Reversal of the Estrogen Receptor—Negative Phenotype in Breast Cancer and Restoration of Antiestrogen Response

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Abstract

Purpose: In breast cancer, the presence of estrogen receptor α (ER) denotes a better prognosis and response to antiestrogen therapy. Lack of ERα correlates with overexpression of epidermal growth factor receptor or c-erbB-2. We have shown that hyperactivation of mitogen-activated protein kinase (MAPK) directly represses ERα expression in a reversible manner. In this study, we determine if inhibition of MAPK in established ERα− breast cancer cell lines and tumors results in reexpression of ERα, and further, if reexpression of ERα in these ERα− tumors and cell lines could restore antiestrogen responses.

Experimental Design: Established ERα− breast cancer cell lines, ERα− breast tumors, and tumor cell cultures obtained from ERα− tumors were used in this study. Inhibition of hyperactive MAPK was accomplished via the MAPK/ERK kinase 1/2 inhibitor U0126 or via upstream inhibition with Iressa or Herceptin. Western blotting or reverse transcription-PCR for ERα was used to assess the reexpression of ERα in cells treated with U0126. Growth assays with WST-1 were done to assess restoration of antiestrogen sensitivity in these cells.

Results: Inhibition of MAPK activity in ERα− breast cancer cell lines results in reexpression of ERα; upstream inhibition via targeting epidermal growth factor receptor or c-erbB-2 is equally effective. Importantly, this reexpressed ERα can now mediate an antiestrogen response in a subset of these ERα− breast cancer cell lines. Treatment of ERα− tumor specimens with MAPK inhibitors results in restoration of ERα mRNA, and similarly in epithelial cultures from ERα− tumors, MAPK inhibition restores both ERα protein and antiestrogen response.

Conclusions: These data show both the possibility of restoring ERα expression and antiestrogen responses in ERα− breast cancer and suggest that there exist ERα− breast cancer patients who would benefit from a combined MAPK inhibition/hormonal therapy.

Breast cancer patients with tumors that do not express estrogen receptors (ERα) have a very poor prognosis. The presence of ERα not only confers increased disease-free survival and overall survival but, more importantly, also predicts for response to hormonal therapies such as tamoxifen (1–4). ERα− breast cancers, which account for approximately one third of all invasive breast cancers, are associated with a more aggressive and metastatic phenotype and overexpression of tyrosine kinase receptors such as the epidermal growth factor receptor (EGFR) or c-erbB-2, with resultant hyperactivation of downstream signaling pathways including p42/44 mitogen-activated protein kinase (MAPK; refs. 5–15). Several questions exist about the biological pathways and alterations involved in determining ERα expression and its regulation during breast cancer evolution. Current evidence suggests that most ERα− breast cancers arise from ERα+ cells that stop expressing the receptor (16). Proposed mechanisms for the origin of ERα− breast cancers include that of pressures being exerted on ERα+ cells by hyperactivation of MAPK due to overexpression of EGFR or c-erbB-2 (17), estrogen withdrawal (18, 19), or hypoxia (20, 21), as well as more permanent epigenetic alterations such as methylation of CpG islands in the ERα gene promoter region (22, 23). Using engineered cell line models of up-regulated growth factor signaling through EGFR, erbB-2, Raf, and MAPK/ERK kinase, we have previously shown that hyperactivation of MAPK directly represses ERα expression and, importantly, that this repression is reversible via inhibition of MAPK (17, 24). Recently, we have established a MAPK gene profile that accurately distinguishes ERα− from ERα+ breast cancer, further showing the link between hyperactive MAPK and the ERα− phenotype (25).

In this study, we have investigated the likelihood of reversing the ERα− phenotype and restoring response to antiestrogens in established ERα− breast cancer lines and in ERα− tumor specimens. The hypothesis that we explore here is that
abrogation of the MAPK pathway by either direct inhibition of hyperactivated MAPK or upstream inhibition of overexpressed growth factor receptor (EGFR and/or erbB-2) signaling will result in reexpression of ERs and, thus, restoration of estrogen dependence and antiestrogen sensitivity in a subset of ERα-breast cancers.

**Materials and Methods**

**Drugs.** U0126 was from Upstate and Hereceptin (trastuzumab) was from Genentech. DMSO and 17β-estradiol [E2] were from Sigma. (Z)-4-Hydroxy-tamoxifen was from Calbiochem (EMD Biosciences) and ICI 182,780 was from Tocris Bioscience. Iressa was from AstraZeneca.

