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Estrogen Suppression of EGFR Expression in Breast Cancer Cells: A Possible Mechanism to Modulate Growth

Ronit I. Yarden, Melissa A. Wilson, and Susan A. Chrysogelos

Department of Oncology, Georgetown University, Washington, DC 20007

Abstract Epidermal growth factor receptor (EGFR) is a transmembrane receptor whose overexpression in breast cancer predicts for poor prognosis and is inversely correlated with expression of estrogen receptor (ER). This study was designed to investigate whether estrogen plays an active role in suppression of EGFR expression in estrogen-responsive breast cancer cell lines expressing low levels of EGFR. Upon withdrawal of estrogen, EGFR mRNA and protein increased 3–6 fold in MCF-7, T47D, and BT474 ER+ breast cancer cells. This was reversible upon addition of estradiol back to the culture media, but only after prolonged treatment. Nuclear run-on assays and studies with the transcription inhibitor actinomycin D demonstrated that regulation is at the transcriptional level. These results indicate that in the presence of estrogen, ER+ breast cancer cells possess active mechanisms to suppress EGFR expression. Up-regulation of EGFR in response to estrogen depletion and growth inhibition could represent an attempt to rescue cell growth by utilizing an alternative pathway. Indeed, we found that estrogen-depleted breast cancer cells are more sensitive to the mitogenic effects of EGF and TGF-α, and simultaneous blockade of both estrogen and EGFR signaling pathways induced cell death. J. Cell. Biochem. Suppl. 36:232–246, 2001.

Key words: EGF receptor; estrogen; estrogen receptor; EGF; TGF-α; anti-estrogens; breast cancer cells; cell proliferation; apoptosis

Growth factors and their receptors play a central role in cell growth, therefore, their expression must be carefully regulated. The epidermal growth factor receptor (EGFR) is part of the tyrosine kinase receptor family that also includes erbB2, erbB3, and erbB4 [Rajkumar and Gullick, 1994] and is important for normal development, differentiation, and cell proliferation [Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995]. Deregulation and overexpression of EGFR can lead to transformation of cells in vitro [Di Fiora et al., 1987] and tumor formation in nude mice in an EGF-dependent manner [Dickson et al., 1986]. In many human cancers the EGFR gene is amplified and/or overexpressed, and this overexpression often correlates with advanced disease and poor prognosis [Gullick, 1991], suggesting a role for EGFR in the malignant process.

Estrogen is the main hormone that controls breast cancer proliferation in its early stages, and it is believed that estrogen mediates its effects at least in part by inducing growth factors and their receptors that act locally in autocrine and/or paracrine pathways. EGFR and its ligands EGF, TGF-α, amphiregulin [Normanno et al., 1994] and related polypeptides are candidates for such an autocrine loop since estrogen up-regulates TGF-α [Bates et al., 1988] and amphiregulin [Martinez-Lacaci et al., 1995] in an ER-dependent manner, and moreover, EGF can partially replace estrogen in inducing tumor formation [Dickson et al., 1986]. Such an autocrine loop utilizing EGFR and its ligands has been observed in various systems including some ovarian [Morishige et al., 1991] and breast cancer cells [Ennis et al., 1989; Reddy et al., 1994] that no longer depend on

For reprints requests, GMBB, NHGRI NIH 49 Convent Drive, #3A35 Bethesda MD, 20892.

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Ronit I. Yarden's present address is Genetic and Molecular Biology Branch, NHGRI, NIH, Bethesda, MD 20892-4470.

*Correspondence to: Ronit I. Yarden, Rm# 3A35 Bldg 49, 49 Convent Dr. NHGRI, NIH, Bethesda, MD, 20892.

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estrogen for their growth. This raises the possibility that EGFR signaling provides an alternative growth pathway that breast cancer cells are able to utilize in the absence of estrogen. Further supporting this theory is the observation that in breast tumors EGFR expression is inversely correlated with expression of estrogen receptor (ER) [Fitzpatric et al., 1984; Koenders et al., 1991; Sharma et al., 1992]. While expression of ER usually predicts for responsiveness to endocrine therapy and overall good prognosis [Osborne et al., 1980], EGFR expression (independent of ER) correlates with lack of response to endocrine therapy, high incidence of metastasis, and poor survival [Nicholson et al., 1990; Koenders et al., 1991; Nicholson et al., 1994].

An inverse correlation between ER and EGFR is also found in breast cancer cell lines, with ER-positive cells expressing very low levels of EGFR [Davidson et al., 1987]. Moreover, many cell regulators such as EGF, TPA, and sodium butyrate have opposite effects on the expression of the two receptors [Lee et al., 1989; Secada et al., 1991; De Fazio et al., 1992], suggesting that the reciprocal expression may be a consequence of a reciprocal control mechanism. Indeed, it has been demonstrated that overexpression of an exogenous EGFR gene in ER-positive MCF-7 breast cancer cells was modulated by the presence of estrogen in the media resulting in down regulation of EGFR expression [Miller et al., 1994]. In another study where a similar approach was undertaken, overexpression of EGFR in ER-positive ZR-75 breast cancer cells led to antiestrogen resistance and loss of ER [Van Agthoven et al., 1992]. While there was a different outcome in each of these studies, they both indicate a lack of compatability between ER expression and EGFR overexpression. It also has been shown that transfection of ER into ER-negative MDA-MB-231 breast cancer cells resulted in a decrease in the high levels of endogenous EGFR expression [Sheikh et al., 1994]. Although all these studies attempt to address the question of the reciprocal expression between ER and EGFR, they did not investigate the mechanisms of interaction between the two endogenous genes.

We have previously shown that estrogen is capable of transiently inducing EGFR expression in ER-positive breast cancer cells [Yarden et al., 1996]. This induction was followed by a rapid decrease to the level of EGFR seen in estrogen-depleted cells, and both up and down regulation were mediated at the transcriptional level [Yarden et al., 1996]. These results imply that estrogen has multiple roles in regulating EGFR expression. In this study we asked whether estrogen is actively involved in maintaining the low basal levels of EGFR expression in breast cancer cells that express ER. We found that upon withdrawal of estrogen, EGFR expression was increased and the long term presence of estrogen was sufficient to confer transcriptional repression of EGFR expression. When this repression was relieved in the absence of estrogen or in the presence of antiestrogens, the cells become more responsive to EGF/TGF-α mediated growth, suggesting that the EGFR pathway may provide an alternative mechanism for the control of breast cancer proliferation, and increased expression of EGFR may play an active role in the progression of breast cancer to hormone independence.

MATERIALS AND METHODS

Cell Culture and Estrogen Depletion

MCF-7, T47D, and BT474 cells were obtained through the Lombardi Cancer Center Tissue Culture Core Facility (MCF-7 cells were originally obtained from Dr. Marvin Rich, Michigan Cancer Foundation, Detroit, MI, and T47D and BT474 cells were from American Type Culture Collection, Rockville, MD). Cells were propagated in IMEM medium supplemented with 10% heat-inactivated fetal calf serum (FCS). All media and sera were purchased from Biofluids Inc., Rockville, MD. For estrogen depletion studies, cells were washed twice each day with phenol-red-free IMEM and cultured in phenol-red-free IMEM supplemented with 10% charcoal-treated calf serum (CCS) for 5 days. Alternatively, cells were treated with 5×10^{-7} M 4-OH-tamoxifen (Sigma, St. Louis, MO) or 10^{-7} M ICI 164,384 (ICI) in the presence of complete media containing 10% FCS for 5 days. For the actinomycin D studies, BT474 cells were grown to subconfluency in IMEM with 10% FCS or CCS, and actinomycin D (Sigma, St. Louis, MO) at 5 μg/ml was added to fresh media.

