Estradiol-Induced Regression in T47D:A18/PKCα Tumors Requires the Estrogen Receptor and Interaction with the Extracellular Matrix

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Abstract
Several breast cancer tumor models respond to estradiol (E2) by undergoing apoptosis, a phenomenon known to occur in clinical breast cancer. Before the application of tamoxifen as an endocrine therapy, high-dose E2 or diethystilbesterol treatment was successfully used, albeit with unfavorable side effects. It is now recognized that such an approach may be a potential endocrine therapy option. We have explored the mechanism of E2-induced tumor regression in our T47D:A18/PKCα tumor model that exhibits autonomous growth, tamoxifen resistance, and E2-induced tumor regression. Fulvestrant, a selective estrogen receptor (ER) down-regulator, prevents T47D:A18/PKCα E2-induced tumor growth inhibition and regression when given before or after tumor establishment, respectively. Interestingly, E2-induced growth inhibition is only observed in vivo or when cells are grown in Matrigel but not in two-dimensional tissue culture, suggesting the requirement of the extracellular matrix. Tumor regression is accompanied by increased expression of the proapoptotic FasL/FasL ligand proteins and down-regulation of the prosurvival Akt pathway. Inhibition of colony formation in Matrigel by E2 is accompanied by increased expression of FasL and short hairpin RNA knockdown partially reverses colony formation inhibition. Classic estrogen-responsive element-regulated transcription of pS2, PR, transforming growth factor-α, C3, and cathepsin D is independent of the inhibitory effects of E2. A membrane-impermeable E2-BSA conjugate is capable of mediating growth inhibition, suggesting the involvement of a plasma membrane ER. We conclude that E2-induced T47D:A18/PKCα tumor regression requires participation of ER-α, the extracellular matrix, FasL/FasL ligand, and Akt pathways, allowing the opportunity to explore new predictive markers and therapeutic targets. (Mol Cancer Res 2009;7(4): 498–510)

Introduction
Currently, tamoxifen is prescribed for the treatment of all stages of estrogen receptor (ER)-α-positive breast cancer and was the first drug approved as a chemopreventive agent for women at high risk for developing the disease (1). Tamoxifen belongs to the class of drugs known as selective ER modulators, exhibiting target site-specific activities in the body (2). Resistance to tamoxifen often occurs as a result of a variety of cellular changes (3) but usually is not a result of loss or mutations within the ER (4-6). Evidence of cross-talk between both nuclear and plasma membrane ER with growth factor receptors is well documented in the literature (7) including involvement of insulin-like growth factor-I receptor (8), HER-2/neu (9-11), epidermal growth factor receptor (HER-1; ref. 12), and PC cell-derived growth factor/GP88 (13). Consequently, secondary signaling effectors are also modulated including Akt (14), PTEN (15), mitogen-activated protein kinase (16, 17), and activator protein-1 (18-20). Increased levels of coactivators such as AIB1 (21) and decreased levels of the corepressor NCoR1 have also been implicated in mediating tamoxifen resistance (22).

Before the introduction of tamoxifen for the treatment of breast cancer, high-dose estradiol (E2) and diethystilbesterol (DES) were often used with response rates similar to those observed with tamoxifen (23, 24). Tamoxifen became the drug of choice due to the lower incidence of side effects. A report comparing DES-treated and tamoxifen-treated patients with a 20-year follow-up indicated a survival advantage for DES-treated patients (25). Another small trial conducted in postmenopausal patients with advanced breast cancer exposed to multiple endocrine therapies reported that 31% of patients achieved complete or partial response to DES therapy (26). Several cell and tumor models exhibiting growth inhibition and apoptosis with E2 have now emerged (27-34). A common mechanism shared by at least three of these models is the involvement of the FasL/FasL ligand (FasL) apoptotic pathway (28, 33, 34). FasL is a member of the death receptor family also known as the tumor necrosis factor superfamily that mediates the extrinsic apoptotic pathway on activation by FasL, binding in many tissues, the most well-studied of which include the immune system and the breast (35, 36).
PKCa Overexpression and E2-Induced Tumor Regression

We previously reported a T47D:A18 cell line stably transfected with protein kinase Cα (PKCa) that is hormone independent in vitro and produces tumors in athymic mice that exhibit autonomous growth, are tamoxifen resistant, and are exquisitely sensitive to E2, resulting in tumor regression and apoptosis (31, 37). We have also reported the potential predictive value of PKCa overexpression in tamoxifen-resistant breast cancer in the clinic (38). PKC is a family of serine/threonine protein kinases that is composed of at least 12 isozymes that are subdivided into three subfamilies: conventional (α, βI, βII, and γ), novel (δ, ε, η, θ, and μ), and atypical (ζ and ι/λ; refs. 39-41). The PKC family of isozymes mediates a multitude of physiologic processes in a cell type- and tissue-specific manner. The inverse relationship of PKC activity and ER status in breast cancers (43). Other laboratories have described the importance of PKCα and other PKC isozymes in MCF-7 breast cancer cells in invasion (44, 45) and tamoxifen resistance (41, 46, 47). Specifically, Frankel et al. (47) documented PKCa overexpression in nine antiestrogen-resistant cell lines and showed that stable overexpression of PKCa in MCF-7 cells resulted in reduced antiestrogen sensitivity. Recently, Assender et al. (46) reported that PKCa expression correlates with poor clinical response to endocrine therapy.

