Activation Function-1 Domain of Estrogen Receptor Regulates the Agonistic and Antagonistic Actions of Tamoxifen

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The antiestrogen tamoxifen has been widely used for decades as selective estrogen receptor (ER) modulator for ERα-positive breast tumors. Tamoxifen significantly reduces tumor recurrence by binding to the activation function-2 (AF-2) domain of the ER. Acquired resistance to tamoxifen in breast cancer patients is a serious therapeutic problem. Antiestrogen-resistant breast cancer often shows increased expression of the epidermal growth factor receptor (EGFR) family members, EGFR and ErbB2. In this report we now show that overexpression of EGFR or activated AKT-2 in MCF-7 cells leads to phosphorylation of Ser167 in the AF-1 domain of ERα, enhanced ER-amplified in breast cancer 1 (ER:AIB1) interaction in the presence of tamoxifen, and resistance to tamoxifen. In contrast, transfection of activated MAPK kinase, an immediate upstream activator of MAPK (ERK 1 and 2) into MCF-7 cells leads to phosphorylation of Ser118 in the AF-1 domain of ERα, inhibition of ER-amplified in breast cancer 1 (ER:AIB1) interaction in the presence of Tam, and maintenance of sensitivity to tamoxifen. Inhibition of AKT by short inhibitory RNA blocked Ser167 phosphorylation in ER and restored tamoxifen sensitivity. However, maximum sensitivity to tamoxifen was observed when both AKT and MAPK were inhibited. Taken together, these data demonstrate that different phosphorylation sites in the AF-1 domain of ERα regulate the agonistic and antagonistic actions of tamoxifen in human breast cancer cells. (Molecular Endocrinology 20: 996–1008, 2006)

The effects of estrogens are mediated primarily via estrogen receptor α and β (ERα and ERβ), which are members of the nuclear hormone receptor superfamily (1). 17β-Estradiol (E2) binding to its receptor induces the ligand-binding domain to undergo a characteristic conformational change, whereupon the receptor dimerizes, binds to DNA, and subsequently stimulates gene expression (2). ERα is stimulated by two distinct activation regions, activation function-1 (AF-1) and AF-2 (3–5). AF-1 is located in the N-terminal A/B domain and is constitutively activated or exerts ligand-independent transcriptional activity (6–8). AF-2 is located in the C-terminal ligand-binding domain and exerts ligand-dependent transcriptional activity (9). AF-1 and AF-2 activate transcription independently or synergistically and act in a promoter-specific and cell-specific manner (10). ER is also activated by ligand-independent mechanisms that involve cross talk from peptide and growth factor signal transduction pathways. Both ligand-dependent and ligand-independent activation of ER are modulated by receptor phosphorylation (11, 12). The major phosphorylation sites of ER residues in the N-terminal domain are serine 104, 106, 118, and 167. Mutations of serine 104, 106, and 118 to alanine result in a general decrease of ER transcriptional activity (12, 13). Phosphorylation of serine 167 was shown to be important in DNA binding by the receptor (14). Numerous signaling pathways regulate ER phosphorylation (6, 13, 15, 16). Interactions with coregulatory proteins are important mechanisms mediating E2 and selective ER modulator action.

Protein kinase B (PKB)/AKT family is composed of three closely related isoforms, AKT-1 (PKBα), AKT-2 (PKBβ), and AKT-3 (PKBγ), which are expressed at mRNA levels in all normal tissues (17). Elevated AKT-1 kinase activity (16, 18) and AKT-2 kinase activity have been reported in breast carcinomas (18, 19). Expression of AKT-3 has been shown to be up-regulated in ER-negative breast cancer tumors, suggesting a role for AKT-3 in this aggressive phenotype of breast cancer (20, 21). Overexpression of AKT is associated with tamoxifen resistance in breast cell lines (22, 23) and human breast tumors (24, 25). The AKT phosphorylation site (RXRXXS/T, where X is any residue) is present in ERα but not in ERβ. This suggests the possibility that AKT-induced changes in ER signaling are mediated through ERα. However, the mechanisms by which activated AKT regulates tamoxifen sensitivity or resistance are not clear at present.