Growth Factors and Antibodies

EGF was purchased from Upstate Biotechnology Inc (Lake Placid, NY) and TGF-α was purchased from Gibco-BRL (Gaithersburg,

MD). EGFR antibody 1005 was purchased from Santa Cruz (CA), IgG528 was purchased from Oncogene Science (MA). Antibody against the phosphorylated form of EGFR was purchased from Transduction Laboratories (WI). EGFR neutralizing antibody IgG225 was kindly provided by Dr. H. Masui (Memorial Sloan-Kettering Cancer Center, NY).

RNA Extraction and Analysis

Total cellular RNA was prepared by the one-step acid-guanidinium method as described by Chomczynski and Sacchi [1987], and 60 μg aliquots were subjected to RNase protection assays as previously described [Yarden et al., 1996]. Radiolabeled RNA probes corresponding to EGFR and 36B4 were generated using SP6 and T7 RNA polymerase respectively with the RPA kit (Promega, Madison, WI) as specified by the supplier. Quantitation of the data was performed with a scanning densitometer using Protein + DNA Imageware (PDI) systems (Huntington Station, NY).

Nuclear Run-on Assay

Nuclei were isolated from BT474 cells cultured in IMEM supplemented with 10% FCS or in estrogen-free conditions as previously described [Yarden et al., 1996]. Nuclear run-on transcription assays were performed using the method of Celano et al. [1989] with modifications we have described previously [Yarden et al., 1996]. Data were analyzed with a phosphorimager and Imagequant software (Molecular Dynamics, CA).

Membrane Extraction and Western Blot Analysis

Cells were cultured in IMEM media containing 10% FCS or CCS and harvested at subconfluency for preparation of crude membrane extracts as previously described [Elashry-Stowers et al., 1988]. The protein concentration of the extracts was determined by the BCA assay (Pierce, Rockford, IL). For Western blot analysis, 100 μg of protein from membrane extracts were separated by electrophoresis in a 7.5% SDS-polyacrylamide gel, transferred to a nitrocellulose Bioblot-NC membrane (Costar, Cambridge, MA) using a semi-dry transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA) and stained with Ponceau-S (Sigma, St. Louis, MO) to check for equal loading. The membrane was probed with 1 μg/ml of the polyclonal antibody 1005 against EGFR (Santa-Cruz Biotechnology, Inc., Santa-Cruz, CA), and EGFR was visualized using HRP-conjugated anti-rabbit IgG, an ECL detection kit (Amersham, Arlington Heights, IL), and exposure to Hyperfilm-ECL (Amersham Co., Arlington Heights, IL). For some experiments, the membrane was probed with 1 μg/ml of a mouse monoclonal antibody that recognizes the phosphorylated form of EGFR exclusively (Transduction Laboratories, Lexington, KY), then stripped according to the manufacturer, and thereafter was reprobed with 1 μg/ml of the polyclonal antibody 1005 against EGFR.

Immunoprecipitation

BT474 cells, cultured in the presence or absence of estrogen, were washed twice with ice cold PBS (Biofluid, Rockville MD) and solubilized for 10 min at 4°C in lysis buffer (20mM Tris-Cl, pH 7.9, 137mM NaCl, 5mM EDTA, 10% glycerol, 1% Triton X-100, 1mM EGTA and a cocktail of protease and phosphatase inhibitors. Cells were scraped off the plate, collected into microfuge tubes and centrifuged at 14,000 rpm for 10 min. The protein concentration of the supernatants containing the cell extracts was determined by the BCA Assay (Pierce, Rockford, IL). For immunoprecipitation, 1 mg of protein from whole cell lysates was incubated with 20 μg of the anti-EGFR mouse monoclonal antibody 528IgG (Oncogene Science, Cambridge, MA) and with 20 μl of protein A plus G Sepharose beads (Oncogene Science, Cambridge, MA) for $2\frac{1}{2}$ h at 4°C with constant rotation. Immunocomplexes were collected by centrifugation in a microfuge tube for 10 min and washed four times in 1 ml of lysis buffer. Proteins were eluted by boiling in sample buffer (120 mM Tris-Cl, pH 6.8, 25 mM EDTA, pH 8.0, 4% SDS, 20% glycerol, 10% βME) prior to electrophoresis in 7.5% SDS-PAGE. Assays were carried out from this point as described above for Western blot analysis.

Immunohistochemistry

BT474 cells were plated in Falcon 2-well chamber slides (Becton Dickinson) at 60% confluence and maintained in IMEM with 10% FBS or 10% CCS, or treated with 10^{-7} M ICI 182,780 (Zeneca Pharmaceuticals) or 5×10^{-7} M 4-hydroxytamoxifen. Treatments were for a total of 5 days and media was changed after 3 days. Cells were then fixed in 10% formalin for 10 min and ice cold acetone for 15 sec. After

washing 3× with PBS, cells were blocked with 1%BSA in PBS at room temperature for 1 h and then incubated with 2 μg/ml mouse monoclonal anti-EGFR antibody Ab-5(Oncogene Research Products) overnight at room temperature. Cells were washed 3× with PBS and then incubated with a goat anti-mouse HRP-conjugated secondary antibody (Bio-Rad) at a 1:300 dilution for 1 h and membrane staining of EGFR was detected using the Vector Purple peroxidase substrate kit (Vector Laboratories, Inc.).

Growth Assays

Cells were grown in T75 cc flasks and were maintained under normal culture conditions or subjected to estrogen depletion. 500 cells/well were seeded in 96 well plates, and on the following day, the cells in the plate corresponding to Day 0 were harvested. All other cells (ten replicates/treatment) were treated with various concentrations of recombinant EGF (UBI, Lake Placid, NY) or recombinant TGF-α (Gibco/BRL, Gaithersburg MD) ranging from 10^{-10} M to 10^{-8} M. At the indicated time points, cells were stained for 15 min with a solution of 0.1% crystal violet (Sigma, St. Louis, MO) and 15% methanol, washed in distilled water, and allowed to air-dry for 24 h. Cells were destained with 0.1 M sodium citrate in 50% ethanol, and absorbence at 540 nm was determined.

Cell Death Assay

BT474 cells were plated at a density of 1×10^5 cells/well in 6 well plates. Two days later, cells were subjected to the following treatments: depletion of estrogen, or blockade of EGFR by EGFR-neutralizing antibody clone 225IgG at a concentration of 15 μg/ml with or without estrogen depletion. Two days later, the treatments were repeated. After a total of 4 days treatment, cells were harvested and assayed for the presence of nucleosomes and fragmented DNA in the cytosol using a Cell Death Detection ELISA (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's protocol.

RESULTS

Estrogen Withdrawal Increases the Level of EGFR mRNA

The well established inverse correlation between EGFR and ER expression led us to hypothesize that estrogen may play an active role in maintaining low levels of EGFR expression in ER-positive breast cancer cells. To determine if estrogen is involved in suppression of EGFR expression in ER-positive breast cancer cells, we measured changes in EGFR mRNA levels in response to estrogen withdrawal in three ER-positive breast cancer cells lines: MCF-7, T47D, and BT474. Cells were depleted of estrogen over five days by replacing the culture media (IMEM supplemented with 10% fetal calf serum [FCS]) daily with phenol red-free IMEM supplemented with 10% charcoal-treated serum (CCS). Levels of EGFR mRNA in estrogen-depleted cells and cells that were maintained in the presence of FCS were analyzed by RNase protection assay and normalized to the level of mRNA for the ribosomal protein 36B4, which is not regulated by estrogen [Masiakowski et al., 1982]. In all three breast cancer cell lines there was a 3–6 fold increase in the level of EGFR mRNA in response to estrogen withdrawal as shown in Figure 1. This increase in EGFR mRNA levels suggests that the estrogen present in the media containing 10% FCS, but absent in the phenol red-free media containing 10% CCS, can suppress EGFR expression. Treatment of estrogen-depleted cells with 10^{-7} M ICI 164,384 (a pure anti-estrogen) for 24 h had no further effect on the level of EGFR mRNA, indicating that the increase in EGFR mRNA levels is not due to residual estrogen in the culture media.