**Cell lines.** Cells were incubated in a 37°C, 5% CO2 forced-air humidified incubator. SUM 149 and SUM 229 cells were grown in F-12 Nutrient Mixture (Ham) with 5% fetal bovine serum, 10 mmol/L insulin, 5 μg/mL gentamicin (all Life Technologies, Inc./Invitrogen), 0.5 μg/mL fungizone (Cambrex), and 1 μg/mL hydrocortisone (Sigma). SUM 130 cells were grown in F-12 Nutrient Mixture (Ham) but without serum and with the following additions: 5 mmol/L ethanolamine, 10 mmol/L HEPES, 0.5 g/L albumin bovine serum fraction V, 10 nmol/L T3 (as 3,3',5-triiodo-l-thyronine sodium salt; all from Sigma), 5 μg/mL transferrin, and 50 μmol/L selenium (Life Technologies). (ca)erbB-2- MCF-7 cells were grown in phenol red-free medium with 1% charcoal-stripped fetal bovine serum (Valley Biomedical).

**Gel electrophoresis and Western blotting.** Whole-cell protein lysates were prepared from cells grown to ~80% confluence using Gold Lysis Buffer [20 mmol/L Tris (pH 7.9), 137 mmol/L NaCl, 5 mmol/L EDTA, 10% glycerol, 1% Triton X-100, supplemented with 77 μg/mL aprotinin, 47 μg/mL leupeptin, 250 μg/mL pepstatin, and 184 μg/mL sodium orthovanadate]. Protein concentrations were obtained using the BCA Protein Assay Kit (Pierce). Protein was denatured in Laemmli sample buffer (Bio-Rad), supplemented with 2-mercaptoethanol, by boiling for 3 min followed by quick chilling on ice. The denatured protein was loaded on 7.5% or 10% Tris-glycine PAGEs Gold Precast gels (Cambrex) and electrophoresed in 1× Tris-glycine-SDS buffer (25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS, pH 8.3; Bio-Rad). Gels were transferred onto Hybond-P polyvinylidene difluoride membrane (Amersham) in 1× Tris-glycine buffer (25 mmol/L Tris, 192 mmol/L glycine, 20% methanol, pH 8.3; Bio-Rad). Following transfer, the membranes were blocked for 1 h at room temperature in blocking buffer [10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.1% Tween 20, 5% bovine serum albumin]. Membranes were incubated overnight at 4°C in primary antibody diluted in blocking buffer. The following primary antibodies were used: mouse anti-ER (1D5; Zymed) and phospho-Thr202/Tyr204 p44/p42 MAPK (Cell Signaling Technology). Membranes were washed with TBS-T [10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.1% Tween 20]. Secondary antibody, diluted in blocking buffer, was added for 1 h at room temperature. The following secondary antibodies were used: enhanced chemiluminescence antimouse IgG horseradish peroxidase-linked whole antibody (from sheep; Amersham) and enhanced chemiluminescence antirabbit IgG horseradish peroxidase-linked whole antibody (from donkey; Amersham). Membranes were washed with TBS-T. Chemiluminescent detection was accomplished using SuperSignal West Pico substrate (Pierce) following the manufacturer’s protocol. Following detection, membranes were stripped with Restore Western Blot Stripping Buffer (Pierce) for 15 min at room temperature, then washed in TBS-T. The stripped membranes were then probed for actin to verify even loading. Actin (1-19) horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology) was used following the above protocol, with the exclusion of the secondary antibody step.

**Growth assays.** Twenty-four hours before adding the treatments, 1,000 to 3,000 cells (depending on cell line) were plated in each well of a 96-well tissue culture plate in 100-μL medium. Treatments were made at 2× concentration in medium and 100 μL were added to the preplated cells. Cells were incubated for 0 (in growth medium only), 2, 4 or 6 days. SUM cell lines were re-fed and re-treated every 24 to 48 h (depending on cell line and dose of inhibitor). At each time point, 1/10 volume of Cell Proliferation Reagent WST-1 (Roche Applied Science) was added to each well and the plates were incubated at 37°C for 60 to 90 min (consistent time per cell line, but differing times for each cell line). Absorbances were read at 450/630 nm on a microplate reader [Dynex Revelation (Dynex Technologies) or Bio-Rad Benchmark Plus].