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Abbreviations: AF-2, Activation function 2; AIB1, amplified in breast cancer 1; DAPI, 4′,6-diamidino-2-phenylindole; DCC, dextran-coated charcoal; E2, 17β-estradiol; EGFR, epidermal growth factor receptor; ER, estrogen receptor; ERE, estrogen response element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PKB/Akt, protein kinase B; MEK, MAPK kinase; myr-Akt, myristoylated Akt; siRNA, short inhibitory RNA.

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Approximately 70% of all breast cancers are dependent for their growth on estrogen and a functional estrogen receptor $\alpha$ (ER$\alpha$). Hence, ER-positive breast cancer is usually treated through hormone reduction using aromatase inhibitors (26, 27) or antiestrogens such as tamoxifen (28). The most commonly used antiestrogen is tamoxifen, and it is beneficial in pre- and postmenopausal women whose tumors are ER positive; the optimal treatment period is 5 yr (29). However, most patients undergoing long-term treatment of breast cancer with tamoxifen eventually experience recurrence of tumor growth. One of the reasons for this treatment failure is the acquisition by the tumor of the ability to respond to tamoxifen as a stimulatory rather than an inhibitory ligand (30, 31). It is widely documented that the inappropriate activation of growth factor-signaling cascades can promote antiestrogen failure in breast cancer cells. This phenomenon has been described for the overexpression of multiple growth factors and their receptors, including epidermal growth factor and TGF-\alpha, acting through the epi-dermal growth factor receptor (EGFR) (32, 33). Furthermore, heregulins are known to act through HER-3 and HER-4 (34, 35), and IGF-I and -II act through the type I IGF receptor (IGF-IR) (36, 37). It is also known that HER-2 contributes to antiestrogen failure either directly when overexpressed (38–40) or indirectly through heterodimerization with other ErbB receptor family members (33). However, the molecular mechanisms by which overexpression of growth factors and their receptors lead to antiestrogen resistance have not been adequately established.

To elucidate the signaling pathways involved in regulating tamoxifen resistance in ErbB-overexpressing cells, we expressed EGFR, myristoylated AKT-2 (myr-AKT-2), constitutively activated MEK-1, or both myr-AKT and activated-MEK in ER-positive MCF-7 human breast tumor cells. Our results show that overexpression of EGFR or the activation of AKT in MCF-7 cells leads to ligand-independent phosphorylation of Ser167 on ER. This phosphorylation event leads to enhanced ER$\alpha$-amplified breast cancer 1 (AIB1) interactions and tamoxifen resistance. However, inhibition of AKT by short inhibitory RNA (siRNA) in EGFR- or MEK/AKT-expressing cells reverses tamoxifen resistance. Simultaneous inhibition of AKT and MAPK by siRNA restores 70–80% of the tamoxifen sensitivity in these cells. Our findings provide insight into the molecular mechanism by which ligand-independent activation of ER$\alpha$ leads to tamoxifen resistance.

RESULTS

ErbB Kinase Activity Is Required for Ligand-Independent Activity of ER$\alpha$ and Tamoxifen Resistance in Vitro

Multiple lines of experimental evidence suggest that overexpression of ErbB receptors confers antiestro-
whereas tamoxifen blocks only AF-2 activation (43). These data suggest that expression of either activated MAPK or AKT can induce increase in the transcriptional activity of ERα. However, amoxifen is unable to block AKT-induced transcriptional activation in vitro.

To determine the physiological significance of MAPK- and AKT-induced phosphorylation of ERs and its effects on the antiestrogen tamoxifen in vitro, we treated the MCF-7, MEK/MCF-7, EGFR/MCF-7, and HER-2/MCF-7 cells with increasing concentration of tamoxifen (4-OHT, 10⁻¹⁰ to 10⁻⁶ M) and determine its effects on cell proliferation. The proliferation of MCF-7 and MEK/MCF-7 cells was inhibited 60–70% by 10⁻⁶ M tamoxifen. In contrast, only 20–30% inhibition was observed in EGFR- and HER-2-overexpressing cells (Fig. 3A). These data suggest that EGFR- or HER-2-overexpressing cells are less sensitive or resistant to tamoxifen treatment in vitro.