Estrogen is Sufficient to Mediate the Suppressive Effect on EGFR Expression

Charcoal treatment of serum is a frequently used method to deplete cells of estrogen [Horwitz and McGuire, 1978]. However, often other small molecules in the serum bind to the dextran-charcoal and are removed from the culture media by this treatment. Therefore, to specifically verify the role of estrogen in the suppression of EGFR expression, two different approaches were undertaken. First, BT474 cells cultured in IMEM supplemented with 10% FCS were treated with the anti-estrogens 4-hydroxytamoxifen (a partial agonist of ER) or ICI 164,384 (a pure antagonist) to specifically block estrogen action and mimic the effect of estrogen withdrawal. Second, the normal growth medium of BT474 cells (IMEM supplemented with 10% FCS) was replaced with phenol-red free IMEM supplemented with 10% CCS and 10^{-8} M 17β-estradiol (E_2). These conditions allow the effect of estrogen on EGFR expression to be

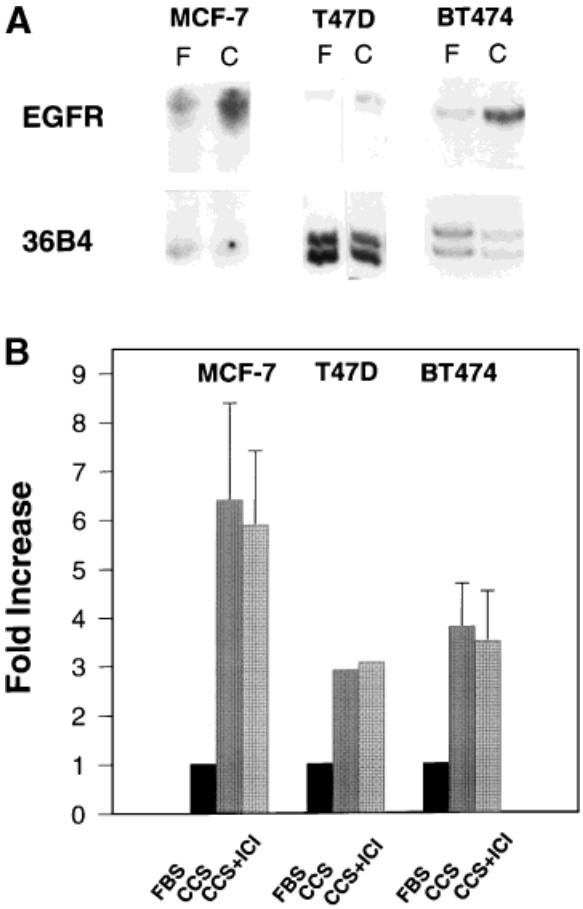

Fig. 1. Estrogen depletion up-regulates EGFR mRNA expression. **A:** ER-positive breast cancer cells MCF-7, T47D, and BT474 were depleted of estrogen and maintained in phenol red-free IMEM supplemented with 10% charcoal-treated calf serum for 5 days (**lanes C**), or were maintained in the continuous presence of IMEM supplemented with 10% complete serum (**lanes F**). Cells were harvested and total RNA was isolated and analyzed by RNase protection assay using ^{32}P-labeled antisense RNA probes for EGFR and an internal control, 36B4. **B:** Autoradiograms from at least three independent experiments per cell line were quantitated by densitometry and EGFR values were normalized to 36B4 as described in Materials and Methods. Normalized levels of EGFR mRNA in cells depleted of estrogen (CCS; CCS + ICI) are presented as fold induction over cells growing continuously in the presence of estrogen (FBS).

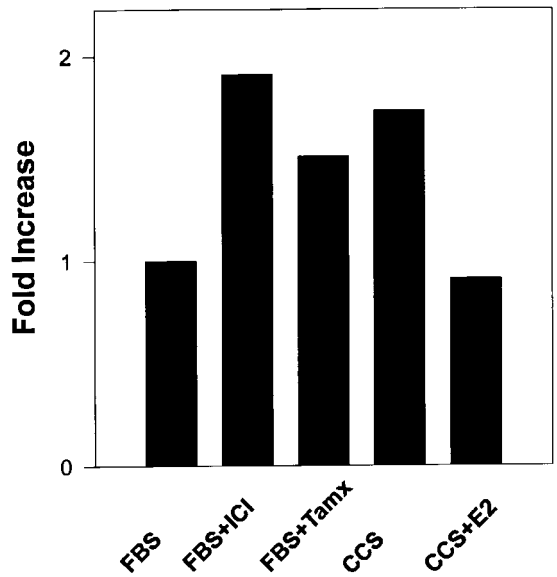

Fig. 2. Anti-estrogens also up-regulate EGFR mRNA. BT474 cells were grown in the presence of IMEM supplemented with 10% complete serum (FBS) and treated with 10^{-7} M ICI 164,384 (FBS + ICI) or 5×10^{-7} M OH-Tamoxifen (FBS+Tamx) for five days. Additionally, cells were either depleted of estrogen (CCS) or cultured in phenol red-free IMEM supplemented with 10% CCS and 10^{-8} M E_2 simultaneously (CCS + E_2) for the same period of time. Total RNA was prepared from the cells and analyzed for EGFR and 36B4 mRNA levels by RNase protection assay. Autoradiograms were quantified by densitometry and normalized levels of EGFR are presented relative to untreated cells as described in Materials and Methods.

evaluated separately from the effect of other components that might have been eliminated from the serum by the charcoal treatment. As shown in Figure 2, when BT474 cells were treated with antiestrogens in the presence of FCS, the level of EGFR mRNA was increased similarly to when cells were depleted of estrogen by the charcoal treatment of the serum. When these cells were cultured in phenol red free-IMEM supplemented with both 10% CCS and E_2, EGFR mRNA did not increase, remaining at the low level found in cells cultured in IMEM supplemented with 10% FCS. Both approaches that were taken indicate that the presence of estrogen alone is sufficient to suppress the expression of EGFR in ER-positive breast cancer cells.