**Ex vivo tumors, tumor dissociation, and tumor cell cultures.** Breast tumors were procured from surgical patients by the Tissue Procurement Core of the University of Michigan Comprehensive Cancer Center, following the Institutional Review Board protocol. The freshly procured tissue was used immediately for experiments or dissociation. For experiments using tissue chunks (ex vivo tumors), tumors were minced into small pieces and randomly divided into treatment groups. Each group was cultured in a 100-mm TC plate containing phenol red-free, modified IMEM with ε-glutamine, without gentamicin sulfate (Life Technologies/Invitrogen), with 10% charcoal-stripped calf serum (Valley Biomedical), 50 μg/mL gentamicin, and the appropriate treatment. Treatments consisted of vehicle control (DMSO) or 10 mmol/L U0126. Samples were incubated at 37°C, 5% CO2 for 20 h before harvest for RNA (except for tumor no. 15, which was incubated for 48 h). To create cell cultures, tumors were minced into small pieces with a scalpel and dissociated in serum-free modified IMEM, with ε-glutamine, without gentamicin sulfate and phenol red, supplemented with 300 units/mL collagenase type 3, 100 units/mL hyaluronidase (both from Worthington Biochemical), 2% bovine serum albumin fraction V, and 5 μg/mL recombinant human insulin (Sigma), at 37°C, 5% CO2 with gentle agitation for 5 to 16 h until the majority of tissue was digested. The dissociated cells were centrifuged at 100 × g for 5 min to pellet the epithelial cells. The epithelial cell pellet was plated in phenol red-containing modified IMEM with ε-glutamine, without gentamicin sulfate (Life Technologies/Invitrogen), with 10% fetal bovine serum (Valley Biomedical), 5 μg/mL recombinant human insulin, and 50 μg/mL gentamicin (Life Technologies/Invitrogen) in tissue culture flasks. Cells were continuously passaged with 0.05% trypsin, 0.53 mmol/L EDTA until the only the epithelial cell population remained. These stable tumor cell populations were named DT5 [ER/ε/progesterone receptor (PR)/H2N designation by pathology], DT6 (ER/ε/PR/H2N designation by pathology), DT13 (ER/ε/PR/H2N designation by pathology), and DT16 (ER/ε/PR/H2N designation by pathology). DT5, DT13, and DT16 were also grown in the same media but minus phenol red and insulin and with 10% charcoal-stripped calf serum.

**Real-time PCR.** Before RNA extraction, tumor chunks were stored in RNALater (Ambion). Tissue chunks were homogenized in TRizol reagent (Life Technologies/Invitrogen) with a glass dounce homogenizer. RNA was extracted according to the TRizol manufacturer’s protocol. RNA from cell lines was also extracted with TRizol reagent per manufacturer’s protocol. RNA was DNase treated using TURBO DNase (RNase-Free; Ambion) following the manufacturer’s protocol. The DNase-treated RNA was then subjected to reverse transcription using TaqMan reverse transcription reagents (Applied Biosystems). RNA was denatured at 65°C for 5 min followed by a quick chill on ice before addition to the reverse transcription reaction. Plus reverse transcription reactions were carried out in a final volume of 10 μL with 0.1 μg RNA, 1× reverse transcription buffer, 5.5 mmol/L MgCl2, 500 μmol/L of each deoxynucleotide triphosphate, 2.5 μmol/L random hexamers, 0.4 units/μL RNase inhibitor, and 3.125 units/μL MultiScribe reverse transcriptase. Minus reverse transcription reactions were carried out in the same manner as for plus reverse transcription reactions, but MultiScribe reverse transcriptase was omitted. The plus and minus reverse transcription reactions were incubated at 25°C for 10 min, 37°C for 60 min, 95°C for 5 min, followed by a 4°C hold in a thermocycler. Real-time PCR was carried out to determine relative quantification.
using the comparative Ct method \((2^{-\Delta\Delta Ct}}\); ref. 26). Plus reverse transcription reactions were run in triplicate per sample per gene. Minus reverse transcription reactions and no template controls were run in duplicate per sample per gene to verify that all amplifications are due to cDNA. Real-time PCR for ERx and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on the tumor chunk RNA was done on the iCycler IQ Real-time PCR Detection System (Bio-Rad) in a 25-μL reaction volume of 1× IQ SYBR Green Supermix (Bio-Rad), 5 ng of reverse transcription reaction, and 300 nmol/L of each ERx primer or 200 nmol/L of each GAPDH primer. Reactions were cycled for 10 min at 95°C, followed by 40 cycles of 95°C for 15 s/60°C for 1 min (during which time data collection and real-time analysis were enabled). The following primer sequences were used: ERx-F, 5′-CCACCAACACTG-CACCAT; ERx-R, 5′-GCTTCTTCTGATCCACCCTTTC (27); GAPDH-F, 5′-CACCAAGGCGCTTCTTAACTCGTGA; GAPDH-R, 5′-CTTGACCCGTGCCATGGAATTGC (GAPDH primer sequences obtained from Bio-Rad Tech Note 2804). Real-time PCR for GREB1 and GAPDH on cell line RNA was done on the Applied Biosystems 7900HT Sequence Detection System in a 25-μL reaction volume of 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 10-ng reverse transcription reaction, and 1× TaqMan Gene Expression Assay. Reactions were cycled at 95°C for 10 min and 40 cycles of 95°C for 15 s/60°C for 1 min. The TaqMan Gene Expression Assays (Applied Biosystems) GREB1 Hs00536409_m1 and GAPDH Hs99999905_m1 were used. Results were calculated using the comparative Ct method \((2^{-\Delta\Delta Ct}}\). Samples were normalized to GAPDH and are relative to vehicle control (DMSO)–treated samples.

