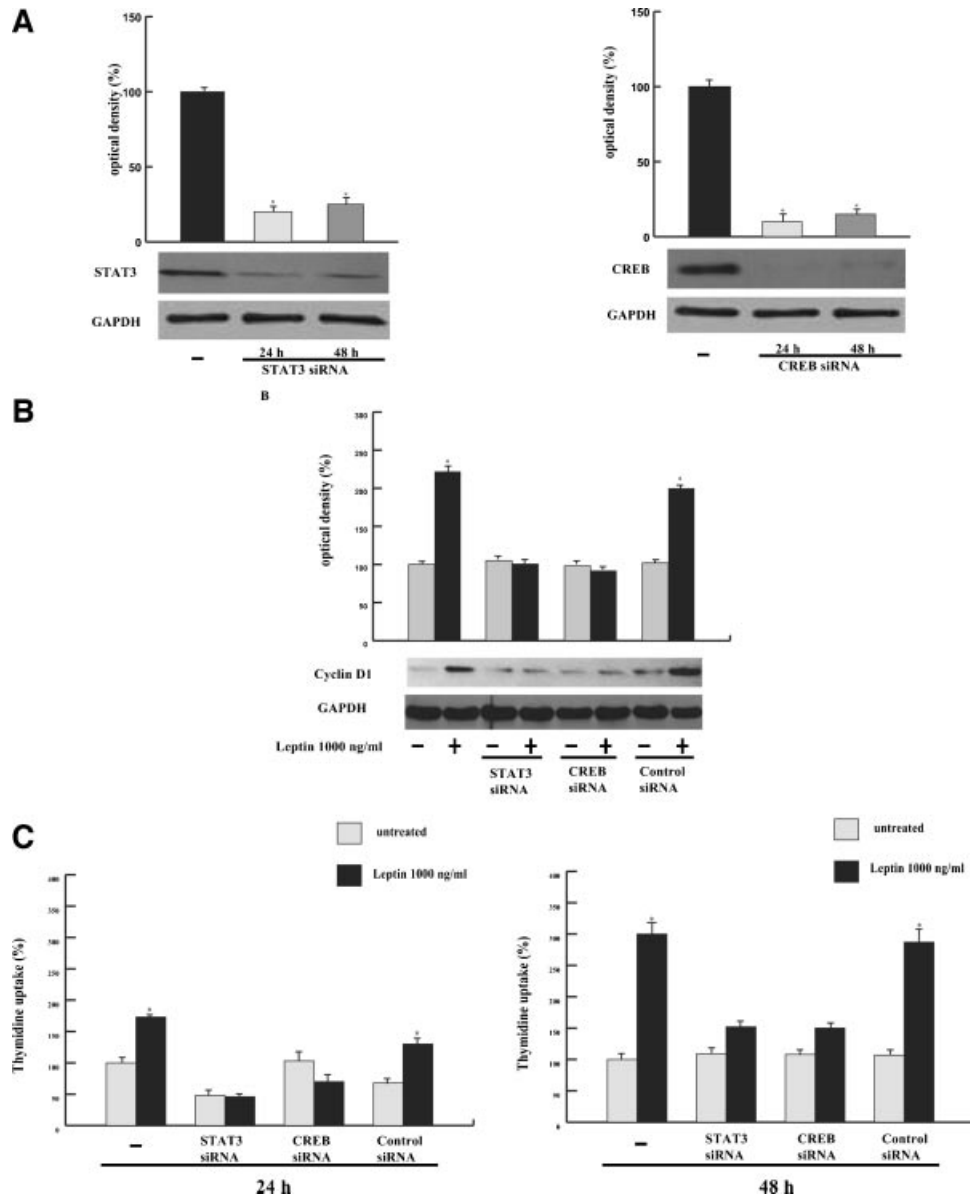
Fig. 6. The effect of STAT3 and CREB silencing on leptin-stimulated cyclin D1 and Ishikawa cells proliferation. A: STAT3 and CREB protein expression (evaluated by Western blotting) in Ishikawa cells not transfected (–) or transfected with RNA interference (RNAi) targeted human STAT3 or CREB mRNA sequence respectively as reported in Materials and Methods Section. GADPH was used as loading control. B: Ishikawa cells were transfected with STAT3, CREB, or control RNAi, and untreated (–) or treated with 1,000 ng/ml of leptin for 24 h. Total protein was extracted and Western blotting analysis was performed to evaluate the expression of cyclin D1. GADPH was used as loading control on the same stripped blot. C: Ishikawa cells were transfected with STAT3, CREB, control RNAi, or untransfected (–) and untreated or treated with 1,000 ng/ml of leptin for 24 and 48 h. Thymidine incorporation assay was performed. The data represent the mean ± SE of three separate experiments. *$P < 0.01$ compared to untreated cells.

we evidenced that leptin signaling via ERK1/2 was able to potentiate estrogen action and aromatase activity promoting breast cancer cell growth (Catalano et al., 2003; Catalano et al., 2004).

It was previously observed that stimulated Ras-MAPK pathway induces activation of CREB kinase, a member of the p90[RSK] family that corresponds to RSK2, and thereby phosphorylates CREB at Ser[133] (Dalby et al., 1998). CREB, belongs to the ATF/CREB transcription family and interacts with CRE site in the cAMP-responsive gene promoter; although

is a major downstream substrate of ERK1/2, it is also classically known as a PKA effector (Delghandi et al., 2005). The relationship of PKA and JAK/STAT-dependent intracellular mechanism of leptin action was previously suggested (Matsuoka et al., 1999) as well as the involvement of PKA in leptin-induced human ovarian proliferation (Sirotkin et al., 2007). These observations well fit with our results showing a significant increase of CREB/ATF-1 phosphorylation upon leptin exposure, which was completely abrogated by specific inhibitors of ERK1/2 and PKA signaling pathways demonstrating

that CREB is a target over which the above-mentioned pathways converge.

Therefore, in order to investigate the potential ability of leptin to modulate the cyclin D1 promoter gene, we performed transient transfection experiments in Ishikawa cells using deleted constructs of the cyclin D1 promoter gene. The results indicated that leptin signalling up-regulates the full-length promoter activity of cyclin D1. Moreover, we documented that the region spanning from −136 to +142, which contains CRE and GAS sites, was required for the responsiveness to leptin. Mutation analyses of the CRE and GAS sites on cyclin D1 promoter showed that both motifs were the mediators of cyclin D1 regulation by leptin since loss of both completely abolished leptin-induced promoter activation.

Other evidences strengthened our observation, since cyclin D1 has been reported to be transcriptionally regulated by STAT3 (Leslie et al., 2006) and CRE (Sabbah et al., 1999) in breast cancer cells. Moreover, GAS and CRE has been shown to be a potential target of leptin signaling. Indeed, a recent work, showed that leptin-activated STAT3 binds to its cognate sites in cyclin D1 promoter leading to hyperacetylation and overexpression of cyclin D1 gene through a recruitment of distinct co-activator complexes (Saxena et al., 2007b). On the other hand, our previous findings reported, in MCF-7 breast cancer cell line, activation of E-cadherin gene promoter by leptin through CRE site (Mauro et al., 2007).

Our EMSA experiments extended the aforementioned observations because nuclear extracts from Ishikawa cells treated with leptin showed an increased binding to the CRE and GAS sequences located in the cyclin D1 promoter region. These findings were supported by ChIP assay demonstrating the ability of leptin to enhance the recruitment of CREB and STAT3 to the promoter of cyclin D1. Finally, the relative contribution of these two transcription factors emerges from our data showing that silencing of STAT3 and CREB gene expression is able to reverse the up-regulatory effect of leptin on cyclin D1 expression and cell growth proliferation.

Taken together our results, for the first time, evidence that STAT3 and CREB are involved in leptin-mediated induction of cyclin D1 and clarify the role of leptin signaling in the progression of endometrial cancer, addressing it as a potential target of pharmacological therapy in obese women.

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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 female nude mice, and an *in vitro* model represented by MCF-7 three-dimensional cultures. Xenograft tumors, obtained only in animals with estradiol (E_2) pellet implants, doubled control value after 13 weeks of leptin exposure. In three-dimensional cultures, leptin and/or E_2 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 E_2 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–responsive 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. [Cancer Res 2007;67(7):3412–21]

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. These combined effects 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 (STAT⁻), was given by Dr. J. Turkson (University of South Florida, College of Medicine, Tampa, FL). pCMV5myc vector containing the cDNA-encoding dominant-negative extracellular signal-regulated kinase 2 K52R (ERK2⁻) was provided by Dr. M. Cobb (Southwestern Medical Center, Dallas, TX).

Site-directed mutagenesis. The E-cadherin promoter plasmid-bearing 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′-AGGGTGGAT-CACCT<u>GAtacCA</u>GGAGTTCCAGACCAGC-3′ and 5′-GCTGGTCTGGAACTC-<u>CTGgtaTCA</u>GGTGATCCACCCT-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% L-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 by caliper measurements along two orthogonal axes: length (L) and width (W). The volume (V) of tumors was estimated by the following formula: $V = L \times (W^2) / 2$. At the time of killing (13 weeks), tumors were dissected out from the neighboring connective 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 100 nmol/L E_2 for 48 h. To block E-cadherin function, the medium was supplemented with E-cadherin antibody (1:100 dilution; Chemicon International, Temecula, CA) or EGTA to a final concentration of 4 mmol/L. To generate three-dimensional

spheroids, the plates were rotated for 4 h at 37°C. The three-dimensional cultures were photographed using a phase-contrast microscope (Olympus, Milan, Italy). The extent of aggregation was scored by measuring the spheroids with an ocular micrometer. The spheroids between 25 and 50, 50 and 100, and >100 μm (in the smallest cross-section) were counted in 10 different fields under ×10 magnification.

Cell number was determined, after trypsinization of spheroids, by direct cell counting at 48 h of treatments.

E-cadherin adhesion assay. MCF-7 cells were pretreated with leptin (1,000 ng/mL) and/or E_2 (100 nmol/L) for 48 h and then plated on six-well plates coated with 1.5 μg/mL recombinant human E-cadherin/Fc chimeric. 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-diphenylte-trazolium 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′-TCTAAGATGAAGGAGACCATC-3′ and 5′-GCGGTAGTAGGACAGGAAGT-TGTT-3′ (cyclin D1), 5′-TGGAATCCAAGCAGAATTGC-3′ and 5′-TATGTGG-CAATGCGTTCTCTATCCA-3′ (E-cadherin), and 5′-CTCAACATCTCCCCC-TTCTC-3′ and 5′-CAAATCCCATATCCTCGT-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).
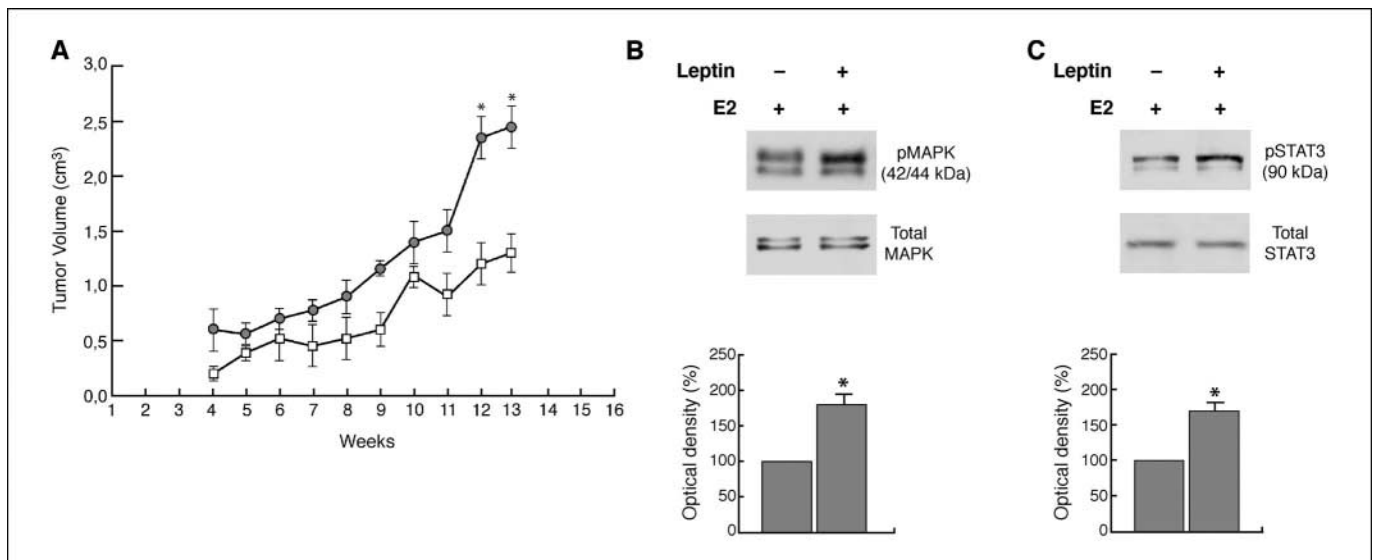