Prolonged Presence of Estrogen Suppresses the Level of EGFR mRNA

Previously, we had observed that upon treatment of estrogen-depleted ER-positive breast cancer cells with 10^{-8} M E_2 there is a further 2–3 fold transient increase in EGFR mRNA levels, followed by a plateauing at 24 h post E_2 addition of EGFR mRNA levels at the same level as in estrogen-depleted cells [Yarden et al., 1996]. Since EGFR mRNA levels in cells maintained in FCS are lower than this plateau reached at 24 h of E_2 treatment, we speculated that the long term presence of estrogen is responsible for the low levels of EGFR expression in cells maintained in FCS. Therefore, we depleted BT474 cells of estrogen, and then added back 10^{-8} M E_2

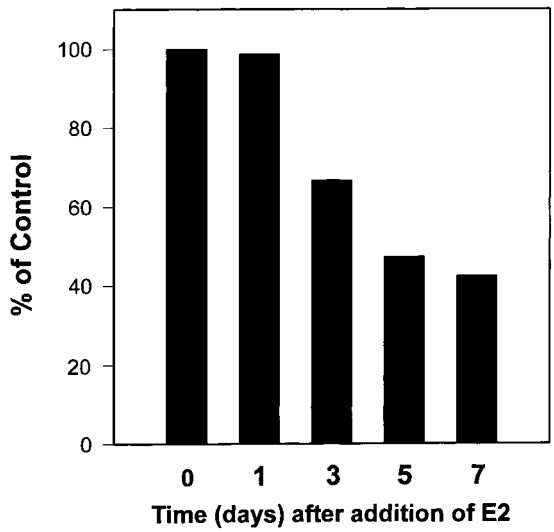

Fig. 3. Long term estrogen treatment down-regulates EGFR mRNA. BT474 cells were depleted of estrogen for 5 days as described in Materials and Methods and then treated with 10^{-8} M 17β-estradiol for up to 7 days. Cells were harvested at Days 1, 3, 5, and 7 following the addition of estradiol, and total RNA was isolated and analyzed for EGFR and 36B4 mRNA levels by RNase protection assay. Normalized levels of EGFR are presented as percent of control, estrogen-depleted cells.

in phenol-red-free IMEM supplemented with 10% CCS for various times. RNA was extracted from cells at the times indicated in Figure 3, analyzed by RNase protection assay, and EGFR mRNA levels were normalized to levels of 36B4 RNA. The results shown in Figure 3 reveal a gradual decrease in EGFR mRNA levels starting at Day 3 following addition of E_2. At seven days of E_2 treatment, EGFR mRNA had decreased to 40% of the level in estrogen-depleted cells, a level similar to that previously observed in cells maintained in the presence of FCS. The same effect was seen when FCS was added back to the media instead of E_2, or when MCF-7 cells were used in place of BT474 cells (data not shown). These results indicate that the long term presence of estrogen has a suppressive effect on EGFR expression.

Estrogen Suppression of EGFR is at the Transcriptional Level

Estrogen suppression of EGFR mRNA levels could result from either rapid turnover of EGFR mRNA, transcriptional repression, or a combination of both. Therefore, we studied the turnover rate of EGFR mRNA in BT474 cells growing in the presence of 10% FCS and in cells depleted of estrogen, by exposing them to the transcription inhibitor actinomycin D. The rate of EGFR mRNA decay was determined by RNase protection assay in a time course up to 4 h. The half-life of EGFR mRNA was found to be 1 h in either the presence or absence of estrogen (Fig. 4A), strongly suggesting that changes in EGFR mRNA stability do not contribute to the difference we have observed in the level of EGFR expression. Therefore, to determine the role of transcription as the mechanism of suppression, nuclear run-on assays were performed using nuclei isolated from BT474 cells maintained in FCS or depleted of estrogen. Nuclear transcripts were hybridized to slot blots containing a genomic fragment encompassing the 5′-untranslated region and the first exon of the EGFR gene as a probe. Fragments of cDNA encoding the progesterone receptor (PR) and pS2 were used as controls for estrogen inducible genes, while a fragment of the 36B4 gene was used as a normalization control and pUC19 DNA was used as a negative control to assess nonspecific binding. As shown in Figure 4B, while transcription of PR was reduced three fold as expected, estrogen withdrawal resulted in a 2–3 fold increase in EGFR gene transcription as determined by Phosphorimager analysis. These results are in good agreement with our findings for steady-state EGFR mRNA levels, and clearly indicate that removal of estrogen results in transcriptional up-regulation of the EGFR gene.

Estrogen Withdrawal Increases Levels of EGFR Protein and Phosphorylation

The above results demonstrate that the removal of estrogen from the growth media of estrogen-dependent, ER-positive breast cancer cells causes an increase in the level of EGFR mRNA as a result of transcriptional induction/release of repression. To determine if this increase is also reflected in the amount of the receptor expressed on the cell surface we employed Western blot analysis. Membrane fractions from BT474 cells maintained in the presence of estrogen or from cells depleted of estrogen were probed with a polyclonal antibody, 1005, directed against EGFR. As shown in Figure 5A, the EGFR protein level also increased in response to estrogen withdrawal, coinciding with the RNA data. This result was confirmed by several independent experiments. We also assessed EGFR levels by immunohistochemistry, and observed an increase in