Statistical analysis. To determine if differences in growth assays between different hormone treatments were significant, a two-tailed, type 1 Student’s t test was chosen because in each growth assay, it was different treatments of the same cells being compared and not different cell types being compared. In each assay, U0126 + tam was compared with control and ICI 182,780 was compared with U0126 + ICI 182,780. For coMCF-7 cells (which express ERx), (a)erbB-2-MCF-7 cell treated with 5 μmol/L Iressa or DMSO for 4 or 24 h and Western blotted for ERx and actin; right, (ca)erbB-2-MCF-7 cells treated with 5 μg/mL Herceptin or 1% benzyl alcohol vehicle (Co) for 1 or 16 h and Western blotted for ERx or actin. coMCF-7 (−100-150 fmol ERx/mg protein) and coMCF-7/16-E2 (−400 fmol ERx/mg protein) were included in some blots for comparison.

Results

Restoration of ERx expression in ERx breast cancer cell lines via direct MAPK inhibition or via targeted inhibition of EGFR and c-erbB-2. We have previously shown the mechanistic link between hyperactivation of MAPK and down-regulation of ERx expression using MCF-7 cell lines stably overexpressing constitutively active Raf-1, constitutively active MAPK/ERK kinase (MEK), constitutively active c-erbB-2, or ligand-activatable (i.e., +EGF) EGFR as models of overexpressed growth factor signaling. The four engineered cell lines, referred to as (ca)Raf, (ca)MEK, (ca)erbB-2, and EGFR + EGF, all show hyperactivation of MAPK, estrogen-independent growth, and loss of ERx expression (17). Importantly, inhibition of this hyperactive MAPK restored not only the expression but also the transcriptional function of ERx (17, 24).

We have now extended these studies to established ERx breast cancer cell lines. Using three ERx SUM breast cancer cell lines [SUM 229, which overexpresses EGFR, SUM 190, which overexpresses both EGFR and erbB-2; and SUM 149, which models inflammatory breast cancer and has high levels of RhoC leading to hyperactivation of nuclear factor κB (NF-κB) in addition to EGFR overexpression], we assessed whether inhibition of MAPK activity could result in restoration of ERx expression. In fact, inhibition of MAPK activity via the pharmacologic MAPK/ERK kinase inhibitor U0126 resulted in significant levels of ERx expression in each of the three cell lines (Fig. 1A-C). In Fig. 1A, the relative ERx expression levels of both coMCF-7 cells (which express −100-150 fmol/mg protein) and coMCF-7/16-E2 cells (coMCF-7 grown long-term in estrogen-depleted media; these cells express ~400 fmol/mg protein) are shown for comparison. ERx reexpression was sustained over the 24-h time period in which U0126 was effective in inhibiting the hyperactivation of each of these cell lines. Upstream inhibition of MAPK via inhibition of the overexpressed EGFR (with Iressa in SUM 190 cells) or erbB-2 (with Herceptin in our constitutively active erbB-2 MCF-7 line) is also effective in restoring ERx expression (Fig. 1D). We also assessed the effects of MAPK inhibition on the expression of two other factors that could be involved in estrogen responses in breast cancer cells, ERβ and GPR30. None of these cell lines exhibited appreciable expression of ERβ, nor did MAPK inhibition have any effect on this expression. All the cell lines, on the other hand, expressed GPR30, but similar to ERβ, MAPK inhibition in these cells had no effect on the GPR30 expression (data not shown).