Figure 1. Effect of leptin on growth of MCF-7 cell tumor xenografts. *A,* xenografts were established with MCF-7 cells in female mice implanted with E_2 pellet. One group was treated with 230 μg/kg leptin (●, *n* = 5) and a second group with vehicle (□, *n* = 5). *, *P* < 0.05, treated versus control 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.
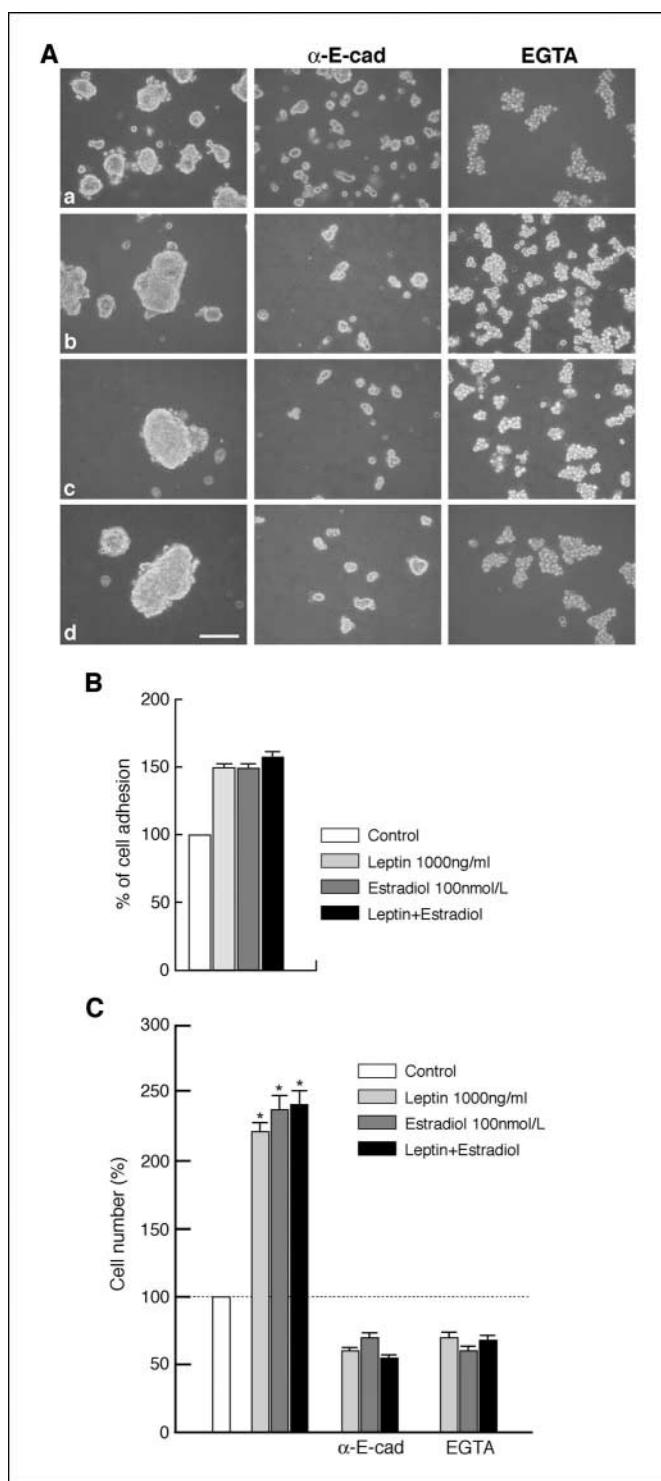
Figure 2. Leptin-enhanced cell-cell adhesion and proliferation depend on E-cadherin function. *A,* E-cadherin–positive MCF-7 cells were seeded in 2% agar–coated plates and cultured as three-dimensional spheroids (*a–d*). To block E-cadherin function, the medium was supplemented with E-cadherin antibody (1:100 dilution; α-E-cad) or EGTA (4 mmol/L). Cells were untreated (*a*) or treated with leptin (*b*), E_2 (*c*), and leptin plus E_2 (*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 E_2 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 E_2 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^{32}P]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′-TGGATCACCTGAGGTCAGGAGTTCCAGACC-3′; Sp1, 5′-ATCAGC-GGTACGGGGGGGCGGTGCTCCGGGG-3′. *In vitro* transcribed and translated CREB protein was synthesized using the T7 polymerase in the rabbit reticulocyte lysate system (Promega). The protein-binding reactions were carried out in 20 mL 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 E_2 for 1 h. The cells were then cross-linked with 1% formaldehyde and sonicated. Supernatants were immunocleared with sonicated salmon DNA/protein A agarose (Upstate Biotechnology, Inc., Lake Placid, NY) and immunoprecipitated with anti-CREB or anti-Sp1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Pellets were washed as reported (26), eluted

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

MCF-7	Spheriods		
	25 ≤ 50 μm	50 ≤ 100 μm	>100 μm
Control	30 ± 1.2	0.6 ± 0.2	0.0 ± 0.0
Leptin	6 ± 0.8	26 ± 1.8	85 ± 2.5
E_2	7 ± 0.6	32 ± 2.1	78 ± 3.2
Leptin + E_2	3 ± 0.9	40.5 ± 2.3*	80.7 ± 2.9

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. 2*A*.
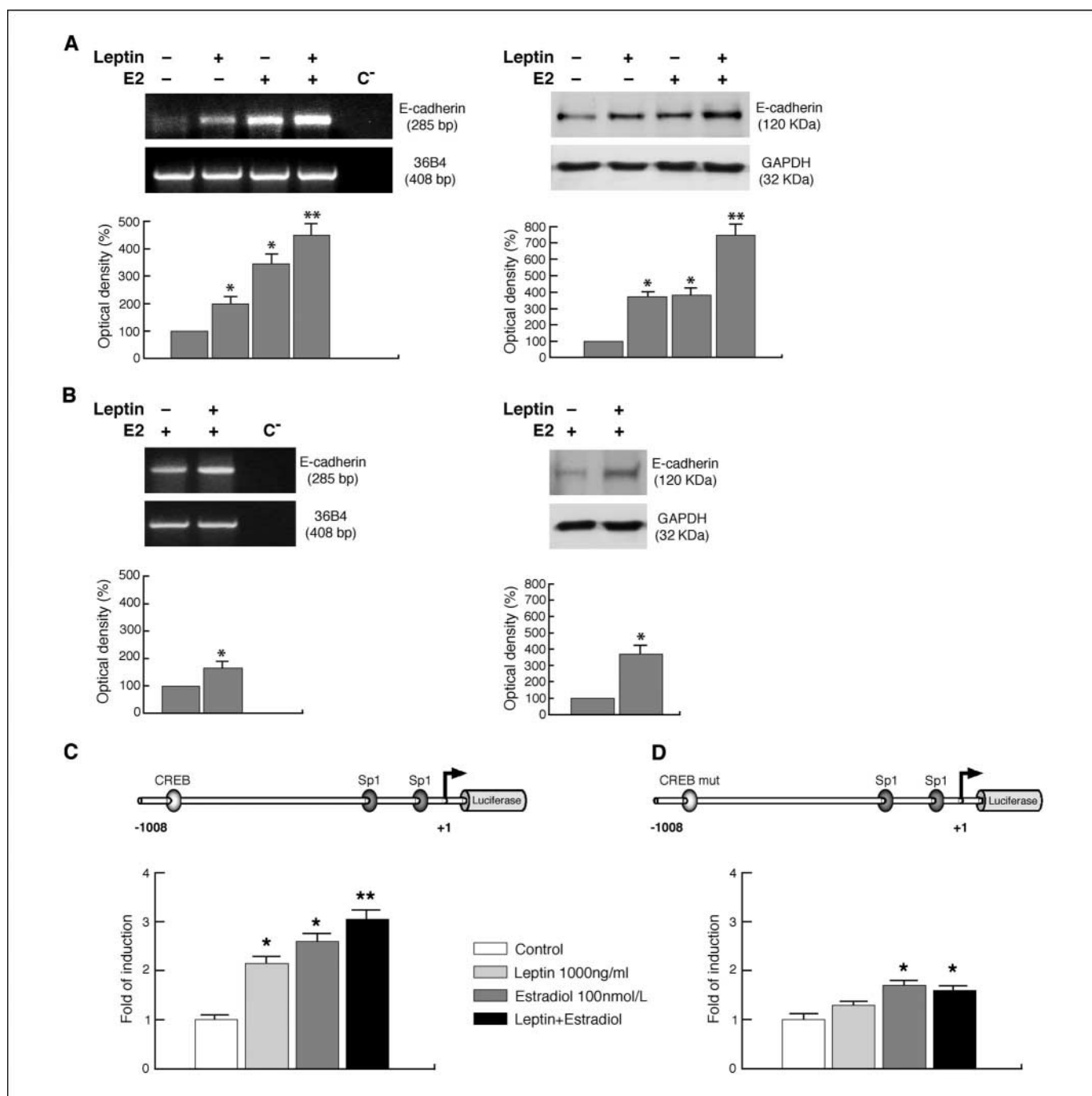*$P < 0.05$ versus leptin and E_2.

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 E$_2$ (*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 E$_2$. *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.

with elution buffer (1% SDS and 0.1 mol/L NaHCO$_3$), 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′-TTGAGACG-GAGTCTCGCTCT-3′) and Sp1 primers (5′-TAGCAACTCCAGGCTAGAGG-3′

and 5′-AACTGACTTCCGCAAGCTCACA-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.

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 E_2 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 *in vivo* biological features of tumors and improve the relevance of *in vitro* studies (27–30).

Our results evidenced that leptin and/or E_2 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 singled suspended.

In addition, the role of E-cadherin was confirmed using an adhesion assay in which cells were allowed to adhere to E-cadherin/Fc protein–coated dishes. This assay showed a greater binding of cells pretreated with leptin and/or E_2 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 E_2 was dependent on E-cadherin.