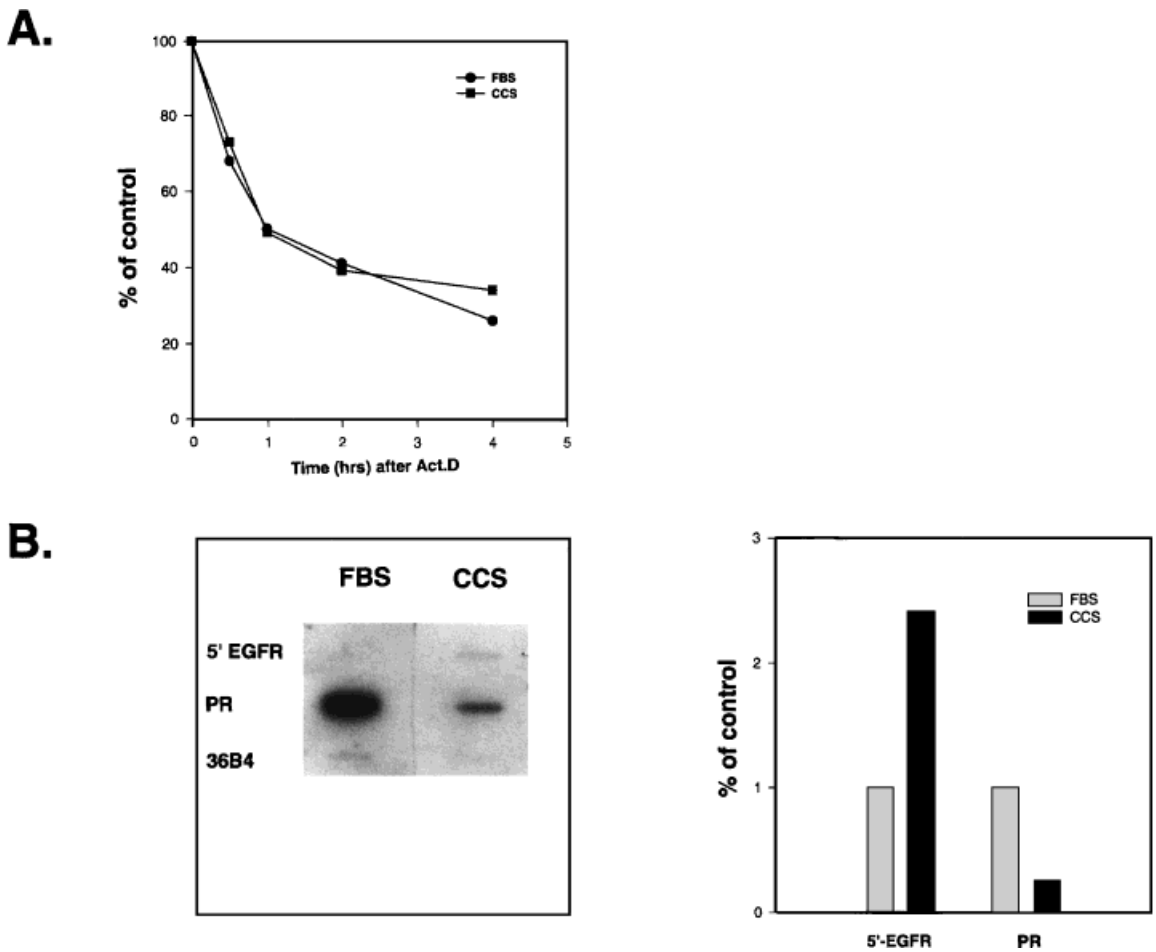

Fig. 4. EGFR Gene Transcription is increased in response to estrogen withdrawal while mRNA stability is unaffected. **A:** 5 μg/ml of the transcription inhibitor actinomycin D were added to BT474 cells that were cultured in the continuous presence of estrogen (FBS) or had been depleted of estrogen (CCS). Total RNA was isolated from cells at the indicated time points, and analyzed by RNase protection. Autoradiograms were quantified by densitometry and EGFR values were normalized to 36B4 and plotted as percent of control, untreated cells for each culture condition. **B:** Nuclei were isolated from BT474 cells cultured in IMEM supplemented with 10% FBS or 10% CCS and run-on assays were performed as described in Materials and Methods. A genomic fragment containing the 5′ untranslated region and the first exon was used as a probe for EGFR, while cDNA corresponding to progesterone receptor (PR) was used as a control, and a fragment corresponding to 36B4 was used for normalization (**left panel**). Blots were analyzed with a phosphorimager and Imagequant software, and normalized levels of EGFR and PR in cells depleted of estrogen (CCS) were expressed as % of the level in control cells (FBS) (**right panel**).

membrane staining for EGFR in BT474 cells upon estrogen withdrawal or treatment with antiestrogens (Fig. 6).

Interestingly, a slightly slower migrating form of the EGFR band that may represent post-translational modifications was consistantly observed by Western blot analysis in cells that were depleted of estrogen. To further investigate the possibility that EGFR is phosphorylated in response to estrogen depletion, we immunoprecipitated EGFR from whole cell lysates of BT474 cells cultured either in 10% FCS or 10% CCS using the monoclonal antibody 528IgG. To determine the phosphorylation state of EGFR we performed Western blot analysis and probed the membrane with a monoclonal antibody that specifically recognizes the phosphorylated form of EGFR. An increase in the amount of EGFR phosphorylation in the absence of estrogen can be seen in Figure 5B. This increase was found to be proportional to the increase in total EGFR protein as detected by reprobing the membrane with the polyclonal antibody for EGFR. Moreover, treatment of estrogen-depleted cells with EGF further increased phosphorylation of

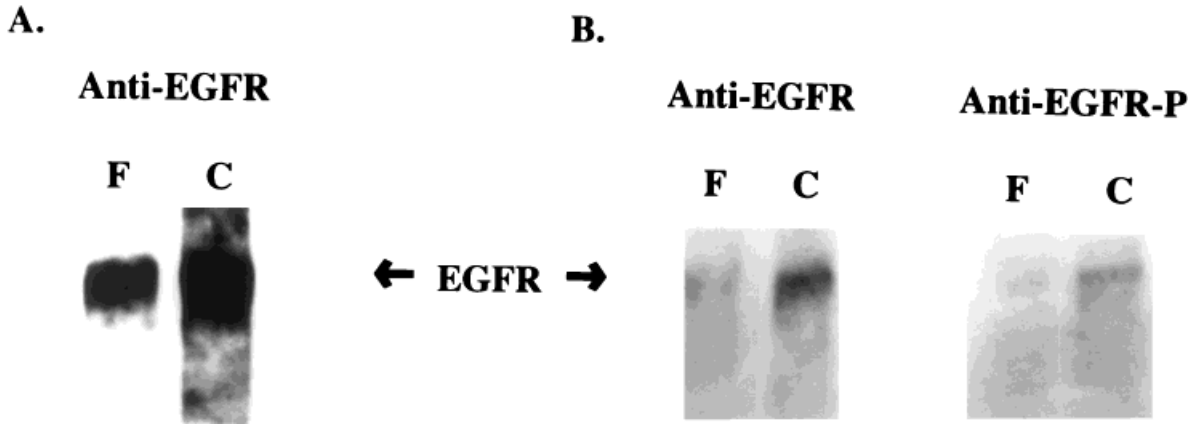

Fig. 5. Effect of estrogen withdrawal on EGFR Protein and Phosphorylation. **A:** BT474 cells were depleted of estrogen (C) or cultured in IMEM supplemented with 10% FBS (F). Membrane fractions of cells were prepared and 100 μg of protein per sample were analyzed by 7.5% SDS-PAGE and Western blot analysis using anti-EGFR polyclonal antibody 1005 as described in Materials and Methods. **B:** Whole cell extracts of BT474 cells cultured as described above were prepared and 1 mg extract for each treatment was used for immunoprecipitation with the monoclonal antibody 528IgG and protein A plus G Sepharose beads. Immunocomplexes were analyzed by 7.5% SDS-PAGE and Western blot analysis using anti-EGFR polyclonal antibody 1005 and anti-EGFR-P (activated form) monoclonal antibody.

EGFR as detected with this assay, indicating that EGFR could be further stimulated (data not shown).

Estrogen Depleted Cells are More Responsive to Growth Factors

Does the increase in EGFR levels in estrogen-depleted cells sensitize these cells to low levels of growth factors and thus provide an alternative pathway for growth? To address this question we performed growth assays that tested the ability of growth factors of the EGF family to affect cell proliferation. BT474 cells were cultured in IMEM supplemented with 10% FCS or in phenol red-free IMEM supplemented with 10% CCS with or without 10^{-8} M E_2. EGF and TGF-α at concentrations from 10^{-10} M to 10^{-8} M were assessed for their ability to increase cell growth under these different culture conditions. In the absence of estrogen, both EGF and TGF-α had a much stronger proliferative effect, stimulating the growth of the cells 2–3 fold that of the effect seen in the presence of estrogen (88–110% increase in cell number relative to control, untreated cells in the absence of E_2 vs. 27–42% increase in the presence of E_2) (Fig. 7, Table I). Both EGF and TGF-α provided a significant growth advantage to estrogen-depleted cells and were nearly able to restore growth to the level seen in the presence of estrogen. Moreover, an increase in the degree of growth stimulation of estrogen

TABLE I. Effect of EGF and TGF-α on the Growth of BT474 Cells in Presence or Absence of Estrogen

	Cell growth as % of control untreated cells								
	EGF treatment						TGF-α treatment		
	10^{-8} M		10^{-9} M		10^{-10} M		10^{-8} M	10^{-9} M	10^{-10} M
Growth condition	6 days	9 days	6 days	9 days	6 days	9 days	9 days	9 days	9 days
FBS	133		123		110		138	134	114
CCS + E_2	143	142	129	135	108	132	127	127	120
CCS	161	219	130	188	128	159	188	174	152
FBS + 5×10^{-7} M Tam	227								
FBS + 1×10^{-8} M ICI	196	284	194	224	164	181			

Summary from Figures 7 and 8 of the maximal growth induction of BT474 cells in response to various concentrations of EGF or TGF-α in the presence and absence of estrogen, expressed as % of untreated control cells for each condition.