ERx reexpression restores responses to the antiestrogens tamoxifen and Faslodex. To assess the ability of this reexpressed ERx to restore antiestrogen responsiveness, SUM 229 cells were analyzed in 6-day WST-1 growth assays. The protein
results shown in Fig. 1A were achieved with 10 μmol/L U0126; however, this dose used every 24 h was extremely growth inhibitory in SUM 229 cells. Therefore, we carried out a dose response assay to determine a dose that would not, on its own, inhibit growth so that we could observe a tamoxifen or Faslodex response if there was one. U0126 at 1 μmol/L was the maximum tolerated daily dose for these cells, so this dose was then assessed for its ability to restore ERα expression. Whereas 1 μmol/L U0126 did not result in the same large increase in ERα expression that 10 μmol/L did, it was still effective in restoring some ERα expression in SUM 229 cells (Fig. 2A). Growth assays were then carried out where the effects of 4-hydroxy-tamoxifen and the pure antiestrogen ICI 182,780 (Faslodex) at 10^{-7} mol/L alone, 1 μmol/L U0126 alone, or the combination of 4-hydroxy-tamoxifen or ICI 182,780 and U0126 on cell proliferation were assessed by a WST-1 assay at 6 days. For comparison, the growth-inhibitory effects of both 4-hydroxy-tamoxifen and ICI 182,780 at 6 days on ERα+ coMCF-7 is shown in Fig. 2B (left). As can be seen in Fig. 2B (right), 1 μmol/L U0126, although not having growth-inhibitory effects on its own in SUM 229 cells, restored the growth-inhibitory effects of both 4-hydroxy-tamoxifen and ICI 182,780. Estrogen addition to ICI 182,780 and U0126 partially reversed the antiestrogen inhibition, indicating that this is a specific ERα-mediated effect; however, estrogen by itself did not further induce growth presumably because these cells were growing at their maximum rate and this dose of U0126 was not inhibiting their growth. When a slightly higher dose of U0126 was used to induce a modest growth suppression, then an estrogen dependence effect on growth can also be observed (data not shown). These data suggest that reexpressed ERα, on inhibition of MAPK activity, is capable of mediating the growth-inhibitory effects of antiestrogens in at least some ERα+ breast cancers. SUM 149 cells, on the other hand, were extremely resistant to the growth-inhibitory effects of U0126 although MAPK activity was inhibited. In this cell line in which 10 μmol/L U0126 resulted in a significant increase in ERα expression (Fig. 1A), this reexpressed ERα could not restore the growth-inhibitory
effects of antiestrogens (Fig. 2C). This could be due to the hyperactivation of NF-κB exhibited by these cells, a well-established inducer of antiestrogen resistance (28–30). These two cell lines thus serve as examples of two different subsets of ERα breast cancers: those in which EGFR/erbB-2–driven MAPK plays a role in cell proliferation, and thus the concomitant inhibition of MAPK and restoration of ERα expression restores ERα-driven growth pathways and antiestrogen sensitivity, and those in which pathways/factors other than growth factor receptor/MAPK drive growth, and thus while inhibition of MAPK restores ERα expression, these other pathways/factors bypass ERα and maintain the antiestrogen resistant phenotype.

Breast cancer cell lines exhibiting a basal phenotype do not exhibit MAPK-dependent reexpression of ERα. We hypothesized that a third subset of ERα breast cancers would exist: those in which inhibition of MAPK would not result in reexpression of ERα. It is well established that a subset of ERα breast cancers exhibit hypermethylation of the ERα promoter resulting in the permanent repression of ERα (23, 31, 32), and thus MAPK inhibition alone would not be expected to restore ERα expression in this case. More recently, breast tumors have been defined as having luminal cell properties or basal cell properties, with the basal cell phenotype correlating with lack of ERα expression, in some cases with BRCA mutation and in many cases with EGFR overexpression (33–35). We therefore examined two ERα breast cancer cell lines (SUM 102 and SUM 159) that have been shown by microarray analyses to display the basal phenotype (36) to further analyze the ability of MAPK inhibition to restore ERα expression. Whereas both cell lines exhibit hyperactive MAPK and U0126 is able to effectively inhibit this MAPK activity, no restoration of ERα expression could be observed in these cells (Fig. 3). These two cell lines, in fact, turn out to exhibit hypermethylation of the ERα promoter (data not shown). These data suggest that an additional mechanism, hypermethylation of the ERα promoter, operates to repress ERα expression in at least two cell lines exhibiting a basal phenotype such that MAPK inhibition alone is not sufficient to restore ERα expression.