In three-dimensional cultures, we also observed a significant increase of cell growth upon leptin and/or E_2 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 E_2 (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 E_2 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 both 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 E_2 for 48 h (Fig. 3C).
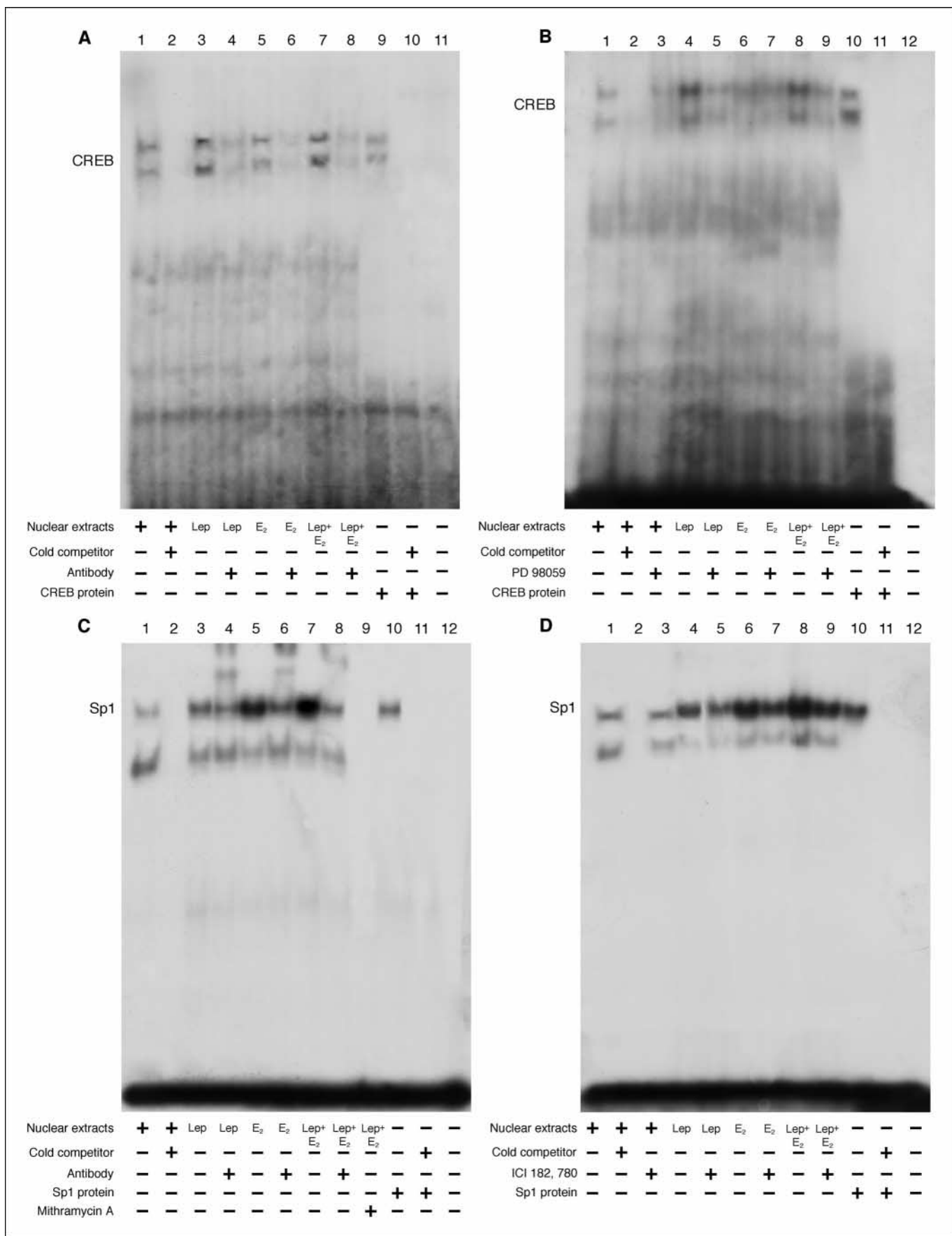
In contrast, we observed that leptin was unable to activate the constructs containing different deleted segments of human E-cadherin promoter (p-164/+49 and p-83/+49) with respect to the full length, whereas E_2 induced activation in the presence of p-164/+49 construct (Supplementary Fig. S2).

Leptin enhances CREB-DNA and Sp1-DNA binding activity to E-cadherin promoter. The role of leptin and E_2 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 E_2 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-responsive 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 E_2 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

Figure 4. Effects of *in vitro* leptin treatment on CREB-DNA and Sp1-DNA binding activity in MCF-7 cells. Nuclear extracts from MCF-7 cells were incubated with a double-stranded CREB-specific (A and B) or Sp1-specific (C and D) consensus sequence probe labeled with [γ-^{32}P]ATP and subjected to electrophoresis in a 6% polyacrylamide gel (lane 1). A, we used as positive control a transcribed and translated *in vitro* CREB protein (lane 9). Competition experiments were done by adding as competitor a 100-fold molar excess of unlabeled probe (lanes 2 and 10). MCF-7 nuclear extracts treated with 1,000 ng/mL leptin (Lep) and/or 100 nmol/L E_2 for 48 h incubated with probe (lanes 3, 5, and 7, respectively). The specificity of the binding was tested by adding to the reaction mixture a CREB antibody (lanes 4, 6, and 8). B, MCF-7 cells were serum starved overnight with 10 μmol/L PD 98059 (lanes 3, 5, 7, and 9). Lanes 11 (A) and 12 (B) contain probe alone. C, Sp1 human recombinant protein was used as positive control (lane 10). Competition experiments were done by adding as competitor a 100-fold molar excess of unlabeled probe (lanes 2 and 11). MCF-7 nuclear extracts treated with 1,000 ng/mL leptin and/or 100 nmol/L E_2 for 48 h incubated with probe (lanes 3, 5, and 7, respectively). The specificity of the binding was tested by adding to the reaction mixture a Sp1 antibody (lanes 4, 6, and 8). The formation of DNA-Sp1 complexes was blocked by the addition of 100 μmol/L mithramycin A (lane 9). D, the pure antiestrogen ICI 182,780 (1 μmol/L) was added in leptin-treated (lane 5) and/or E_2-treated (lanes 7 and 9) MCF-7 nuclear extracts. Lane 12 contain probe alone.

A

Nuclear extracts	+	+	Lep	Lep	E₂	E₂	Lep+E₂	Lep+E₂	−	−	−
Cold competitor	−	+	−	−	−	−	−	−	−	+	−
Antibody	−	−	−	+	−	+	−	+	−	−	−
CREB protein	−	−	−	−	−	−	−	−	+	+	−

B

Nuclear extracts	+	+	+	Lep	Lep	E₂	E₂	Lep+E₂	Lep+E₂	−	−	−
Cold competitor	−	+	−	−	−	−	−	−	−	−	+	−
PD 98059	−	−	+	−	+	−	+	−	+	−	−	−
CREB protein	−	−	−	−	−	−	−	−	−	+	+	−

C

Nuclear extracts	+	+	Lep	Lep	E₂	E₂	Lep+E₂	Lep+E₂	Lep+E₂	−	−	−
Cold competitor	−	+	−	−	−	−	−	−	−	−	+	−
Antibody	−	−	−	+	−	+	−	+	−	−	−	−
Sp1 protein	−	−	−	−	−	−	−	−	−	+	+	−
Mithramycin A	−	−	−	−	−	−	−	−	+	−	−	−

D

Nuclear extracts	+	+	+	Lep	Lep	E₂	E₂	Lep+E₂	Lep+E₂	−	−	−
Cold competitor	−	+	−	−	−	−	−	−	−	−	+	−
ICI 182, 780	−	−	+	−	+	−	+	−	+	−	−	−
Sp1 protein	−	−	−	−	−	−	−	−	−	+	+	−

of mithramycin A (100 μmol/L), which binds to GC boxes and prevents sequential Sp1 binding, to nuclear extracts treated with leptin and E_2 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 E_2 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 E_2 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. Stemming 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 effects of E_2 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, E_2 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 progres-
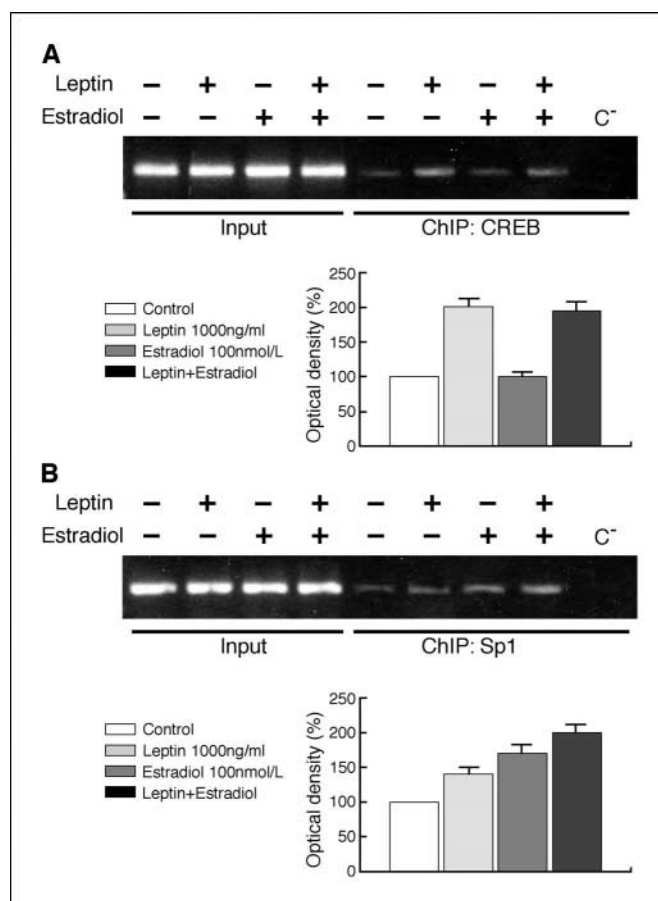

Figure 5. Recruitment of CREB and Sp1 to the E-cadherin promoter in MCF-7 cells. The cells were treated for 1 h with 1,000 ng/mL leptin and/or 100 nmol/L E_2 or left untreated. The precleared chromatin was immunoprecipitated with specific antibodies [i.e., anti-CREB for CREB immunoprecipitates (A) or anti-Sp1 for Sp1 immunoprecipitates (B)]. E-cadherin promoter sequences containing CREB or Sp1 sites were detected by PCR with specific primers, as detailed in Materials and Methods. To determine input DNA, the E-cadherin promoter fragment was amplified from 5 μL purified soluble chromatin before immunoprecipitation. PCR products obtained at 30 cycles. ChIP with non-immune IgG was used as negative control (C^-). This experiment was repeated three times with similar results. Most representative experiment. *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.

sion, 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 E_2-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 E_2 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 E_2 on E-cadherin/Fc protein–coated dishes.