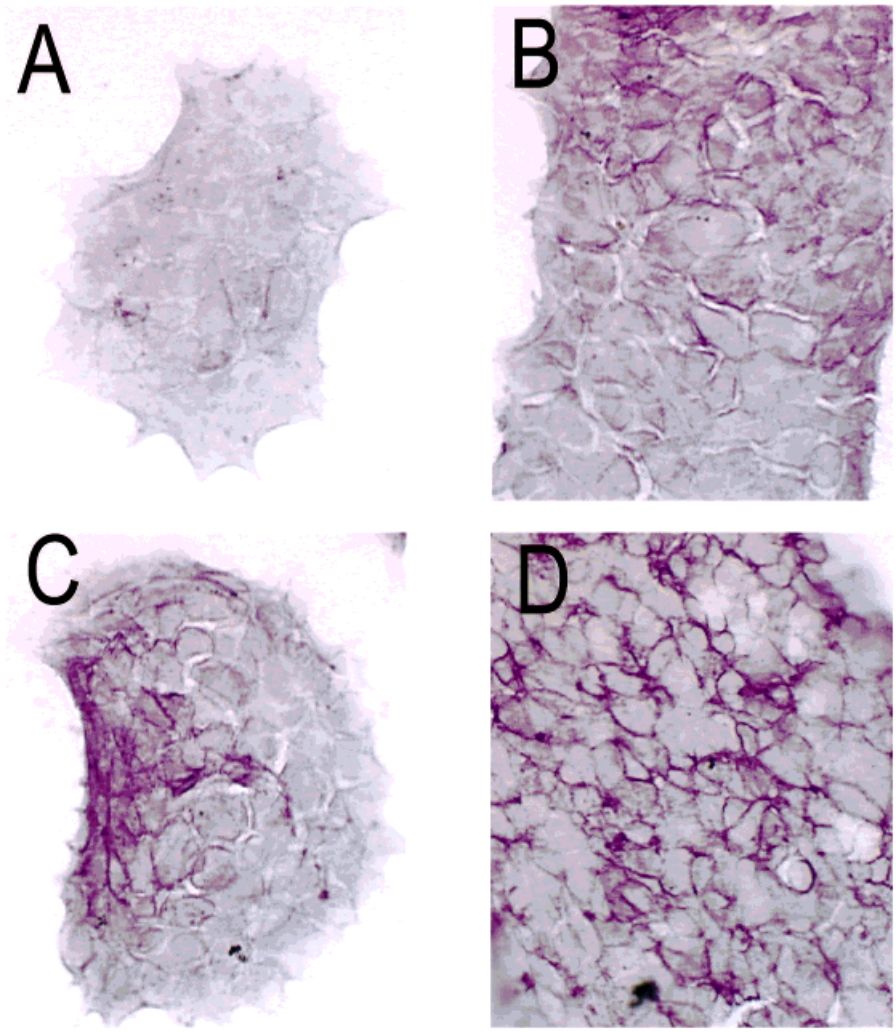

Fig. 6. Immunohistochemical analysis of the effect of estrogen-depletion on EGFR Protein. BT474 cells were (**A**) maintained in 10% FBS, (**B**) estrogen-depleted (stripped), (**C**) treated with 10^{-7} M ICI 182,780, or (**D**) treated with 5×10^{-7} M OH-tamoxifen for 5 days. Cells were then stained using anti-EGFR monoclonal antibody Ab-5 and Vector Purple. Magnification = 100X.

depleted cells was seen even at low concentrations of EGF or TGF-α indicating that estrogen depleted cells (which express higher levels of EGFR on their surface) are more sensitive to EGFR ligands. To further address this hypothesis, we performed growth assays with EGF in which BT474 cells were cultured in IMEM supplemented with 10% FCS and the antiestrogens ICI 164,384 or 4-hydroxytamoxifen. Treatment with these antiestrogens results in cell growth arrest and an increase in EGFR expression (see Fig. 2). As shown in Figure 8, EGF strongly induced cell growth under these conditions, again suggesting that estrogen-depleted cells have a higher sensitivity to EGFR ligands and that signaling through EGFR can provide these cells with an alternative growth pathway.

EGFR Up-Regulation in Response to Growth Inhibition: A Survival Mechanism for ER-Positive Breast Cancer Cells?

In the absence of estrogen, estrogen-dependent ER positive breast cancer cells are growth arrested [Lippman et al., 1976]. The results in the previous sections indicate that signaling through EGFR in the absence of estrogen induces cells to re-enter the cell cycle, and can provide an alternative growth stimulation. We hypothesize that the cells up-regulate EGFR as a survival mechanism to avoid cell death. Therefore, the question that arises is whether

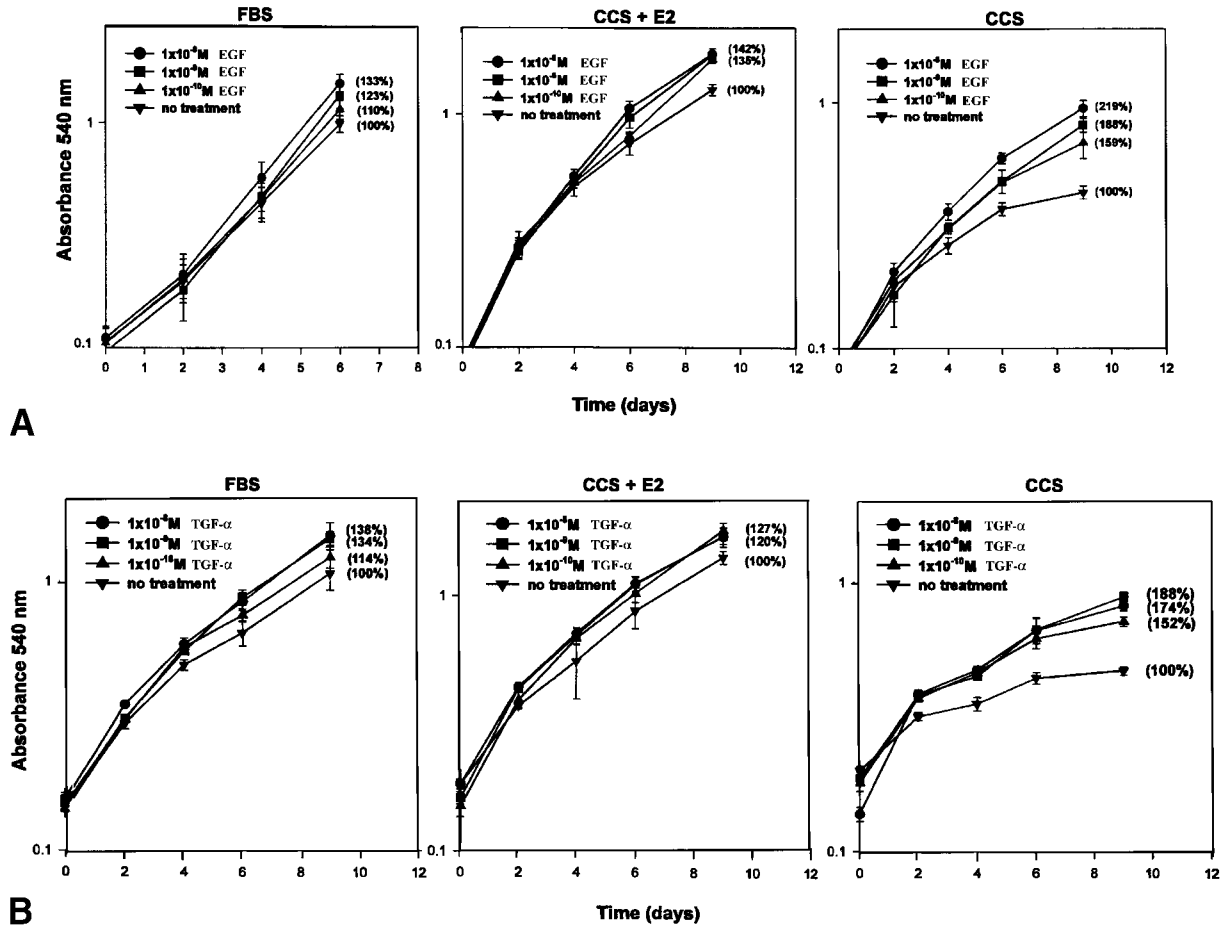

Fig. 7. Estrogen-depleted cells are more responsive to EGF and TGF-α. BT474 cells were plated at 500 cells/well in IMEM supplemented with 10% FBS or in phenol-red free media supplemented with 10% CCS in the presence or absence of estrogen. Human recombinant EGF (**A**) or TGF-α (**B**) was added at various concentrations (10^{-10} M to 10^{-8} M) to the cells on the following day with each treatment consisting of ten replicates. Cells were harvested at the indicated times, stained with crystal violet, and relative growth was measured by absorbance at 540 nm.