To further determine whether EGFR overexpression leads to tamoxifen resistance in vivo, we injected MEK/MCF-7 and EGFR/MCF-7 cells subcutaneously on either side of an ovariectomized SCID mouse in the presence or absence of 17β-estradiol (0.72 mg/pellet, 90-d slow-release pellet). Once xenografts had reached a volume of 2200 ± 128 (mean ± se), tumor-bearing mice were randomly allocated to no treatment or treatment with tamoxifen. In MEK/MCF-7 tumors, E₂-stimulated tumor growth (6137 ± 210, mean ± se)
and tamoxifen significantly blocked E2-induced tumor growth (2983 ± 180, mean ± se) (P < 0.05). However, in EGFR/MCF-7 cells, estrogen significantly enhanced tumor growth (8476 ± 380, mean ± se) whereas tamoxifen was unable to inhibit the tumor growth significantly (646 ± 460, mean ± se) (P = 0.23) (Fig. 3B). The above data suggest that overexpression of EGFR leads to tamoxifen resistance both in vitro and in vivo.

Fig. 2. AKT and MAPK Increase Ligand-Independent Activity of ERα
MCF-7 cells were transiently transfected with activated MEK (panel A) or myr-AKT-2 (panel B) along with EREtkLuc (the ERE-containing reporter plasmid) or tkLuc (the corresponding empty vector with no ERE) and cotransfected with pRL (an internal reporter plasmid to control for transfection efficiency). Then cells were treated with E2 (10⁻²)M, tamoxifen (10⁻⁶), ICI 182,780 (10⁻⁷), or ethanol vehicle for 48 h and then harvested. Cell extracts were prepared and analyzed for luciferase activity, as described in Materials and Methods. The magnitude of activation obtained from tkLuc-transfected cell extracts (as determined after normalization to pRL activity) was used to calculate reporter activity. Transactivation is reported as the fold induction relative to the basal level of luciferase activity in cells transfected with the empty tkLuc reporter vector, which is arbitrarily set at 1.0-fold. Cotransfection of activated MEK or myr-AKT led to increases in estrogen-independent activity and further increases on estrogen-stimulation. Tamoxifen significantly inhibited MEK-induced transcriptional activity. However, in myr-AKT-transfected cells tamoxifen was unable to block AKT-induced transcriptional activation. ICI-182,780 blocked both MEK and AKT-induced transcriptional activity. The data shown here are from three separate experiments, represented as the means ± se. Tam, Tamoxifen; ICI, ICI 182,780.

Fig. 3. Overexpression of EGFR Leads to Tamoxifen Resistance Both in Vitro and in Vivo
A, Effect of MEK, EGFR, or HER-2 overexpression on tamoxifen resistance in vitro. MCF-7 (control cells), MEK/MCF-7, EGFR/MCF-7, and HER-2/MCF-7 cells were grown as described in Fig. 1. These cells were treated with increasing concentrations of tamoxifen. Cells were counted on d 5. MCF-7 and MEK/MCF-7 cells were inhibited by 50–70% in the presence of tamoxifen at 10⁻⁶ M compared with control cells. However, EGFR or HER-2 overexpression cells were inhibited by 20–30% compared with control cells. These results are representative of three independent experiments. B, EGFR overexpression leads to tamoxifen resistance in vivo. EGFR/MCF-7 cells formed large tumors compared with MEK/MCF-7 cells in the presence of slow-release E2 pellet in ovariectomized SCID mice. In EGFR/MCF-7 cells, tamoxifen was unable to inhibit tumor growth significantly (P = 0.23). However, in MEK/MCF-7 cells, tamoxifen significantly inhibited tumor growth (P < 0.05) (panel B). TAM, Tamoxifen.
AKT-Induced Phosphorylation of Ser167 in AF-1 Domain of ERα Plays a Major Role in Tamoxifen Resistance