In the same experimental conditions, an increased proliferative rate was observed upon leptin or E_2 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 E_2 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 E_2 signals. It is well documented how leptin and E_2 through nongenomic effects are able to activate the MAPK pathway that induces activation of CREB kinase, a member of the $p90^{RSK}$ family that corresponds to RSK2 and thereby phosphorylates CREB Ser^{133} (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 E_2 maintained an activatory

effect although in a lower extent with respect to the intact promoter. The latter data suggest that the activatory effect of E_2 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 E_2, 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 E_2 has been well documented in neoplastic mammary tissues and breast cancer cell lines (15, 25, 46, 47). For instance, E_2 up-regulates leptin expression in MCF-7 cells (15), whereas leptin is an amplifier of E_2 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
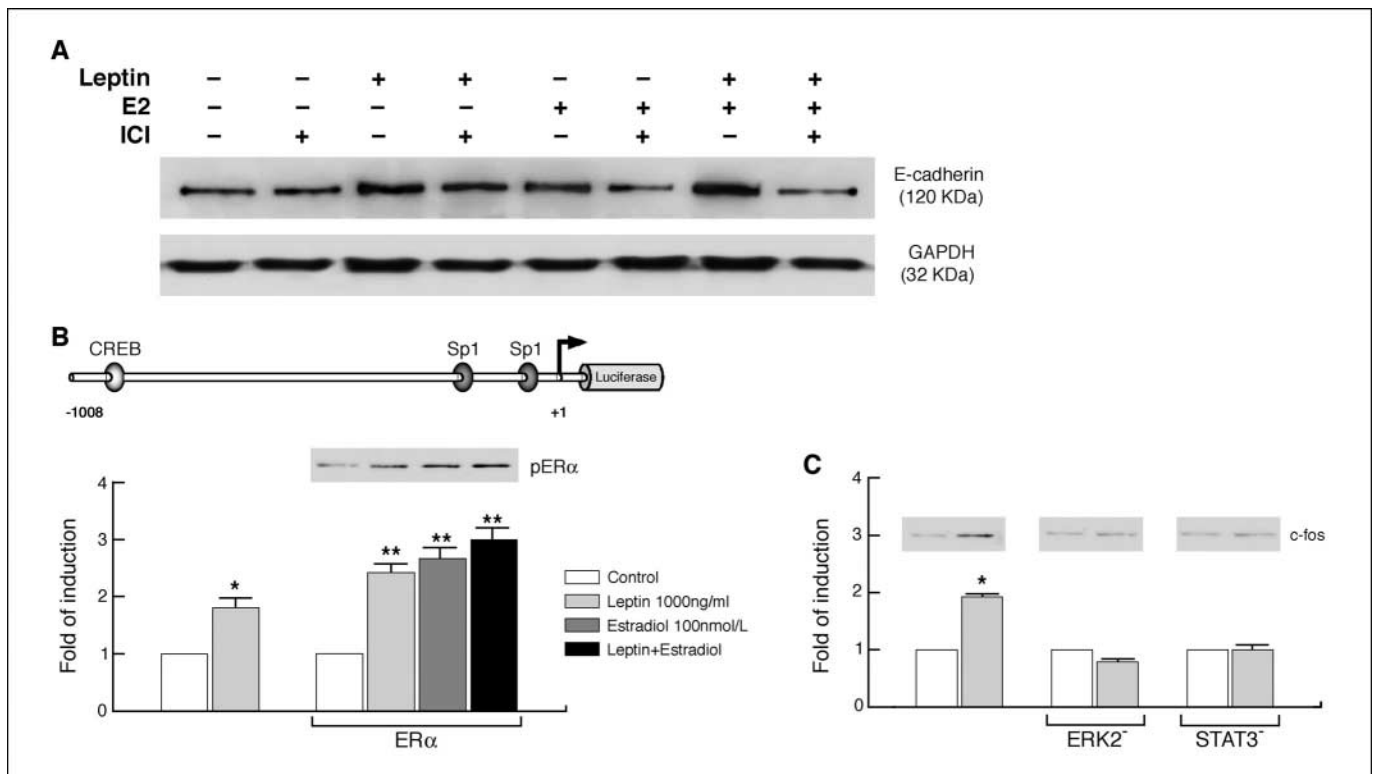

Figure 6. Influence of ERα on leptin-induced upregulation of E-cadherin expression. *A,* MCF-7 spheroids were preincubated with 1 μmol/L ICI 182,780 (*ICI*) for 1 h and then treated with leptin (1,000 ng/mL) and/or E_2 (100 nmol/L) for 48 h. Total proteins (50 μg) were immunoblotted with a specific antibody 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 E_2 (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 (Ser^{118}). *, $P < 0.05$; **, $P < 0.01$, 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.
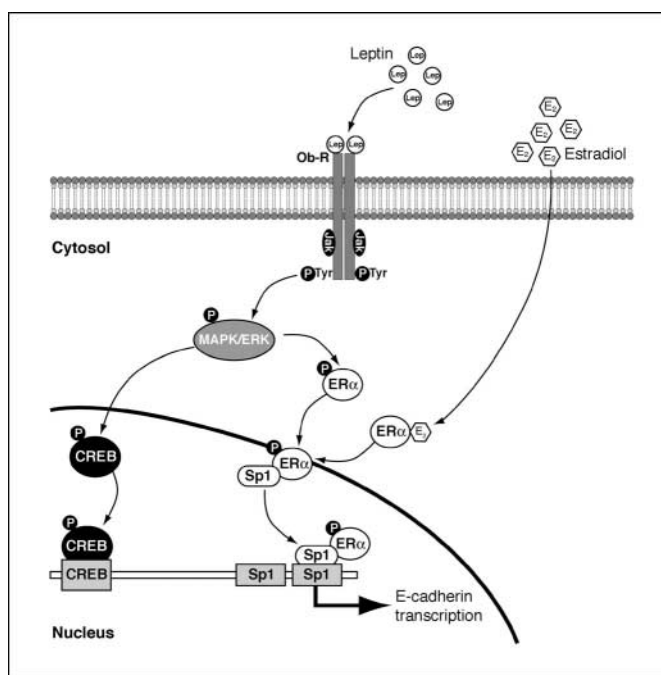
Figure 7. Hypothesized model of leptin signaling in modulating E-cadherin expression in breast cancer. Interaction of leptin (*Lep*) with its specific receptor (ObR) induces, through MAPK activation, phosphorylation of CREB and its transactivation. Leptin may potentiate the transactivation of ERα, which in turn may interact with Sp1 and bind DNA in a nonclassic way. Both CREB and Sp1 transcriptional factors bind on E-cadherin promoter at specific responsive sequences and induce an enhanced E-cadherin expression.

leptin signaling is involved in enhancing E-cadherin expression. These latter data are supported by Western blotting analysis done in BT-20 cells lacking of ERα in which ERK2 and STAT3 dominant negative reversed leptin-enhanced E-cadherin protein content. The up-regulatory effect induced by E_2 on E-cadherin expression in the presence of ectopic ERα seemed inhibited in the presence of ERK2 and STAT3 dominant negative. The latter findings may be a consequence of the enhanced expression of leptin receptor upon E_2 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 E_2 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 Ser[133] 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 E_2 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 E_2 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.

Acknowledgments

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Fas Ligand Expression in TM4 Sertoli Cells is Enhanced by Estradiol "In situ" Production

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The testis is an immunologically privileged site of the body where Sertoli cells work on to favor local immune tolerance by testicular autoantigens segregation and immunosuppressive factors secretion. Fas/Fas Ligand (FasL) system, expressed prevalently in Sertoli cells, has been considered to be one of the central mechanisms in testis immunological homeostasis. In different cell lines it has been reported that the proapoptotic protein FasL is regulated by 17-β estradiol (E2). Thus, using as experimental model mouse Sertoli cells TM4, which conserve a large spectrum of functional features present in native Sertoli cells, like aromatase activity, we investigated if estradiol "in situ" production may influence FasL expression. Our results demonstrate that an aromatizable androgen like androst-4-ene-3,17-dione (Δ4) enhanced FasL mRNA, protein content and promoter activity in TM4 cells. The treatment with $N^6,2'$-O-dibutyryladenosine-3'-5'-cyclic monophosphate [(Bu)₂cAMP] (simulating FSH action), that is well known to stimulate aromatase activity in Sertoli cells, amplified Δ4 induced FasL expression. Functional studies of mutagenesis, electrophoretic mobility shift (EMSA) and chromatin immunoprecipitation (ChIP) assays revealed that the Sp-1 motif on FasL promoter was required for E2 enhanced FasL expression in TM4 cells. These data let us to recruit FasL among those genes whose expression is up-regulated by E2 through a direct interaction of ERα with Sp-1 protein. Finally, evidence that an aromatizable androgen is able to increase FasL expression suggests that E2 production by aromatase activity may contribute to maintain the immunoprivilege status of Sertoli cells.

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The immunoprivilege of male gonad lies on blood-testis barrier, prevalently maintained by Sertoli cell functions. This physical barrier between the general circulation and testicular tissue probably conceals antigens from the immune system and prevents effector cell access (Filippini et al., 2001; Bart et al., 2002; Ferguson et al., 2002). This immune protective function together with the secretion of hormonal and nutritive factors produced by Sertoli cells, under FSH control, substain germ cells functional maturation along all spermatogenesis process (Griswold et al., 1988; De Cesaris et al., 1992).

The Fas/FasL system was first identified in T cells (Suda et al., 1993; Lynch et al., 1995) where it plays a key role in eliminating T cell populations following antigenic stimulation and clonal proliferation. This system is also functional in the testis (Bellgrau et al., 1995; Sanberg et al., 1996) and in a variety of other tissues in which these proteins are constitutively expressed to maintain their immunoprivilege, such as eyes (Griffith et al., 1995), placenta (Guller, 1997; Uckman et al., 1997) and brain (Saas et al., 1997).

FasL is a type II trans-membrane protein that belongs to the tumor necrosis factor (TNF) family of cytokines and induces apoptosis in cells expressing Fas receptors (Suda et al., 1993). Fas (CD95, APO-1) is a transmembrane receptor protein, sharing a high degree of homology with the tumor necrosis factor/nerve growth factor receptor family (TNF/NGF-Rs) (Watanabe-Fukunaga et al., 1992; Nagata and Goldstein, 1995). It is characterized by an intracellular domain called "death domain" responsible for the activation of the intracellular signaling pathway following Fas-FasL interaction (Nagata and Goldstein, 1995).

The Fas/FasL expression during testicular development and its cell specific localization within the testis is still a matter of debate, but it is generally assumed that FasL is predominantly expressed in Sertoli cells (Suda et al., 1993; Bellgrau et al., 1995; French et al., 1996; Lee et al., 1997; Francavilla et al., 2000; D'Abrizio et al., 2004).

Among the different factors influencing FasL, it has been reported that 17-β estradiol (E2) is able to regulate the expression of this proapoptotic protein in human endometrial cells (Selam et al., 2001) and human ovarian tissue (Sapi et al., 2002). Moreover, estrogen treatment increases FasL expression in monocytes through the interaction of estrogen receptor with FasL promoter (Mor et al., 2003).

It has been well established that the estrogens biosynthesis, in the testis, is catalyzed by the enzyme complex referred to as aromatase cytochrome P450, which aromatizes the A ring of C19 androgens to the phenolic A ring of C18 estrogens (Armstrong and Dorrington, 1977; Van der Molen et al., 1981). The enzyme aromatase is composed of two polypeptides: an ubiquitous non-specific flavoprotein NADPH-cytochrome P450 reductase and a specific form of cytochrome P450 (P450arom encoded by the CYP19 gene) (Simpson et al., 1994). In the testis an age-related change has been observed in the cellular localization of the aromatization event, primarily in Sertoli cells in immature animals, but located in Leydig and germ cells in adults (Levallet et al., 1998; Andò et al., 2001). Besides, the synthesis of estrogens is regulated at the level of the

aromatizing enzyme system by Follicole-Stimulating Hormone (FSH) and cyclic AMP (Dorrington and Armstrong, 1975). In the mouse Sertoli cell line TM4 we previously demonstrated P450arom immunocytochemical localization together with its enzymatic activity (Catalano et al., 2003).

In the present study, we investigated if an aromatizable androgen like androst-4-ene-3,17-dione (Δ4), after its conversion to E2, can modulate FasL expression in TM4 cells. Our results demonstrate that estradiol "in situ" production enhanced FasL mRNA, protein content and promoter activity. Many transcription factors have been reported to regulate FasL promoter by DNA-protein interaction upon diverse biological signals in different cells and tissues (Latinis et al., 1997; Kasihatla et al., 1998; Matsui et al., 1998; Mittelstadt and Ashwell, 1998; Kavurma et al., 2001; Kirschhoff et al., 2002; Kavurma and Khachigian, 2003).