blocking the two pathways simultaneously will cause the cells to undergo apoptosis. To test this hypothesis, we used a cell death detection ELISA. This assay takes advantage of the release of DNA and nucleosomes into the cytoplasm due to nuclear membrane breakdown that occurs in apoptotic cells. The data in Figure 9 demonstrates that depleting the cells of estrogen or blocking signaling of EGFR with a neutralizing antibody alone did not result in a significant increase in apoptotic cell death compared to control untreated cells. However, simultaneously depleting the cells of estrogen and blocking EGFR signaling resulted in a three fold increase in apoptosis as detected by the presence of mono and oligonucleosomes in the cytoplasmic fraction of the cells. These results strongly suggest that up-regulation of EGFR in response to estrogen depletion is a survival mechanism the cells are inducing to avoid cell death.

DISCUSSION

The inverse correlation between EGFR and ER expression is well established in breast cancer both in primary tumors and in cell lines [Fitzpatric et al., 1984; Lee et al., 1989; Koenders et al., 1991; Secada et al., 1991; Sharma et al., 1992]. In this study, we investigated the role of estrogen as an active mediator of this inverse correlation between the two receptors. We show here that the EGFR/ER inverse correlation is not due to a casual relationship but is a result of an active mechanism by which estrogen suppresses EGFR

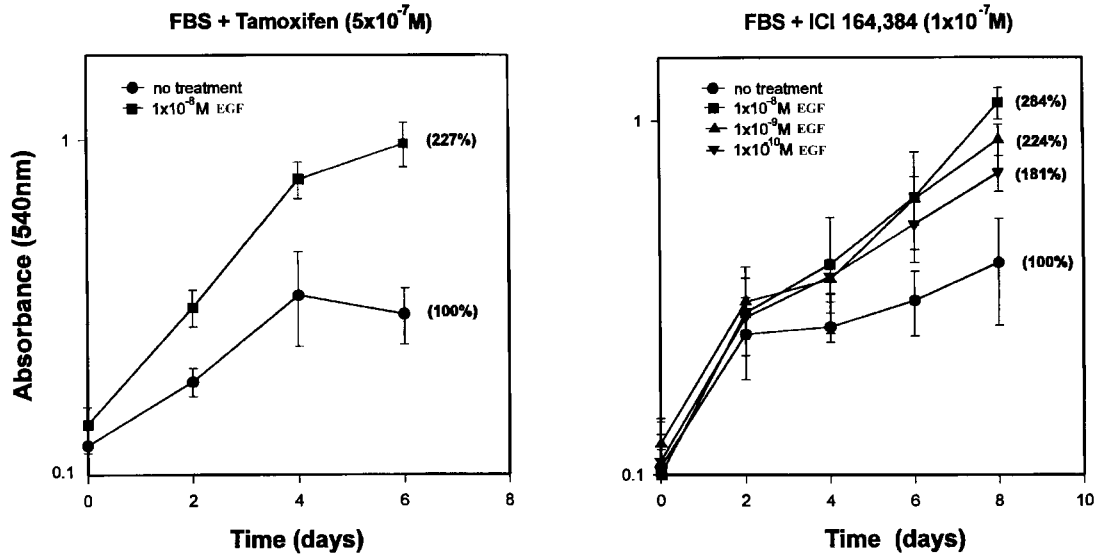

Fig. 8. EGF can alleviate growth inhibition mediated by antiestrogens. BT474 cells were plated at 500 cells/well in IMEM supplemented with 10% FBS in the presence of 10^{-7}M ICI or 5×10^{-7}M 4-hydroxytamoxifen. On the following day human recombinant EGF was added to the cells at various concentrations (ten replicates for each treatment). Cells were harvested at the indicated times, stained with crystal violet, and relative growth was measured by absorbance at 540 nm.

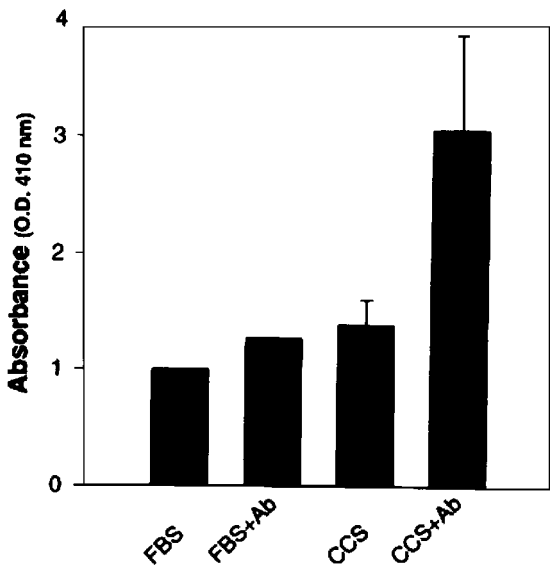

Fig. 9. Effect of estrogen withdrawal and EGFR signaling blockade on cell death. BT474 cells were plated at 1×10^5 cells/well in six well plates in IMEM supplemented with 10% FBS. On the following day, the cells were depleted of estrogen (CCS) and/or treated with 15 μg/ml of the EGFR neutralizing antibody 225IgG as indicated. Fresh treatments were added to the cells after 48 h, and an additional 48 h later the cells were harvested. Cytoplasmic lysates were prepared and analyzed for DNA and nucleosome content by the cell death ELISA as described in Material and Methods. Cell death (expressed as absorbance at 410 nm) is presented for the various treatments as % control, untreated cells (FBS).

expression. This suppression is mediated at the transcriptional level, and is reflected in both EGFR mRNA and protein levels. Abrogation of estrogen action either by its depletion from the culture media or by the use of antiestrogens results in increased expression of EGFR, most likely due to release of a repressive mechanism.] We and others previously have shown in a variety of systems that estrogen is also capable of inducing EGFR at the level of mRNA, protein, and EGF binding sites. This induction, both in breast cancer cells [Yarden et al., 1996] and in the rat uterus [Mukku and Stancel, 1985; Lingham et al., 1988], is transient in nature, and EGFR returns to the level seen in untreated cells within a few hours, indicating that although EGFR is an estrogen inducible gene, its expression is suppressed in the long term presence of estrogen.

The specific role of estrogen in the suppression of EGFR was demonstrated by culturing breast cancer cells with charcoal-treated serum and 10^{-8} M E_2 simultaneously. The lack of induction of EGFR expression under these conditions indicates that the presence of estrogen alone, as opposed to other growth factors and hormones that may have been eliminated from the serum by the dextran-charcoal treatment, is sufficient to suppress EGFR expression.