Activation of ErbB receptors leads to activation of multiple downstream signaling molecules such as AKT, MAPK, signal transducer and activator of transcription, Cyclin D, etc. (44, 45). This could lead to activation of ERα through other signaling molecules in addition to AKT and MAPK. To determine precisely whether AKT- and/or MAPK-induced activation of Ser167 and Ser118 in ER plays a major role in tamoxifen resistance, we generated a series of stable clones in MCF-7 cells that express myristoylated AKT (myr-AKT) alone, or express both myr-AKT and activated MEK. These clones were confirmed by Western immunoblot analysis (Fig. 4A). The various clones identified by Western blot analysis were further tested to determine whether activated AKT and/or MAPK can cross talk and phosphorylate ERα by immunofluorescence using specific antibodies directed against phospho-Ser118 and phospho-Ser167 on ERα. The data in Fig. 4B show that MAPK phosphorylates Ser118 (green) in MEK/MCF-7 cells and that AKT phosphorylates Ser167 (red) in AKT/MCF-7 cells. However, in MEK/AKT/MCF-7 cells, both Ser118 (by MAPK) and Ser167 (by AKT) are phosphorylated. We further determined the effect of estrogen and tamoxifen on cell proliferation in MCF-7 cell lines expressing activated MEK alone, AKT alone, or both MEK and AKT. MCF-7 cells transfected with empty vector were used as control cells. The proliferation of MCF-7 control cells was inhibited by 65–70% in the presence of E2 plus tamoxifen compared with E2 alone. However, in AKT/MCF-7 cells, E2 plus tamoxifen inhibited cell proliferation by 25%, in MEK/MCF-7 cells by 50%, and in MEK-AKT/MCF-7 cells by only 8% (Fig. 4C). These data suggest that activated AKT or AKT-MAPK pathways play a major role in the regulation of antiestrogen resistance to tamoxifen in ER-positive MCF-7 breast tumor cells.

It has been proposed that E2 signaling is mediated by recruiting transcriptional coactivators to the ER (46). AIB1, also called steroid receptor coactivator 3, receptor-associated coactivator 3, acetyltransferase, and p300/cAMP response element-binding protein binding protein interacting protein, is an ER coactivator that was previously shown to play an important role in hormone-mediated breast cancer progression (45, 47). Tamoxifen inhibits coactivator recruitment and promotes corepressor association with ER to inhibit estrogen-dependent gene transcription in tamoxifen-sensitive breast cancer cells (48). To evaluate the effect of cross talk on ER-coactivator interactions, we determined the interaction of ER-AIB1 by coimmunoprecipitation and Western blot analysis in MCF-7, MEK/MCF-7, AKT/MCF-7, and EGFR/MCF-7 cells in the presence or absence of E2 and/or Tam. In the MCF-7 and MEK/MCF-7 cells, ER-AIB1 interactions...
increased in the presence of E2 compared with control and decreased in the presence of E2 plus tamoxifen (Fig. 5, A and B); these cells are sensitive to tamoxifen (Fig. 2A). However, in AKT/MCF-7 and EGFR/MCF-7 cells, both E2 and E2 plus tamoxifen increased ER-AIB1 interactions (Fig. 5, C and D), and these cells are resistant to tamoxifen.

To further examine whether serine-167 is important for ERα transcriptional activity and to determine whether it alters ER-AIB1 interaction, we transiently transfected human wild-type ER or ERα mutant(s) (S167E or S167A) into HeLa cells. Studies have shown that well-characterized ER-negative HeLa cells exhibited 70–80% transfection efficiency (data not shown). Substitution of serine with glutamic acid at Ser-167 of ER mimics ER phosphorylation at 167, whereas substitution with alanine eliminates it. HeLa cells were transiently transfected with human ERα mutant(s) for 48 h after which the transcriptional activity and ER-AIB1 interaction were determined by coimmunoprecipitation and Western blot analysis as described previously (6). Transfection with the phosphorylation mimic ERα mutant (S167E) resulted in approximately 2-fold increase in ERE-reporter activity compared with control, and E2 further enhanced ERE-tkLuc activity to approximately 3.5-fold. However, tamoxifen was unable to block S167E mutant activity (data not shown).