Functional studies of mutagenesis, electrophoretic mobility shift analysis and ChIP assay lead us to demonstrate that the up-regulatory effects induced by E2 on FasL expression are mediated by a direct interaction of Estrogen Receptor alpha (ERα) with Sp-1 protein.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12), Triazol Reagent and 100 bp DNA ladder by Invitrogen (Carlsbad, CA), L-Glutamine, penicillin, horse serum, Eagle's non-essential amino acids, calf serum (CS), streptomycin, bovine serum albumine (BSA), phosphate-buffered saline (PBS) were purchased from Eurobio (Les Ullis Cedex, France). FuGENE 6, Sephadex G50 spin columns and poly (dl-dC) by Roche (Indianapolis, IN). GoTaq DNA polymerase, T4 polynucleotide Kinase, TNT master mix, Dual luciferase kit, Sp-1 human recombinant protein and TK renilla luciferase plasmid were provided by Promega (Madison, WI). The RETROscript kit and DNase I were purchased from Ambion (Austin, TX). Aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, androst-4-ene-3,17-dione (Δ4), 7α,19α-dimethyl-19-nortestosterone (mibolerone), formaldehyde, NP-40, proteinase K, tRNA, Tamoxifen (Tam), $N^6,2'$-O-dibutyryladenosine-3'-5'-cyclic monophosphate [(Bu)$_2$cAMP] and 1,3,5-Tris(4-Hydroxyphenyl)-4-propyl-1H-pyrazole (PPT) by Sigma (Milan, Italy). Antibodies against ERα, ERβ, β-actin, Sp-1, and polymerase II (N20) were provided by Santa Cruz Biotechnology (Santa Cruz, CA) whereas anti-FasL antibody by BD biosciences (San Josè, CA). ECL System and [γ^{32}P]ATP were purchased by Amersham Pharmacia (Buckinghamshire, UK). Letrozole was provided by Novartis Pharma AG (Basel, Switzerland), Mithramycin by ICN Biomedicals, (Shelton, CT). Salmon sperm DNA/protein A agarose by UBI (Chicago, IL). Diarylpropionitrile (DPN) and ICI 182,780 were purchased from Tocris chemical (Bristol, UK). ABI Prism 7000 Sequence Detection System, TaqMan Ribosomal RNA Reagent kit, TaqMan Ribosomal RNA Control Reagent kit and SYBR Green Universal PCR Master Mix by Biosystems (Forster City, CA).

Cell cultures

The TM4 cell line, derived from the testis of immature BALB/c mice, was originally characterized based on its morphology, hormone responsiveness, and metabolism of steroids (Mather, 1980). This cell line was obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and cultured in DMEM-F12 containing 2.5% fetal CS, 5% horse serum, 1 mg/ml penicillin–streptomycin. Human uterin cervix adenocarcinoma (HeLa) cells were obtained from the ATCC. HeLa cells were cultured in DMEM/F12 containing 5% CS, 1% L-Glutamine, 1% Eagle's non essential amino acids and 1 mg/ml penicillin–streptomycin.

Western blot analysis

TM4 cells were grown in 10 cm dishes to 70–80% confluence and lysed in 500 μl of 50 mM Hepes (pH 7.5), 150 mM NaCl, 1.5 mM MgCl$_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, a mixture of protease inhibitors (Aprotinin, PMSF and Na-orthovanadate). Equal amounts of total proteins were resolved on a 11% SDS-polyacrylamide gel and then electroblotted onto a nitrocellulose membrane. Blots were incubated overnight at 4°C with: (1) mouse monoclonal ERα antibody, (2) rabbit polyclonal ERβ ??antibody, (3) mouse monoclonal FasL antibody, (4) mouse monoclonal β-actin antibody. The antigen-antibody complex was detected by incubation of membranes 1 h at room temperature with peroxidase-coupled goat anti-rabbit IgG or goat anti-mouse IgG and revealed using the ECL System. Blots were then exposed to film and bands of interest were quantified by densitometer (Mod 620 BioRad, USA). The results obtained as optical density arbitrary values were transformed to percentages of the control (percent control) taking the samples from cells not treated as 100%.

Real-time RTPCR

Total cellular RNA was extracted from TM4 cells using "TRIAZOL Reagent" as suggested by the manufacturer. All RNA was treated with DNase I and purity and integrity of the RNA were confirmed spectroscopically and by gel electrophoresis prior to use. Two micrograms of total RNA was reverse transcribed in a final volume of 50 μl using a RETROscript kit as suggested by the manufacturer. cDNA was diluted 1:5 in nuclease free water, aliquoted and stored at −20°C. The cDNAs obtained were further amplified for FasL gene using the following primers: forward 5′-CGAGGAGTGTGGCCCATTT-3′ and reverse 5′-GGTTCCATATGTGTCTTCCCATTC-3′.

PCR reactions were performed in the ABI Prism 7000 Sequence Detection System, using 0.1 μM of each primer, in a total volume of 30 μL reaction mixture following the manufacturer's recommendations. SYBR Green Universal PCR Master Mix for the dissociation protocol was used for FasL and 18S. Negative control contained water instead of first-strand cDNA. Each sample was normalized on the basis of its 18S ribosomal RNA content. The 18S quantification was performed using a TaqMan Ribosomal RNA Reagent kit following the method provided in the TaqMan Ribosomal RNA Control Reagent kit. The relative FasL gene expression levels were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as n-fold differences in FasL gene expression relative to 18S rRNA and calibrator, calculated following the ΔΔCt method, as follows:

$$\text{n-fold} = 2^{-(\Delta Ct_{sample} - \Delta Ct_{calibrator})}$$

where ΔCt values of the sample and calibrator were determined by subtracting the average Ct value of the 18S rRNA reference gene from the average Ct value of the different genes analyzed.

Transfection assay

Transient transfection experiments were performed using pGL$_2$ vectors containing different deleted segments of human FasL gene promoter (p-2365: −2365/−2; p-318: −318/−2; p-237: −237/−2) ligated to a luciferase reporter gene (kindly provided by Dr. Paya, Department of Immunology, Mayo Clinic Rochester, Minnesota, USA). Deletion of Sp-1 sequence in FasL gene promoter was generated by PCR using as template p-318 construct. The resulting plasmid encoding the human Fas-L gene promoter containing the desired deletion was designed p-280 Sp-1 and the sequence was confirmed by nucleotide sequence analysis.

FuGENE 6 was used as recommended by the manufacturer to transfect TM4 cells plated in 3.5 cm^2 wells with pGL$_2$ FasL promoter constructs (0.5 μg/well).

Another set of experiments was performed in HeLa cells cotransfecting p-318 FasL promoter (−318/−2) (0.5 μg/well) and the wild-type human ERα expression vector (HEGO) (0.5 μg/well) (Tora et al., 1989) or pCMV5-hERβ, containing human ERβ gene (0.5 μg/well) (a gift from JA Gustafsson).

Empty vectors were used to ensure that DNA concentrations were constant in each transfection. TK renilla luciferase plasmid (25 ng/well) was used to normalize the efficiency of the transfection. Twenty-four hours after transfection, the medium was changed and TM4 cells were treated in serum free medium (SFM) in the presence of Δ4, (Bu)$_2$cAMP, mibolerone, letrozole, PPT and DPN. HeLa cells, 24 h after transfection, were treated in the presence or absence of E2 for 24 h. The firefly and renilla luciferase activities were measured using Dual Luciferase Kit. The firefly luciferase data for each sample were

normalized on the basis of transfection efficiency measured by renilla luciferase activity.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from TM4 as previously described (Andrews and Faller, 1991). Briefly, TM4 cells plated into 60 mm dishes were scraped into 1.5 ml of cold PBS. Cells were pelleted for 10 sec and resuspended in 400 μl cold buffer A (10 mM HEPES-KOH pH 7.9 at 4°C, 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 mM leupeptin) by flicking the tube. The cells were allowed to swell on ice for 10 min and then vortexed for 10 sec. Samples were then centrifuged for 10 sec and the supernatant fraction discarded. The pellet was resuspended in 50 μl of cold Buffer B (20 mM HEPES-KOH pH 7.9, 25% glycerol, 1.5 mM MgCl$_2$, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 mM leupeptin) and incubated on ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation for 2 min at 4°C and the supernatant fraction (containing DNA binding proteins) was stored at −70°C. The yield was determined by Bradford method (Bradford, 1976). The probe was generated by annealing single stranded oligonucleotides and labeled with [γ^{32}P] ATP and T4 polynucleotide kinase, and then purified using Sephadex G50 spin columns. The DNA sequences used as probe or as cold competitor are the following (the nucleotide motifs of interest are underlined and mutations are shown as lowercase letters): Sp1 5′-AAATTGTGGGCGGAAACTTCCAGGGG-3′, mutated Sp-1 5′-AAATTGTGttCGGAAACTTCCAGGGG-3′. Oligonucleotides were synthesized by Sigma Genosys. The protein binding reactions were carried out in 20 μl of buffer (20 mM HEPES pH 8, 1 mM EDTA, 50 mM KCl, 10 mM DTT, 10% glicerol, 1 mg/ml BSA, 50 μg/ml poli dI/dC) with 50,000 cpm of labeled probe, 10 μg of TM4 nuclear protein and 5 μg of poly (dI-dC). The above-mentioned mixture was incubated at room temperature for 20 min in the presence or absence of unlabeled competitor oligonucleotide. For experiments involving Sp-1, ERα and ERβ antibodies, the reaction mixture was incubated with these antibodies at 4°C for 12 h. For in vitro mithramycin treatment, mithramycin (100 nM) was incubated with the labeled probe for 30 min at 4°C before the addition of nuclear extract. As positive controls we used Sp-1 human recombinant protein (1 μl) and in vitro transcribed and translated ERα protein (1 μl) synthesized using T7 polymerase in the rabbit reticulocyte lysate system as direct by the manufacturer. The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel in 0.25 X Tris borate-EDTA for 3 h at 150 V. Gel was dried and subjected to autoradiography at −70°C.

Chromatin immunoprecipitation (ChIP)

According to the ChIP assay procedure previously described (Shang et al., 2000), TM4 cells were grown in 60 mm dishes to 50–60% confluence, shifted to SFM for 24 h and then treated with E2 (100 nM), ICI 182,780 (10 μM), E2 + ICI for 1 h. Thereafter, the cells were washed twice with PBS and crosslinked with 1% formaldehyde at 37°C for 10 min. Next, cells were washed twice with PBS at 4°C, collected and resuspended in 200 μl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1) and left on ice for 10 min. Then, cells were sonicated four times for 10 sec at 30% of maximal power (Sonics, Vibra Cell 500W) and collected by centrifugation at 4°C for 10 min at 14,000 rpm. The supernatants were diluted in 1.3 ml of IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 16.7 mM NaCl) and immunocleared with 80 μl of sonicated salmon sperm DNA/ protein A agarose for 1 h at 4°C. The precleared chromatin was immunoprecipitated with a specific anti-Sp-1, anti ERα and anti polymerase II antibodies and with a normal mouse serum IgG (Nms) as negative control. At this point, 60 μl of salmon sperm DNA/protein A agarose were added and precipitation was further continued for 2 h at 4°C. After pelleting, precipitates were washed sequentially for 5 min with the following buffers: Wash A (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), Wash B (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), and Wash C (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1), and then twice with TE buffer (10 mM Tris, 1 mM EDTA). The immunocomplexes were eluted with elution buffer (1% SDS, 0.1 M NaHCO$_3$), reverse crosslinked by heating at 65°C and digested with proteinase K (0.5 mg/ml) at 45°C for 1 h. DNA was obtained by phenol/chloroform/isoamyl alcohol extraction. Two microliters of 10 mg/ml yeast tRNA were added to each sample and DNA was precipitated with 70% EtOH for 24 h at −20°C, and then

washed with 95% EtOH and resuspended in 20 μl of TE buffer. One microlitre of each sample was used for PCR amplification with the following primers flanking Sp-1 sequence present in the Fas-L promoter region: 5′-GCAACTGAGGGCCTTGAAGGC-3′ (forward) and 5′-GCAGCTGGTGAGTCAGGCCAG-3′ (reverse). The PCR conditions were 1 min at 94°C, 1 min at 65°C, and 2 min at 72°C. The amplification products obtained in 25 cycles were analyzed in a 2% agarose gel and visualized by ethidium bromide staining.