MAPK inhibition of ERα breast tumors ex vivo results in reexpression of ERα mRNA. To extend these data to more clinically relevant models, we have been obtaining specimens of ERα tumors from the tissue procurement core. We have assessed specimens from 10 ERα tumors. On receipt, tumors were minced and divided randomly into tissue culture dishes containing estrogen-depleted medium supplemented with 10 μmol/L U0126 or vehicle control (DMSO). The minced tumor specimens in medium plus or minus U0126 were incubated at 37°C, 5% CO2 for 20 h. RNA was prepared from all treatment groups and analyzed for ERα mRNA level by real-time PCR. Of the 10 tumors, 6 displayed increased ERα mRNA levels after treatment with U0126 (Fig. 4A).

ERα reexpression upon MAPK inhibition of dissociated tumor cells from ERα breast tumors. To ascertain that the reexpressed ERα mRNA exhibited by ERα tumors ex vivo corresponded to reexpression of ERα protein in tumor cells, we have established cultures from breast tumors acquired from the tissue procurement core. These cultures have been propagated over many passages, and the cell culture established from an ERα tumor displays high expression of ERα whereas the cell culture from an ERα tumor does not (Fig. 4B).

Similarly, the cell culture from the ERα tumor exhibits significant hyperactivation of MAPK whereas the cell culture from the ERα tumor has relatively low basal MAPK activation, similar to control MCF-7 cells.

Treatment of three of these ERα cell cultures with 10 μmol/L U0126 does result in inhibition of MAPK activity: for DT5, this occurs at 1 and 4 h, but by 8 h, MAPK activity is returning and is almost back to basal levels by 24 h (Fig. 4C); for DT13 and DT16, this also occurs by 1 h and is sustained through 8 h with modest return of MAPK activity occurring by 24 h (Fig. 4C, DT13; data not shown). In all three ERα cell cultures, this inhibition of MAPK activity (even the relatively short inhibition in DT5) is sufficient to restore ERα expression in these cells (Fig. 4C). In DT13 and DT16, in which the MAPK inhibition is mostly sustained through 24 h, ERα levels are also sustained through 24 h, but in DT5, ERα levels increase through 8 h and then have dropped back down by 24 h when the MAPK activity is almost fully restored. Re-treatment of these cells with U0126 every 8 h for a 24-h period results in maintenance of the ERα levels observed with 8 h of MAPK inhibition, showing that ERα expression can be maintained for the duration of MAPK inhibition (data not shown). In DT13, which overexpresses ErbB-2, Herceptin is also effective in restoring ERα expression (Fig. 4D) although the inhibition of MAPK by Herceptin occurs early and does not last much beyond 4 h. Similar to the SUM breast cancer cell lines, these DTs exhibited no significant expression of ERβ but did express GRPR30, and MAPK inhibition had no effect on these expression profiles (data not shown).

We next assessed whether this reexpressed ERα was transcriptionally active by assessing the ability of U0126-mediated restoration of ERα to result in increased expression of the estrogen inducible gene GREB1. DT13 and DT16 were treated with U0126 for 24 h or for 24 h in the presence of 10 nmol/L E2 for the last 20 h of U0126 treatment (Fig. 4E). In both cell cultures, the return of ERα expression at 24 h of U0126 treatment results in slight increases in GREB1 expression, whereas estrogen treatment along with U0126 is able to further induce the expression of GREB1.

ERα reexpression in tumor cell cultures restores responses to estrogen and the antiestrogens tamoxifen and Faslodex. Finally, the ability of restored ERα to mediate an antiestrogen response in these ERα cell cultures from tumors was assessed in DT16 cells in a 6-day growth assay in which cells were treated with 10 μmol/L U0126 every 48 h. As seen with the established ERα cell lines, in these dissociated ERα tumor cells, reexpression of ERα on inhibition of MAPK does restore...
responses to antiestrogens (Fig. 5). These antiestrogen responses are specific because estrogen at $10^{-8}$ mol/L $E_2$ is able to partially reverse the 4-hydroxy-tamoxifen and ICI 182,780 effects.