Fig. 5. AKT Activation Enhances Interaction between ER and AIB1 in the Presence of Tamoxifen
The coimmunoprecipitation studies in tamoxifen-sensitive MCF-7 and MEK/MCF-7 cells show that E2 enhanced ERα-AIB1 interactions, and tamoxifen reduced the ERα-AIB1 interactions (A and B). However, in tamoxifen-resistant EGFR/MCF-7 and AKT/MCF-7 cells both E2 and tamoxifen enhanced the ERα-AIB1 interactions (C and D). These experiments were performed on at least on three independent occasions. C, Control; TAM, tamoxifen.

Inhibition of AKT Sensitizes the Resistant Cells to Tamoxifen
To further validate the significance of AKT and MAPK in the regulation of the agonistic and antagonistic effects of tamoxifen in MCF-7 cells, we examined the effects of inhibiting AKT and/or MAPK by siRNA in EGFR/MCF-7 and in MEK-AKT/MCF-7 cells. We transfected AKT or MEK siRNA to block AKT and/or MAPK activation in EGFR/MCF-7 cells. Our data show that MEK siRNA significantly blocked phosphorylated MAPK levels, and the inhibition persisted up to 120 h. However, MEK siRNA had no effect on total MAPK levels (data not shown). Random siRNA, which was used as a control, had no effect on phosphorylated MAPK levels, as shown by immunoblot analysis (Fig. 7A). Similarly, AKT siRNA significantly blocked phosphorylated AKT levels, and the inhibition persisted up to 120 h. By contrast, random siRNA had no effect on phosphorylated AKT levels (Fig. 7B). These cells were further tested to determine whether inhibition of AKT and/or MAPK by siRNA blocks AKT- or MAPK-induced ERα phosphorylation by immunofluorescence...
assay using specific antibodies directed against phospho-Ser118 or phospho-Ser167 on ER. Our data show that inhibition of MEK by siRNA specifically blocks Ser118 phosphorylation in AF-1 domain of ER/H9251; random siRNA had no effect (Fig. 7C, top panel). Similarly, inhibition of AKT by siRNA specifically blocked Ser167 phosphorylation; random siRNA had no effect (Fig. 7C, bottom panel). We further evaluated the effect of tamoxifen (10^{-6} M) in the presence of MEK siRNA and/or AKT siRNA on cell proliferation. EGFR/MCF-7 cell proliferation was inhibited by 15–20% in the presence of E2 plus tamoxifen compared with E2 treatment (Fig. 7D). However, when cells are treated with MEK siRNA plus E2 and tamoxifen, inhibition increased to 20–25% compared with the E2 group. When cells are treated with AKT siRNA plus E2 and tamoxifen, inhibition increased to 50–55%. Finally in the presence of AKT and MEK siRNA plus E2 and tamoxifen, inhibition increased to 70–75% (Fig. 7D). To further confirm whether MEK and AKT play a major role in antiestrogen resistance, we blocked AKT and/or MEK with siRNA in MEK-AKT/MCF-7 cells. The data obtained with S167E and S167A mutants and AKT siRNA suggest that, in a Tam-resistant MCF-7 system, AKT is the dominant pathway responsible for antiestrogen resistance. Taken together the above data suggest that different serine phosphorylation sites in AF-1 domain of ER/H9251 significantly influence the agonistic and antagonistic action of tamoxifen.