Statistical analysis

Each datum point represents the mean ± SE of three different experiments. Data were analyzed by ANOVA test using the STATPAC computer program.

Results

Estradiol "in situ" production, by aromatase activity, enhances FasL expression in TM4 cell line

In TM4 cells, which exibit a spectrum of features in common with native Sertoli cells, like the presence of aromatase activity, we investigated if an aromatizable androgen Δ4, through its conversion into E2, may influence FasL mRNA and protein content by Real-time RT-PCR and Western blot analysis. Since aromatase expression and activity, in Sertoli cells, is under FSH control (Dorrington and Armstrong, 1975) we also evaluated the treatment with (Bu)$_2$cAMP (simulating FSH action) on FasL expression.

As shown in Figure 1A the treatment with Δ4 (100 nM) for 24 h resulted in an increase of FasL mRNA expression more than 1.9-fold. The simultaneous treatment with (Bu)$_2$cAMP (1 mM) and Δ4, further enhanced FasL mRNA expression compared with Δ4 treatment alone (2.4-fold), suggesting that (Bu)$_2$cAMP stimulates E2 "in situ" production by its action on aromatase activity. These up-regulatory effects were reversed by addition of the aromatase inhibitor letrozole (1 μM) (90%), while no significant difference was observed in the presence of a non-aromatizable androgen mibolerone (100 nM) with or without (Bu)$_2$cAMP.

Next, we performed Western blot analysis using a monoclonal antibody anti FasL. We detected a band of 37 kDa which intensity was increased upon Δ4 treatment. Exposure to (Bu)$_2$cAMP combined with Δ4 enhanced the effect induced by Δ4 alone. The addition of letrozole reversed these up-regulatory effects (Fig. 1B,C).

To evaluate whether E2 "in situ" production was able to activate FasL promoter we transiently transfected TM4 cells with vector containing human FasL promoter fused to the luciferase reporter gene. The treatment for 24 h with Δ4 or Δ4 + (Bu)$_2$cAMP displayed a significant increase of the basal promoter activity that was reversed by letrozole (Fig. 1D).

Effects of Δ4 on expression of human FasL promoter/ luciferase reporter gene constructs in TM4 cells

To delimit the cis-elements involved in FasL transcriptional activation by Δ4, we transiently transfected TM4 cells with plasmids containing different deleted segments of human FasL promoter. Schematic representation of constructs is shown in Figure 2A. Transfected cells were untreated (C) or treated with 100 nM of Δ4 and 1 μM of letrozole.

p-318 plasmid showed a higher basal activity when compared with the other plasmids (p-2365, p-237) (Fig. 2B) suggesting the presence of a DNA sequences upstream from −318 to which transcription factors with repressor activity bind. These data well fit with previous results demonstrating that FasL gene promoter region, located between −318 and −237, plays a major role in promoting basal transcription in TM4 Sertoli cells (McClure et al., 1999).

In TM4 cells transfected with p-2365 and p-318 plasmids the treatment with Δ4 induced a significant increase of the basal
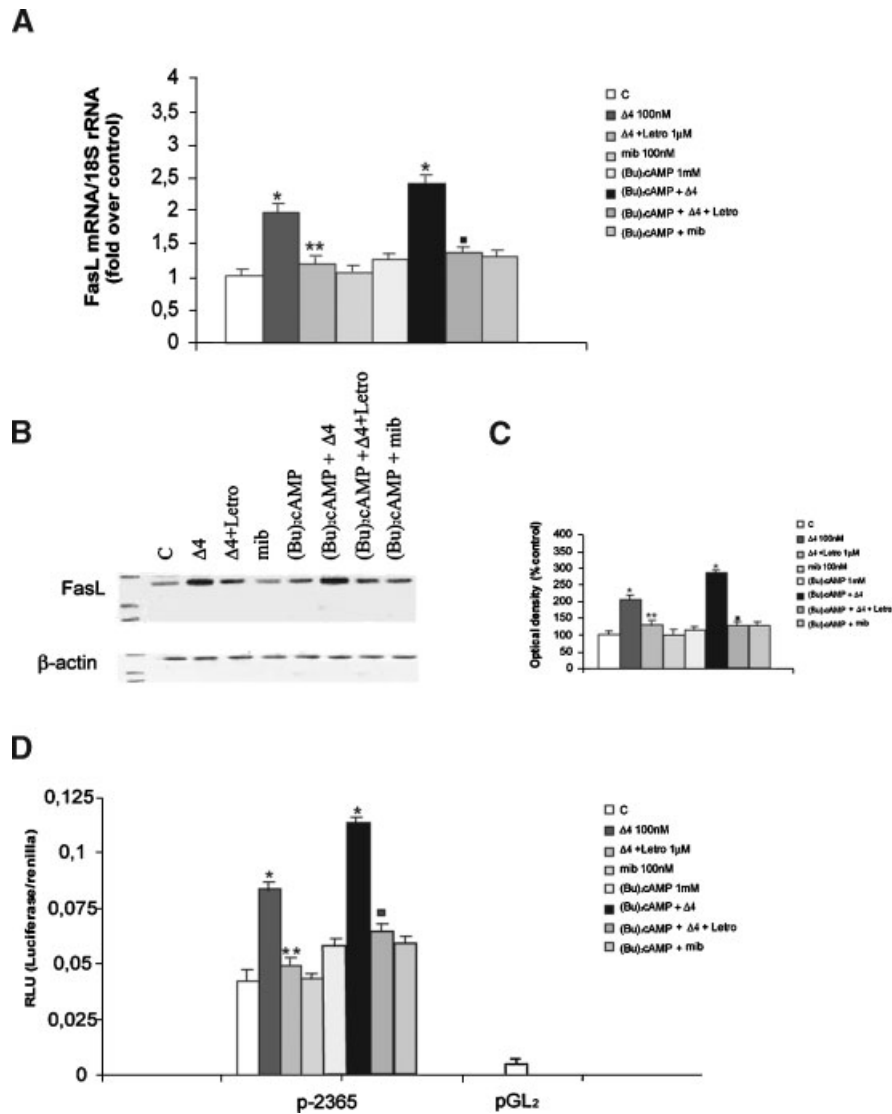
Fig. 1. Effects of Δ4 on FasL expression. A: Total RNA was obtained from TM4 cells untreated (control, C) or treated for 24 h with Δ4 (100 nM) mibolerone (mib 100 nM), $(Bu)_2cAMP$ (1 mM), $(Bu)_2cAMP + Δ4$ and $(Bu)_2cAMP + mib$. One micromolar of aromatase inhibitor letrozole (Letro) was used. Real time RT-PCR was performed to analyze mRNA levels of FasL. Data represent the mean ± SE of values from three separate RNA samples. Each sample was normalized to its 18S ribosomal RNA content. Final results are expressed as n-fold differences of gene expression relative to calibrator (control) calculated with the $ΔΔCt$ method as indicated in the "Material and Methods" section. $*P < 0.01$ compared to control. $**P < 0.01$ compared to $Δ_4$ treated samples; ■ $P < 0.01$ compared to $(Bu)_2cAMP + Δ4$ treated samples. **B:** Immunoblot of FasL from TM4 cells treated in the absence (C) or in the presence of the above-mentioned treatments. **C:** The histograms represent the mean ± SE of three separate experiments in which band intensities were evaluated in term of optical density arbitrary units and expressed as percentage of the control assumed as 100%. $*P < 0.01$ compared to control; $**P < 0.01$ compared to $Δ_4$ treated samples; ■ $P < 0.01$ compared to $(Bu)_2cAMP + Δ4$ treated samples. **D:** Transcriptional activity of TM4 cells transfected with p-2365 construct is shown. TM4 cells were treated in the absence (C) or in the presence of Δ4 (100 nM), mibolerone (mib 100 nM), $(Bu)_2cAMP$ (1 mM), $(Bu)_2cAMP + Δ4$ and $(Bu)_2cAMP + mib$. One micromolar of aromatase inhibitor letrozole was used. The values represent the means ± SE of three different experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. pGL₂: basal activity measured in cells transfected with pGL₂ basal vector. $*P < 0.01$ compared to control. $**P < 0.01$ compared to Δ4 treated samples; ■ $P < 0.01$ compared to $(Bu)_2cAMP + Δ4$ treated samples.

promoter activity that was completely reversed by letrozole. In contrast, Δ4 was unable to activate p-237 construct eliciting, in the region from −318 to −237, the presence of cis-element involved in estrogen responsiveness. In fact, this region contains Sp-1 site, a potential target of ER. In order to explore the role of the Sp-1 binding site in the regulation of FasL expression by Δ4, functional experiments were performed using the Sp-1 deleted plasmid (p-280 Sp-1). Luciferase assay revealed that the inducibility by Δ4 on FasL promoter was totally lost (Fig. 2D).

These results suggest that the up-regulatory effects of estradiol production by aromatase activity require Sp-1 sequence motif.

ERβ is not involved in E2-modulating FasL expression

Before exploring more closely the possible interaction between E2/ER complex to Sp-1 and the role of this binding in modulating FasL expression, we set out to determine which functional ER(s) isoform was present in TM4 cells. By Western blotting
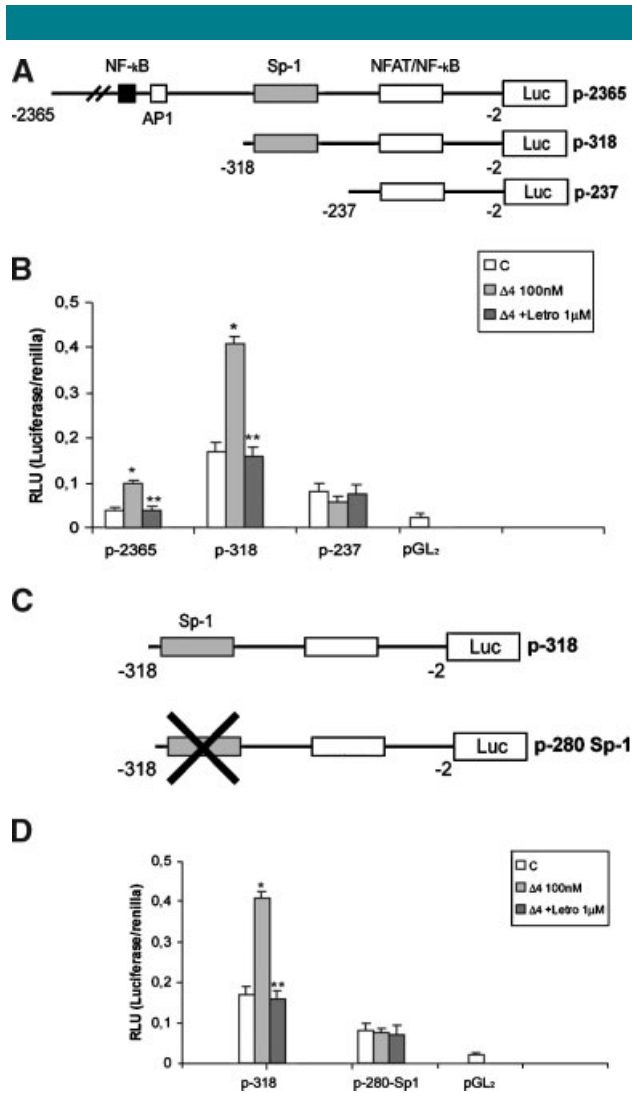
Fig. 2. Effects of estradiol "in situ" production on expression of human FasLpromoter/luciferase reporter gene construts in TM4 cells. **A:** Schematic map of the FasL promoter fragments used in this study. All of the promoter constructs contain the same 3′ boundary (−2). The 5′ boundaries of the promoter fragments varied from −237 to −2365. Each fragment was subcloned into the pGL₂ vector. **B:** Transcriptional activity of TM4 cells with promoter constructs is shown. TM4 cells were treated in the absence (C) or in the presence of Δ4 (100 nM), and Δ4 + letrozole (1 μM) for 24 h. The values represent the means ± SE of three different experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. pGL₂: basal activity measured in cells transfected with pGL₂ basal vector. *P < 0.01 compared to control; **P < 0.01 compared to Δ4 treated samples. **C:** Schematic representation of the p-318 and p-280 Sp-1 constructs. The deletion of Sp-1 sequence is present in p-280 Sp-1 construct containing the region from −318 to −2 of FasL promoter gene. Each fragment was subcloned into the pGL₂ vector. **D:** Transcriptional activity of TM4 cells with p-280 Sp-1 construct is shown. TM4 cells were treated in the absence (C) or in the presence of Δ4 (100 nM), and Δ4 + letrozole (1 μM) for 24 h. The values represent the mean ± SE of three different experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. *P < 0.01 compared to control; **P < 0.01 compared to Δ4-treated samples.