**Discussion**

In this study, we have shown the ability to reexpress ERα in ERα− breast cancer cells via the inhibition of hyperactive MAPK resulting from overexpression of EGFR or erbB-2 in both established ERα− breast cancer cell lines and ERα− tumors. This reexpression of ERα can be achieved via either direct inhibition of MAPK or inhibition of the upstream growth factor receptor (EGFR or erbB-2) that is driving its hyperactivation. Furthermore, we have established, for the first time, that the restoration of ERα expression is sufficient to induce antiestrogen responses in a subset of these ERα− breast cancer cells.

The reexpression of ERα in established ERα− breast cancer cell lines has only been previously shown via inhibition of DNA methylation or histone deacetylation in those cell lines in which the ERα promoter has been shown to be methylated (23, 31, 37). The methylation of ERα promoter is presumably a means of permanent repression secondary to some other down-regulating event. The down-regulation of ERα expression by hyperactive MAPK is a more direct mechanism and is dynamic and reversible (i.e., the down-regulation is reversed by the inhibition of MAPK activity and occurs again shortly after return of MAPK activity). And as we found with the two cell lines in which the ERα promoter turned out to be hypermethylated, despite the very high levels of MAPK exhibited by these cells and the effectiveness of U0126 in inhibiting MAPK, ERα expression could not be restored. Our data indicate that in addition to hypermethylation of the ERα promoter, hyperactivation of MAPK resulting from overexpression of EGFR or

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**Fig. 4.** Inhibition of MAPK in ERα− breast tumors restores both ERα mRNA and protein expression. **A,** ERα mRNA in ex vivo tumors after incubation with $10^{-6}$ mol/L U0126 or vehicle (DMSO) for 20 h (except for tumor no. 15, which was incubated for 48 h) by reverse transcription-PCR. **B,** ERα, p-MAPK, and actin loading control in DT5 (passage 7) and DT6 (passage 6) with MCF-7 cell lysate as a positive control. **C,** p-MAPK, ERα, and actin loading control in DT5 (left) and DT6 (right) treated with $10^{-6}$ mol/L U0126 for indicated times. **D,** p-MAPK, ERα, and actin loading control in erbB-2− overexpressing DT13 treated with 500 ng/mL Herceptin or the vehicle 1.1% benzyl alcohol (Co) for indicated times. **E,** GREB1 relative mRNA expression in DT13 and DT16 cells treated for 24 h with DMSO (vehicle control), $10^{-6}$ mol/L U0126, or $10^{-6}$ mol/L U0126 + $10^{-8}$ mol/L $E_2$ (for final 20 h of treatment) by real-time PCR.
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erB-2 can also be directly responsible for the lack of ER\textalpha expression in ER\textalpha\textsuperscript{+} tumors. Importantly, this MAPK-mediated down-regulation of ER\textalpha expression can be targeted to result in reexpression of ER\textalpha. In fact, it has recently been shown that in a small study of 10 ER\textalpha\textsuperscript{+}/erbB-2\textsuperscript{+} patients treated for various lengths of time with Herceptin, 3 patients reexpressed ER\textalpha (38). A more recent study by Massarweh et al. suggests that this mechanism can also be exploited in ER\textalpha\textsuperscript{+}/erbB-2\textsuperscript{+} tumors that lose ER\textalpha expression during treatment. They found that resistance to estrogen deprivation/fulvestrant in an ER\textalpha\textsuperscript{+}/erbB-2\textsuperscript{+} MCF-7 xenograft model was accompanied by up-regulation of MAPK activity and loss of ER\textalpha expression, and subsequent cotreatment with Iressa resulted in inhibition of MAPK activity and increased ER\textalpha expression (39).