It was previously shown that the AF-1 region of ER/H9251 contains phosphorylation sites for a number of kinases including MAPK, AKT, and cyclin A/cdk2 (13, 15, 49). Some of these sites are conserved between ER/H9251 and...
ERβ (50). However, the AKT phosphorylation site is present in ERα (50) but not in ERβ, suggesting the possibility that AKT-induced agonistic effects of tamoxifen are mediated through ERα. The precise details of how the EGFR/HER-2 kinase pathway modulates ER function remain to be elucidated. We currently favor a model in which ERα is a substrate of ErbB-induced AKT. The strongest evidence in favor of this model is that AKT-induced phosphorylation of Ser-167 in ERα leads to enhanced ERα-AIB1 interaction and tamoxifen resistance both in EGFR/MCF-7 and AKT/MCF-7 cells. Our experimental data with AKT and MEK siRNA showed that reduction of AKT alone was sufficient to reverse tamoxifen resistance by 50–70% in EGFR/MCF-7 and MEK-AKT/MCF-7 tamoxifen-resistant cells. However, inhibition of both AKT and MAPK further enhanced sensitivity to tamoxifen by 70–80% (Figs. 7 and 8). In addition, other members of the ER signaling pathway may be substrates of MAPK and AKT-mediated signaling. There is ample precedent for phosphorylation of steroid hormone receptor coactivators and corepressors by growth factor-mediated signaling (46, 51). To date, MAPK has been strongly implicated in modulating coregulator function, but there is no knowledge about such regulation through the AKT pathway in breast cancer cells. Further experiments are required to determine whether AKT signaling can affect ER function through phosphorylation of coregulators.

EGFR/HER-2 are overexpressed in a variety of human tumors, including breast cancers, and are associated with a poor prognosis (52) and resistance to chemo- and endocrine therapy (33, 40, 53). In vitro studies have implicated EGFR in acquiring resistance to antiestrogen therapy (32, 54). Similarly, EGFR signaling has been linked to the progression of androgen-responsive prostate cancer to androgen-independent/hormone-refractory tumors (55). Unfortunately, most
of the drugs, especially against the EGFR/HER-2 pathway, have failed to elicit a significant response in many solid tumors including breast cancer (41). One of the reasons for the limited success of drugs targeted against EGFR/HER-2 to reverse or prevent the antiestrogen resistance may be due to constitutive activation of downstream signaling molecules such as phosphatidylinositol 3-kinase/AKT-, Ras/MAPK-, or protein kinase C-signaling pathways that support growth in the presence of tamoxifen (23, 56). However, a recent study suggests that breast and prostate cancer cells can acquire resistance to gefitinib (ZD1839/Iressa an EGFR inhibitor) by increasing other signaling pathways such as IGF-1 receptor pathway (57). Because AKT is one of the major downstream signaling molecules for ErbB and IGF-mediated signaling, inhibiting AKT could prevent endocrine therapy resistance.

We have previously shown that MAPK-induced Ser118 phosphorylation lowers the estrogen requirement for optimal tumor growth, yet these tumors are still sensitive to antiestrogens such as tamoxifen and ICI 182,780 (6). Consistent with our data, clinical studies also suggest that Ser118 phosphorylation of ERα is associated with better disease outcome in women treated with tamoxifen (58, 59). In contrast, clinical data also show that the presence of activated AKT in breast cancer predicts a worse outcome among endocrine-treated patients (24, 25). Taken together, our data show that different serine phosphorylation sites in AF-1 domain of human ERα significantly influence the agonist and antagonist actions of tamoxifen.

MATERIALS AND METHODS

Cell Lines and Reagents

DMEM, phenol red-free DMEM, recombinant human insulin, and fetal bovine serum were from Life Technologies (Gaithersburg, MD). Charcoal-treated fetal bovine serum was from Cocalico Biologicals (Cocalico, PA). The E2 and 4-hydroxytamoxifen were from Sigma Chemical Co. (St. Louis, MO). The E2 pellets (0.72-mg pellet, 90-d release) and tamoxifen (5 mg pellet, 90-d release) were obtained from Innovative Research of America (Sarasota, FL). Phospho-MAPK antibody was

![Fig. 8. Inhibition of AKT in MEK-AKT/MCF-7 Cells Reverses Tamoxifen Resistance](image)
from New England Biolabs (Beverly, MA). phospho-Akt (Ser 473) rabbit polyclonal antibody was from Cell Signaling Technology (Beverly, MA). ER antibody was from NeoMarkers (Fremont, CA). AIB1 antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) rabbit polyclonal was obtained from Trevigen (Gaithersburg, MD). Human breast cancer MCF-7 cells were purchased from American Type Culture Collection (Manassa, VA). The cells were grown in DMEM supplemented with 10 µg/ml insulin, 5% fetal calf serum, and antibiotics (penicillin/streptomycin).