analysis, we demonstrated in TM4 protein extracts the presence of both ER(s) (Fig. 3A, lane 2), As positive control, the breast cancer cell line MCF-7 (ERα positive) and human prostate cancer cell line LNCaP (ERβ positive) were used (Fig. 3A, lane 1).

In the presence of the two different ER antagonists ICI 182,780 (10 μM) and tamoxifen (10 μM) (Tam) the up-regulation of E2 on FasL expression was abrogated demonstrating that this effect was specifically dependent by ER (Fig. 3B,C).

To specify which isoforms of ER were mainly involved in FasL transactivation, we cotransfected HeLa cells (ER negative) with p-318 FasL promoter and the wild type human ERα or ERβ expression vector. The treatment with E2 (100 nM) for 24 h showed an increased transcriptional activation of FasL promoter only in cells cotransfected with ERα (Fig. 3D). Finally, to demonstrate further the direct involvement of ERα in FasL transactivation we used 100 nM of the selective agonists of ERα [1,3,5-Tris(4-Hydroxyphenyl)-4-propyl-1H-pyrazole (PPT)] and ERβ [diarylpropionitrile (DPN)] in TM4 cells transiently transfected with p-318 FasL promoter. The treatment with PPT showed an increase of FasL promoter activity while no change was observed in the presence of DPN (Fig. 3E).

Effects of 17-β estradiol treatment on Sp1 DNA binding activity in TM4 cells

On the basis of the evidences that the up-regulatory effects of E2 on FasL require the crucial presence of Sp-1-RE, EMSA was performed using synthetic oligodeoxyribonucleotides corresponding to the putative Sp-1 binding site. In the presence of TM4 nuclear extracts (10 μg) we observed the formation of a specific complex (Fig. 4A, lane 1), which was abrogated by a 100-fold molar excess of unlabeled probe (Fig. 4A, lane 2). This inhibition was not observed when a mutated Sp-1 oligonucleotide was used as competitor (Fig. 4A, lane 3). E2-treatment induced a strong increase in Sp-1 DNA binding activity (Fig. 4A, lane 4) compared with basal levels. In the presence of ICI 182,780 the Sp-1 DNA binding activity was drastically reduced (Fig. 4A, lane 5). The addition of mithramycin (100 nM), that binds to GC boxes and prevents sequential Sp-1 binding, decreased the binding of E2 treated TM4 nuclear extracts on Sp-1 DNA sequence (Fig. 4A, lane 6). In a cell free system we observed in the presence of Sp-1 reconbinant protein a single band that causes the same shift respect to the complex revealed in TM4 nuclear extracts (Fig. 4A, lane 7) which was abrogated by 100-fold molar excess of unlabeled probe (Fig. 4A, lane 8). Transcribed and translated in vitro ERα protein did not bind directly to Sp-1 probe (Fig. 4A, lane 9). When the nuclear extracts from TM4 cells treated with E2 were incubated with either anti-Sp-1 or anti-ERα antibody, the original band DNA-protein complex was immunodepleted (Fig. 4B, lanes 3 and 4), whereas anti-ERβ antibody gave no effects (lane 5).

Taken together these results suggest that ERα is recruited by Sp-1 in our DNA binding complex.

17-β Estradiol enhances recruitment of Sp-1/ERα to the promoter region of FasL gene in TM4 cells

Interaction of ERα and Sp-1 with the FasL gene promoter was also investigated using a ChIP assay. After sonication and immunoprecipitation by anti ERα or anti Sp-1 antibodies, PCR was used to determine binding of ERα/Sp-1 protein to the −318 to −2 DNA region of the FasL gene promoter. Our results indicated that treatment with E2 induced an increased recruitment of Sp-1/ERα complex to the FasL promoter. The latter event was reduced in the presence of E2 + ICI. The enhanced recruitment of Sp-1/ERα was correlated with greater association of polymerase II to the FasL regulatory region
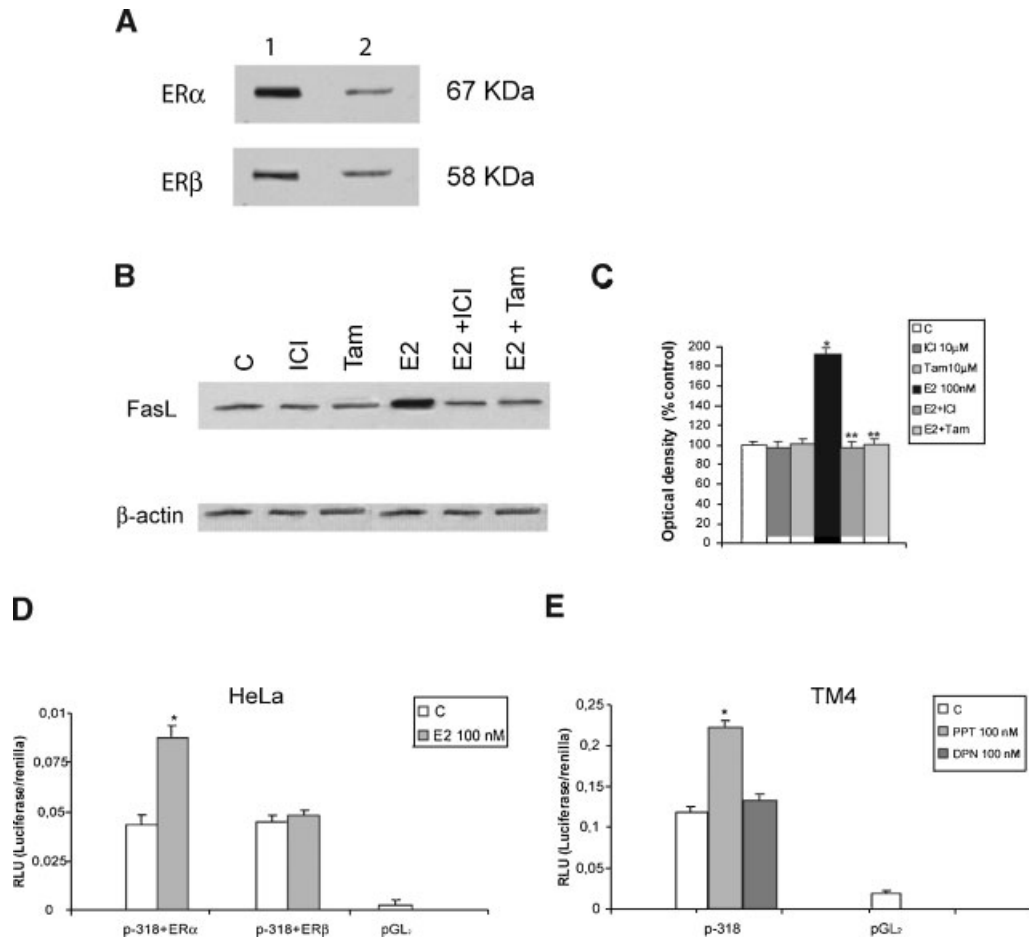
Fig. 3. 17β-Estradiol enhances FasL transcriptional activity through ERα. A: Lysates from TM4 cells were used to evaluate by Western blot analysis the expression of ERα and ERβ (lane 2). The human breast cancer cell line MCF-7 and human prostate cancer cell line LNCaP were used as positive control for ERα and ERβ respectively (lane 1). B,C: Immunoblot of FasL from TM4 cells treated in the absence (C) or in the presence of E2 (100 nM) for 24 h. The pure anti-estrogen ICI 182,780 (10 μM) and tamoxifen (Tam 10 μM) were used. The histograms represent the means ± SE of three separate experiments in which band intensities were evaluated in term of optical density arbitrary units and expressed as percentage of the control assumed as 100%. *$P < 0.01$ compared to control; **$P < 0.01$ compared to E2 treated samples. D: HeLa cells were transiently cotransfected with p-318 FasL promoter construct (−318/−2) and ERα or ERβ plasmids. The cells were untreated (C) or treated with E2 (100 nM) for 24 h. The values represent the means ± SE of three different experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. *$P < 0.01$ compared to control. E: TM4 cells transfected with p-318 FasL promoter construct were untreated (C) or treated with PPT (100 nM) and DPN (100 nM) for 24 h. *$P < 0.01$ compared to control.

(Fig. 5A). No PCR product was observed using DNA immunoprecipitated with normal mouse serum IgG.

Discussion

In testis, Fas/FasL interaction has been thought to play an important role in the establishment of immunoprivilege. Several reports have demonstrated that Sertoli cells through FasL may trigger apoptotic cell death of sensitive lymphoid cells, which express on their cell surface Fas receptor. This has provided new insights into the concepts of tolerance and immunoprivilege (Bellgrau et al., 1995; Sanberg et al., 1996; Ferguson and Griffith, 1997). For instance, testis grafts from mice expressing FasL survived when transplanted into allogeneic animals. On the contrary, grafts derived from "gld" mice, which lack functional FasL, were rejected (Bellgrau et al., 1995).

In the present report, for the first time, we have provided evidences that, in TM4 cell line, an aromatizable androgen Δ4

induces a strong increase in FasL mRNA, protein content and promoter activity. These effects are reversed by addition of letrozole, an aromatase inhibitor, addressing how E2 "in situ" production by aromatase activity plays a crucial role in modulating the immunoprivileged status of these somatic cells. A further support to the specificity of the above described results raises from the evidence that no noticeable effect was produced by mibolerone, a non-aromatizable steroid.