Regardless of the different potential mechanisms for down-regulating/restoring ER\textalpha expression, the reexpressed ER\textalpha must not only be functional on reexpression (i.e., induce the regulation of estrogen-responsive genes) but must also be able to regulate growth in response to estrogen/antiestrogens to be clinically relevant. In studies wherein demethylation of the ER\textalpha promoter or use of histone deacetylase inhibitors restored ER\textalpha expression, this ER\textalpha was functional in that it could regulate ERE-luciferase activity as well as the expression of specific estrogen-regulated genes such as the progesterone receptor (37, 40, 41). In addition, in both our previous studies in our hyperactive MAPK cell line models, wherein reexpression of ER\textalpha on inhibition of MAPK also restored ER\textalpha transcriptional activity (17, 24), and our current study, wherein the reexpressed ER\textalpha in both DT13 and DT16 was able to induce the expression of GREB1 (Fig. 4E), an estrogen-induced gene, we show the restoration of ER\textalpha function. Importantly, for clinical applicability, the ability of the reexpressed ER\textalpha on MAPK inhibition to mediate the growth inhibition of antiestrogens in both established ER\textalpha\textsuperscript{+} breast cancer cell lines and dissociated tumor cell cultures shows clearly for the first time the potential for a novel therapeutic strategy for ER\textalpha breast cancer. In these studies, two different cell line types were observed. In the SUM 229 cell line, which was quite sensitive to MAPK inhibition in terms of growth inhibition, restoration of ER\textalpha expression correlated with restoration of response to both 4-hydroxy-tamoxifen and ICI 182,780 (fulvestrant, Faslodex); however, in the SUM 149 cell line, which also exhibits hyperactivation of NF-\kappaB and RhoC overexpression (42–44), the reexpressed ER\textalpha was not able to restore responses to either antiestrogen. This is most likely due to the hyperactivation of NF-\kappaB, which is known to result in antiestrogen resistance in breast cancer cells (28–30, 45). Thus, although the MAPK repression of ER\textalpha mechanism is operative in these cells and can thus be targeted to allow for reexpression of ER\textalpha, the cells have additional cell signaling alterations that allow them to bypass ER\textalpha and remain antiestrogen resistant although now ER\textalpha. Indeed, these cells were very resistant to growth inhibition induced by MAPK inhibition whereas even modest inhibition of NF-\kappaB significantly affected their proliferation (data not shown). Not surprisingly, the level of reexpressed ER\textalpha necessary to restore antiestrogen effects does not need to be as high as that observed in MCF-7 cells, ~100 to 150 fmol/mg protein (Figs. 2 and 4). There are several established ER\textalpha breast cancer cell lines with varying ER\textalpha levels, all lower than that of MCF-7, which are estrogen dependent and exhibit full estrogen responsiveness, such as T47D, ZR75.1, and BT474.

Together, these data are suggestive of a number of important possibilities for the treatment of ER\textalpha breast cancer (Fig. 6). First, it is clear that in the large majority of ER\textalpha breast tumors, hyperactivation of MAPK by upstream overexpressed/hyperactive EGFR or c-erbB-2 represses ER\textalpha expression, and thus can be targeted to allow for reexpression of ER\textalpha. This targeting can be at the level of MAPK activity itself or via upstream inhibition of EGFR/erbB-2 signaling. In the subset of ER\textalpha tumors exhibiting hypermethylation of the ER\textalpha promoter, such targeting alone is not successful in restoring ER\textalpha expression but would most likely be necessary to maintain ER\textalpha expression after demethylation of the promoter because these tumors also exhibit high MAPK activity. Importantly, restoration of ER\textalpha expression

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\includegraphics[width=\textwidth]{fig5.png}
\caption{MAPK inhibition in DT16 cells restores antiestrogen sensitivity. Proliferation of DT16 cells treated with indicated concentrations of U0126 and/or estradiol, 4-hydroxy-tamoxifen, or ICI 182,780 measured by WST-1 assay at day 6.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6.png}
\caption{Model of ER\textalpha breast cancer subsets with potential mechanisms and therapies for each.}
\end{figure}
simultaneously restores estrogen/antiestrogen responses in those ERα tumors in which MAPK signaling seems to be the predominant mediator of proliferation. However, where alternative signaling pathways, such as NF-κB, seem to be the predominant mediator proliferators, concomitant inhibition of the alternate signaling pathway would be necessary to allow the restored ERs to mediate antiestrogen responses. Furthermore, in those tumors exhibiting hypermethylation of the ERα promoter, in which it has recently been shown that inhibitors of histone deacetylases are equally effective in relieving the repression of ERα transcription (37), a combination of a histone deacetylase inhibitor and MAPK inhibition may be an effective means of restoring antiestrogen responses. Finally, these data indicate that ERα status, rather than being solely positive or negative, is a dynamic process strongly affected by the signaling environment of breast cancer cells.

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**References**

Reversal of the Estrogen Receptor–Negative Phenotype in Breast Cancer and Restoration of Antiestrogen Response

Jill Bayliss, Amy Hilger, Prakash Vishnu, et al.


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