This conclusion was further supported by studies with the antiestrogens hydroxytamoxifen and ICI 164,384, both of which were able to induce EGFR expression when added to cells cultured in complete serum. In our previous studies, we have shown that the rapid repression of EGFR following estrogen induction requires de novo protein synthesis and results in EGFR mRNA levels at 24 h post-E_2 treatment that are equivalent to the level in estrogen-depleted cells. The length of time that is required for E_2 to be present in the culture media to suppress EGFR to basal level (days) compared to the time that is required to counteract the transient induction (hours) suggests the involvement of an intermediate protein to mediate the full suppressive effect of estrogen. However, it is difficult to directly assess this since long incubation times with cycloheximide at concentrations that block de-novo protein synthesis result in cell death. We propose that suppression of EGFR in the long-term presence of estrogen is due to a different mechanism and/or a different regulatory protein than the short-term down-regulation that follows estrogen treatment of breast cancer cells as we described previously [Yarden et al., 1996].

Our results demonstrate that estrogen suppresses EGFR expression at the protein, mRNA and transcript levels, but does not affect the turn-over rate of EGFR mRNA. One potential mechanism by which estrogen could reduce the level of EGFR protein is through the induction of secreted peptide growth factors that are ligands for EGFR. Binding of these growth factors to EGFR can cause internalization of the receptor and decrease binding sites on the cell surface [Stoscheck and Carpenter, 1984]. However, this mechanism cannot explain the reduction in EGFR mRNA and transcription since it has been shown that EGF and TGF-α up-regulate the mRNA level for their receptor [Clark et al., 1985; Bjorge and Kudlow, 1987; Clarke et al., 1989]. As for direct transcriptional regulation by steroid hormones such as estrogen, mechanisms of gene repression are poorly understood compared to gene activation. Activation is mediated by direct binding of the receptor to hormone responsive elements typically located upstream of the regulated gene. Indeed we found within the EGFR promoter 3 putative imperfect EREs, at least two of which can bind estrogen receptor [Yarden et al., 1996]. Although negative regulatory sequences have been described for steroid hormones, there is no consensus among them [Saatcioglu et al., 1994], and in fact other repressive mechanisms have been identified that are not mediated by direct interaction of the receptor with DNA.

First, access of ER to its binding site(s) may be limited by the binding of other estrogen-induced transcription factors to nearby sequences. Since the EREs in the EGFR gene are not conserved and have reduced affinity for the ER, this mechanism is an attractive possibility, particularly for the first repressive step that counteracts the estrogen induction of EGFR. Similarly, the orphan receptor COUP-TF was reported to repress ER-mediated activation of the mouse lactoferrin gene by competing for binding at an overlapping site [Liu et al., 1993], and retinoic acid receptor was also found in some cases to interfere with binding of ER to its response element [Lee et al., 1995]. A second mechanism involves antagonism of transcription activation through protein–protein interactions independent of DNA binding. Such a case is the mutual antagonism observed between AP-1 and several steroid receptors [Pfahl, 1993]. The classical example is the repression of Fos/Jun activity by the glucocorticoid receptor (GR), in which heterocomplexes between GR and Fos/Jun interfere with binding to AP-1 sites and down-modulate transcriptional activity [Jonat et al., 1990; Lucibello et al., 1990, Schule et al., 1990, Yang-Yen et al., 1990]. A similar mechanism has been reported as well for repression of AP-1 activity by retinoic acid and thyroid hormone receptors [Pfahl, 1993], and has been implicated in the estrogen-mediated repression of the choline acetyltransferase gene [Schmitt et al., 1995]. The IL-6 gene provides another example of estrogen mediated transcriptional repression that involves protein–protein interactions between different classes of transcription factors. Estrogen mediated repression of IL-6 requires a promoter sequence that contains binding sites for C/EBPβ and NF-κB, and results from physical interaction of ER with C/EBPβ and NF-κB [Stein and Yang, 1995].

The EGFR promoter sequence is typical of a housekeeping gene; it contains no TATA or CAAT boxes and is rich in GC sequences, resulting in several binding sites for transcription factor Sp1 [Ishii et al., 1985; Johnson et al., 1988]. Additionally, a negative regulator of EGFR expression that binds GC rich sequences and is thus termed GC Factor (GCF) has been

reported [Kageyama and Pastan, 1989]. The binding sites of GCF were mapped to between −274 to −265, and between −236 to −227 relative to the major transcriptional start site. These regions are located between the putative EREs in the EGFR promoter and the site of transcription initiation and therefore may provide a physical obstacle for activation by ER. We have found that the GCF protein is more abundant in breast cancer cells maintained in the continuous presence of estrogen than in those depleted of estrogen (R. Yarden and S. Chrysogelos, unpublished data), suggesting that GCF may contribute to the transcriptional repression of EGFR in the presence of estrogen. Whether GCF itself is regulated by estrogen is currently under investigation.

In previous work we identified DNase I hypersensitive sites at the 5′ end of the first intron of the EGFR gene that were present only in ER-positive breast cancer cells expressing low levels of EGFR as compared to ER-negative cells expressing high EGFR levels [Chrysogelos, 1993]. This region could potentially bind a repressor protein that could interfere with EGFR gene expression either through interaction with the basal transcription machinery or by blocking transcriptional elongation. Control of EGFR expression at the level of transcriptional elongation has been demonstrated to occur in A431 cells and the site of the block was mapped to the first intron [Haley and Waterfield, 1991].

Reports of cross-talk between the EGFR and ER signaling pathways have suggested that estrogen-induced growth may be mediated by growth factor activation, and conversely, that EGF may exert its effects through activation of ER [Reddy et al., 1992; Ignar-Trowbridge et al., 1993]. The finding of active estrogen repression of EGFR further suggests that the interactions between these pathways may serve to modulate as well as induce growth. That is, through a balance of cross-activation and cross-repression, limited growth is permitted and uncontroled growth is prevented. This is demonstrated by the limited growth stimulation afforded by exogenously added EGF or TGF-α in the presence of estrogen (20–40% above untreated controls). In the absence of estrogen both EGF and TGF-α had a more profound effect on growth (60–120% above untreated controls), effectively "rescuing" cell growth. This increase in growth stimulation was observed for each concentration of growth factor used, suggesting that estrogen depleted cells become more sensitive to growth factors that can provide an alternative pathway for growth. Furthermore, we observed that EGF can alleviate growth inhibition mediated by either ICI 164,384 or hydroxytamoxifen in the presence of complete serum, implying that exogenously added growth factor is not simply compensating for factors other than estrogen that are lacking in charcoal-treated calf serum, but rather is providing an alternate growth pathway that allows the cells to bypass the requirement for estrogen.

These results raise the question of whether EGFR up-regulation is a general mechanism of cell survival in response to stress stimuli. Growth factors of the EGF family have been implicated as survival factors that can overcome apoptosis in several systems [Merlo et al., 1995]. An increase in EGFR levels and sensitization to low concentrations of growth factors could provide a means for estrogen-dependent breast cancer cells to avoid cell death in the face of estrogen-depletion. This premise is supported by our finding that blockade of EGFR signaling with a neutralizing antibody simultaneous with estrogen depletion significantly induces apoptosis as compared to either treatment alone.

In summary, our studies present evidence that estrogen is actively involved in suppression of EGFR expression in ER positive breast cancer cells, providing an additional level of cross talk between growth factor receptors and steroid receptors. Taken together with the well-documented inverse correlation between ER and EGFR in breast cancer, one can suggest a role for up-regulation of EGFR in the progression of breast cancer from hormone-dependence to hormone-independence. Treatment of breast cancer with antiestrogenic drugs could give rise to a population of cells with increased levels of EGFR expression that ultimately can bypass the requirement for estrogen and become nonresponsive to treatments. Therefore, targeting EGFR or its downstream effectors in combination with traditional hormone therapy could potentially be of benefit by increasing tumor cell death and preventing signaling through this alternate growth pathway.

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