It is well known that postnatal development and function of testicular Sertoli cells is regulated primarily by FSH, a glycoprotein hormone secreted by the pituitary gland (Dorrington and Armstrong, 1975). In the prepubertal testis, FSH is required for Sertoli cells proliferation to achieve the adult number of these cells (Griswold, 1998). This proliferative stage of Sertoli cells development is also characterized by the presence of FSH-dependent cytochrome P450 aromatase activity (Carreau et al., 2003; Sharpe et al., 2003). In our recent work (Catalano et al., 2003) we have documented in TM4 cell line a strong dose-dependent stimulation of aromatase activity
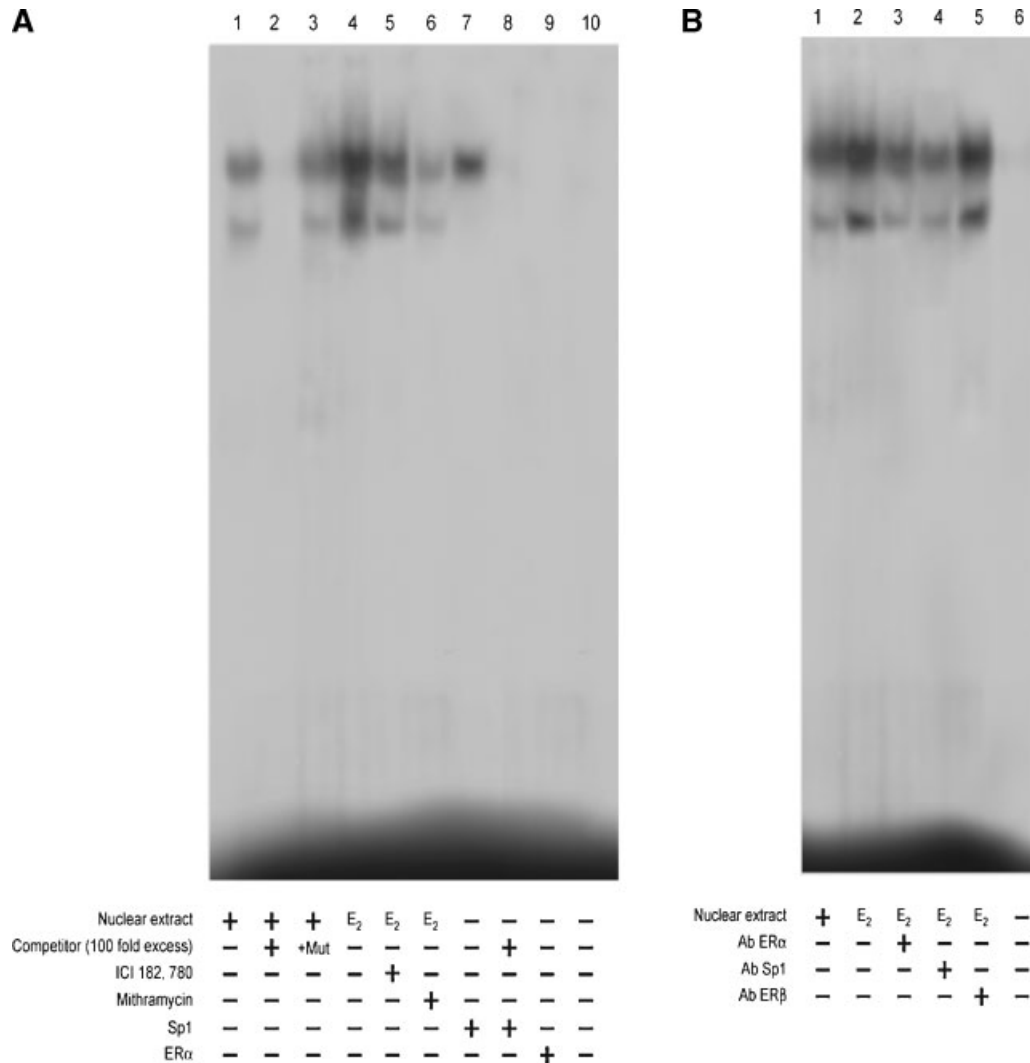
Fig. 4. Electrophoretic mobility shift assay of the Sp-1 binding site in the FasL promoter region. A: Nuclear extracts from TM4 cells were incubated with a double-stranded Sp1-specific consensus sequence probe labeled with [γ-^{32}P] ATP and subjected to electrophoresis in a 6% polyacrilamide gel (lane 1). Competition experiments were perfomed adding as competitor a 100-fold molar excess of unlabeled probe (lanes 2 and 8) or a 100-fold molar excess of unlabeled oligonucleotide containing a mutated Sp-1 (lane 3). Nuclear extracts were obtained from TM4 cells treated with 100 nM of E2 (lane 4), E2 + ICI 182,780 (10 μM) (lane 5), E2 + mithramycin (100 nM) (lane 6) for 24 h. As control we used human Sp-1 recombinant protein and transcribed and translated in vitro ERα protein (lane 7 and 9). Lane 10 contains probe alone. B: Anti-ERα, anti Sp-1 and anti-ERβ antibodies (lanes 3–5) were incubated with E2-treated TM4 nuclear extracts. Lane 6 contains probe alone.

induced by (Bu)$_2$cAMP similar to that described previously in immature Sertoli cells (Andò et al., 2001). In the present study it is worth to emphasize that FSH induced an increased FasL expression through the enhancement of aromatase activity. To elucidate the molecular mechanism involved in Δ4 enhanced FasL expression, we transiently transfected TM4 cells with different constructs containing deleted segments of the human FasL promoter.

A maximal constitutive reporter gene activity was observed with p-318 construct, containing the region between −318 and −2 bp from the transcriptional start site of the human FasL promoter. This is in agreement with previous results demonstrating that FasL gene promoter region from 318 to −237 bp plays a major role in promoting basal transcription in TM4 cells (McClure et al., 1999). Moreover, the induced activation by Δ4 was not observed in cells transfected with p-237 construct (−237 to −2) suggesting that the region

between −318 and −237 bp contains elements that mediate the potentiating effects of estrogen on FasL expression.

A broadening number of transactivating factors has been identified as regulators of FasL gene expression (Kavurma and Khachigian, 2003), as nuclear factor in activated T cells (NF-AT) (Latinis et al., 1997), nuclear factor-kappa B (NF-KB) (Matsui et al., 1998), activator protein-1 (AP-1) (Kasihatla et al., 1998), interferon regulatory factor-1 (IFN-1) (Kirschhoff et al., 2002), early growth response factor (Egr) (Mittelstadt and Ashwell, 1998) and specificity protein-1 (Sp-1) (Kavurma et al., 2001). Sp-1 is involved in the transcriptional regulation of many genes and has also been identified to be important in the regulation of FasL gene expression and apoptosis. Indeed, this transcription factor is able to activate FasL promoter via a distinct recognition element, and inducible FasL promoter activation is abrogated by expression of the dominant-negative mutant form of Sp-1 (Kavurma et al., 2001). In addition, it has been recently
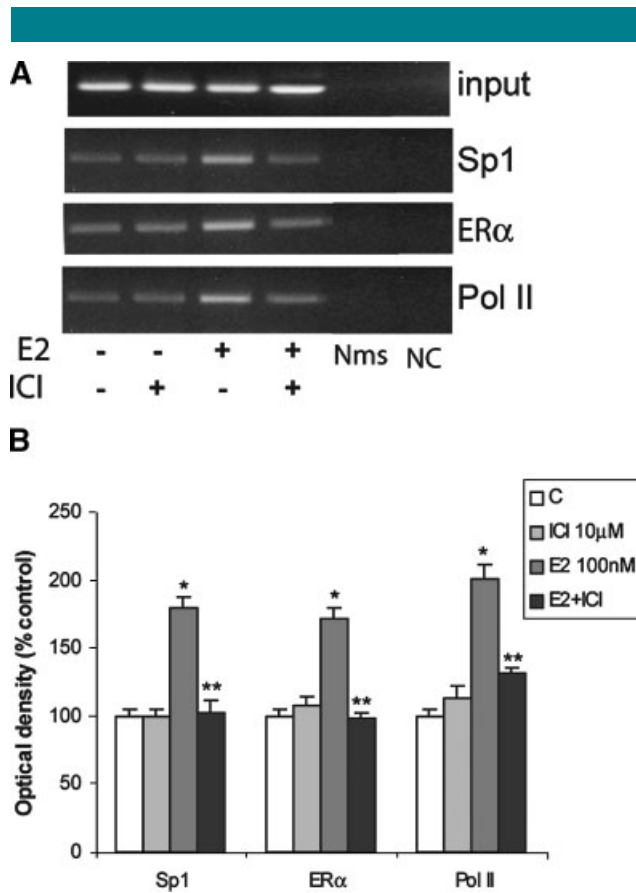
Fig. 5. 17β-Estradiol increases Sp-1/ERα recruitement to FasL promoter. A: Soluble precleared chromatin was obtained from TM4 cells treated for 1 h with 100 nM E2, 10 μM ICI and E2 + ICI or left untreated **(C)** and immunoprecipitated **(IP)** with an anti-Sp-1, anti ERα, anti polymerase II antibodies and with a normal mouse serum **(Nms)** as negative control. **The FasL promoter sequences containing Sp-1 were detected by PCR with specific primers, as described in "Materials and Methods". To control input DNA, FasL promoter was amplified from 30 μl of initial preparations of soluble chromatin (before immunoprecipitations). PCR products obtained at 25 cycles are shown. Sample without the addition of DNA was used as negative control (NC). This experiment was repeated three times with similar results. B:** The histograms represent the means ± SE of three separate experiments in which band intensities were evaluated in term of optical density arbitrary units and expressed as percentage of the control assumed as 100%. $^*P < 0.01$ compared to control; $^{**}P < 0.01$ compared to E2-treated samples.

demonstrated that nuclear extracts of TM4 Sertoli cells contain high levels of Sp-1 and Sp-3 that specifically bind to the GGGCGG consensus sequence present in the FasL gene, and overexpression of Sp-1 but not Sp-3 is able to increase the basal transcription of the FasL promoter (McClure et al., 1999). The latter observation fits with our functional studies demonstrating that Sp-1 is a crucial effector of estradiol signal in enhancing FasL gene expression. For instance, it is well known that ERs can transactivate gene promoters without directly binding to DNA but instead through interaction with other DNA-bound factors in promoter regions lacking TATA box. This has been most extensively investigated in relationship to protein complexes involving Sp-1 and ERα at GC boxes, which are classic binding sites for members of the Sp-1 family of transcription factors. Sp-1 protein plays an important role in the regulation of mammalian and viral genes, and recent results have shown that E2 responsiveness of c-fos, cathepsin D, retinoic acid receptor α1 and insulin-like grow factor-binding

protein 4 gene expression in breast cancer cells is linked to specific GC rich promoter sequences that bind ER/Sp-1 complex in which only Sp-1 protein binds DNA (Krishnan et al., 1994; Cowley et al., 1997; Porter et al., 1997; Sun et al., 1998; Qin et al., 1998; Saville et al., 2000).

In our work, the interaction between ERα and Sp-1 is clearly evidenced by gel mobility shift analysis and chromatin immunoprecipitation assay. Besides, the functional assays performed in ER-negative HeLa cells showed that ERα and not ERβ mediates the estrogen-induced increase in FasL gene expression. The specificity of ERα to induce transcription of FasL in TM4 was demonstrated using selective agonists for the ER subtypes. For instance we evidenced that only PPT was able to enhance FasL promoter activity.

Our results stemming from functional analysis, EMSA and ChiP assays led us to recruit FasL among those genes whose expression is upregulated by E2 through a direct interaction of ERα with Sp-1 protein.

In conclusion, the present study demonstrates that aromatizable steroids, normally present in the testicular milieu, through their conversion into E2 by aromatase activity, are able to increase FasL expression in TM4 Sertoli cells. The aromatase enzyme assures that estrogens through a short autocrine loop maintain Sertoli cells proliferation before their terminal differentiation. Thus, we propose that at the latter crucial maturative stage, FasL may achieve an intracellular content sufficient to protect Sertoli cells from any injury induced by Fas expressing immunocells, then potentiating the immunoprivileged condition of the testis.

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