Transfection and Establishment of Stable Cell Lines

Constitutively activated MAPK kinase (MEK) cDNA was kindly provided by Dr. Ahn (University of Colorado, Boulder, CO). In brief, constitutively activated MEK was constructed by combining a deletion of a helix encompassing residues 32–51 with the substitution of glutamic or aspartic acid for Ser218 (61, 62) or the corresponding empty vector, tkLuc containing a Vitellogenin A2-derived ERE as described elsewhere (61, 62) or the corresponding empty vector, tkLuc containing a Vitellogenin A2-derived ERE as described elsewhere. MEK/MCF-7 cells (10^6 cells) were seeded into 24-well plates at a density of 1 x 10^5 cells/ml. After mixing 0.4 ml of cells with DNA (1 µg of pMCL hemagglutinin-tagged MEK/cytomegalovirus promoter) and electroporating in a Bio-Rad Gene Pulsar (Bio-Rad Laboratories, Hercules, CA) at 950 µF and 0.22 kV/cm (t = 20–30 msec), cells were allowed to stand at room temperature for 10 min before the addition of 5 ml of complete medium and incubation for 48 h. Cells were then split and grown in the presence of hygromycin to isolate at least 20 clonal hygromycin-resistant cell lines. Immunoblot analysis and kinase assay were done to select the constitutively activated MEK expressing (MEK/MCF-7) cell and control MCF-7 cells lines.

MCF-7 cells were stably transfected with full-length EGFR cDNA (pRCMV3 vector obtained from Dr. Gordan Gill, University of California, San Diego, CA), myr-AKT-2 by electroporation using a Bio-Rad gene pulsar at 950 µF and 0.22 kV/cm (t = 12–14 msec). Stable transfectants were selected in the presence of 250 µg/ml G418 (Life Technologies) for 2–3 wk. Individual antibiotic-resistant colonies were isolated and screened for the expression of the corresponding protein by immunoblot analysis using anti-EGFR antibody (clone-528) or AKT antibody. All cell lines were routinely tested for mycoplasma contamination and found to be negative.

ER-Dependent Reporter Transcriptional Activity

MCF-7 cell lines were propagated in DMEM containing 5% dextran-coated charcoal (DCC)-stripped fetal bovine serum for 3 d before the onset of experiments. For experiments, the cells were seeded into 24-well plates at a density of 1 x 10^5 cells per well and allowed to reach approximately 60% confluence. Using Superfect (QIAGEN, Valencia, CA), according to the manufacturer’s instructions, the cells were then transfected with 1.0 µg/well of either the estrogen-responsive reporter, ERE-tkLuc, containing a Vitellogenin A2-derived ERE as described elsewhere (61, 62) or the corresponding empty vector, tkLuc (with no ERE), along with 0.1 µg/well of the internal control plasmid pRL, used to correct for transfection efficiency. After 4 h, the transfected cells were washed and then incubated in fresh DMEM containing 5% DCC, supplemented with E2 (10^-8 M), ICI 182,780 (10^-7 M), 4-hydroxytamoxifen (10^-6 M), and/or 0.1% ethanol vehicle alone for 48 h. The cells were harvested and the cell extracts were assayed for luciferase activities using the dual-luciferase reporter system (Promega Corp., Madison, WI) according to the protocol specified by the manufacturer. The magnitude of activation in tkLuc-transfected cells was determined after normalization to the activity of pRL and then taken as 1.0-fold. This value was used to calculate the relative (fold) change in transcriptional activities of ERE-tkLuc-transfected cells, after normalization to pRL activity. All data have been normalized as the ratio of raw light units to pRL units corrected for pRL activity and are shown as the means ± SE from three separate experiments (performed in triplicate).

The Immunofluorescence Analysis

Cells were plated on coverslips in a 24-well dish and grown in 5% DCC in DMEM phenol red-free medium for 48 h and then treated as indicated in the figure legend. The cells were washed with PBS and were fixed with 100% methanol for 5 min. The fixed cells were blocked using 5% BSA/PBS solution for 30 min and incubated overnight with primary ER-Ser118 antibody diluted (1:50) and/or primary Ser167 antibody diluted (1:50) in Tris buffer saline [50 mM Tris-HCl (pH 7.4), 150 mM NaCl] (Cell Signaling Technology). Cells were incubated with Alexa 488 goat antioimmune or antirabbit antibody, and DAPI (Molecular Probes, Eugene, OR) for 1 h. The coverslips were mounted on slides with Slow Fade reagent (Molecular Probes), and images were captured using fluorescence microscopy.

Western Immunoblot Analysis

Western blotting was performed as described previously (63) using a standard protocol. Crude protein extracts were obtained by lysing 5 x 10^6 cells in a buffer [50 mM Tris-HCl (pH 7.6), 1% Nonidet P-40, 2 mM EDTA, 0.5% sodium deoxycholate, 1 mM sodium ortho-vanadate, 5 mM MgCl2, 2 mM EGTA, 4 mM sodium p-nitro phenyl phosphate, 100 mM sodium fluoride] supplemented with protease inhibitors [leupeptin (0.5%), aprotinin (0.5%), and phenylmethylsulfonyl fluoride (0.02%)]. Samples containing 50 µg of total protein were electrophoresed on 7.5 or 10% sodium dodecyl sulfate-polyacrylamide gels and transferred on to nitrocellulose membrane by electrobobting. To determine an ER-AIB1 association, 1 µg of cell lysate was precipitated with the AIB1 antibody and protein G-Sepharose (Sigma, St. Louis, MO) followed by immunoblot analysis for ERα and/or coactivators. Membranes were probed with antibodies as indicated, followed by horseradish peroxi-
dase-conjugated mouse or rabbit secondary antibodies and enhanced chemiluminescence detection (Amersham Biosciences Corp, Piscataway, NJ). Intensities of the bands were quantified using Unscan-it software (Silk Scientific, Inc., Orem, UT) and normalized to the corresponding GAPDH levels.

RNA Interference Assay

Cells were plated in 24-well tissue culture plates (Corning Laboratories, Houston, TX), at a density of 1 x 10^4 cells per well, in DMEM containing 5% fetal bovine serum. After 24 h, the seeding medium was removed, cells were washed twice with PBS, and the medium was replaced with phenol red-free DMEM with 5% charcoal-stripped serum. To suppress AKT-2 and/or MEK-1 expression, cells were transfected with 0.8 µg of the Smart-pool siRNA duplexes (AKT-2 and/or MEK-1) from Upstate Biotechnology, Inc. (Lake Placid, NY) using Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Cells were harvested and extracts prepared for immunoblot analysis at 0, 48, 72, and 120 h.

Tumorigenesis in Scid-Beige Mouse

MEK/MCF-7 cells (1 x 10^5 cells per mouse) or EGFR/MCF-7 cells were suspended in matrigel and injected sc on either
side of the mouse in Scid-beige ovariectomized mice (Taconic Farms, Germantown, NY). The estrogen-treated mice received sc implantation of E2 pellets (E2=0.72 mg/biodegradable carrier-binder pellet) at the time of cell inoculation. Pellets containing the antiestrogen Tamoxifen (5 mg/biodegradable carrier-binder pellet) were implanted after 30 d. All pellets were 90-d biodegradable slow-release pellets obtained from Innovative Research of America (Sarasota, FL). Five animals were included in each group in two sets of independent experiments. Tumor growth was monitored by caliper measurements twice weekly by measuring the length (a) and width (b) of tumor and volumes calculated as (a x b^2)/2.

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The authors have nothing to declare.

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