Our T47D:A18/PKCα tumor model is unique because PKCα overexpression may be a useful tumor marker to identify patients likely to respond to an E2-like treatment regimen. We reported previously that E2 causes tumor regression in vivo but does not inhibit cells grown on two-dimensional plastic (31). In this study, we explored the mechanism of E2-induced tumor regression in the T47D:A18/PKCα model by focusing on the contribution of the tumor microenvironment, involvement of the Fas/FasL pathway, and the role of ER-α.

Results

Role of the Fas/FasL Pathway in E2-Induced Regression

We showed previously that regression of T47D:A18/PKCα tumors by E2 is accompanied by apoptosis (31). To determine the signaling pathways that may mediate the apoptotic process induced by E2, T47D:A18/PKCα tumors were established in 30 untreated athymic mice. Following 9 weeks, mice were randomized to continue without treatment (NT group, 10 mice) or were implanted with a 1.0 cm E2 capsule (E2 group, 20 mice) and the experiment was continued for an additional 15 days. Tumor stabilization was observed in the E2-treated mice until day 7 followed by tumor regression (Fig. 1A). The size of the E2-treated tumors was significantly reduced compared with the NT tumors at day 7 and all time points up until day 15 (P < 0.001). Tumors were collected at various time points from NT group and post-E2-treated mice on days 1, 2, 4, 5, 7 to 9, 12, and 15 following capsule implantation (Fig. 1A). Apoptosis was assessed by TUNEL assay as described in Materials and Methods in tumors from both NT and E2 groups on days 2, 4, 8, and 15. The percentage of apoptotic cells was significantly

![Figure 1](https://example.com/image1.jpg)

**FIGURE 1.** E2 induces tumor regression, apoptosis, up-regulation of Fas/FasL, and down-regulation of p-Akt/T-Akt in T47D:A18/PKCα tumors. A. Tumor growth of T47D:A18/PKCα in vivo. T47D:A18/PKCα cells were bilaterally injected into the mammary fat pads of 30 athymic mice. Mice were left untreated for 9 wk until the mean tumor cross-sectional area reached 0.5 cm² and then randomized to two treatment groups: continued no treatment (control, 10 mice) and 1.0 cm E2 capsule (control + E2, 20 mice). Mean ± SE tumor cross-sectional area (upper limit only is shown for each point). Tumors were collected from both control and control + E2 groups on 2, 4, 8, and 15 d post-E2 capsule implantation. B. Apoptotic effect of E2 on T47D:A18/PKCα tumors. Apoptosis in tumors was assessed by the TUNEL method from both control and control + E2 groups excised 8, 12, and 15 d post-E2 capsule implantation (corresponds to data shown in A). Percentage ± SE apoptotic cells in each treatment groups. *** P < 0.001 compared with Control treatment group. Statistical test was two-sided. C. Western blot analysis of FasL and Fas protein expression in T47D:A18/PKCα tumors. D. Western blot of T-Akt and p473-Akt protein expression in T47D:A18/PKCα tumors. Tumors were excised from both control and control + E2 groups on days 2, 4, 8, and 12 post-E2 capsule implantation. β-Actin was used as a loading control. Western blots are representative of two independent tumors, with three replicate experiments, all with similar results.


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higher at all three time points in the E2 group compared with the NT group (Fig. 1B). These results are in agreement with our previous study and suggest that apoptosis contributes to E2-induced tumor regression (31).

Previous reports have indicated the involvement of the Fas/FasL pathway in mediating apoptosis by E2 in other model systems (28, 33, 48). To determine the participation of Fas/FasL in mediating apoptotic cell death in T47DA18/PKCα tumors, Fas/FasL protein expression was compared in NT and E2-treated tumors (Fig. 1A). Initially, the E2-treated tumors express reduced Fas protein on days 2 and 4 compared with the NT tumors. However, at later time points, Fas is significantly increased in the E2-treated tumors compared with the NT tumors (Fig. 1C). The same biphasic pattern of FasL regulation is observed with an initial decrease of FasL expression in E2-treated tumors relative to NT tumors on days 2 and 4 followed by increased FasL expression in E2-treated tumors compared with NT tumors on days 8 and 12. Therefore, up-regulation of both Fas and FasL is temporally related with E2-induced T47D:A18/PKCα tumor regression and apoptosis (Fig. 1A and C). However, because up-regulation of Fas/FasL is not observed before tumor regression, it is likely that Fas/FasL-induced apoptosis may not be the sole pathway responsible for E2-induced tumor regression.

Akt/Protein Kinase B Pathway Is Down-Regulated in E2-Regressing Tumors

Because the Fas/FasL pathway is not likely to be the only mechanism whereby E2-induced tumor regression occurs, we examined the possible involvement of the prosurvival Akt/protein kinase B pathway. We find that both total and p473-Akt are reduced in the E2-treated tumors compared with the untreated control at all time points (Fig. 1D). These results suggest that the prosurvival, antiapoptotic Akt pathway is inactivated by E2 treatment and therefore contributes to E2-induced tumor regression. These results suggest that repression of the prosurvival, antiapoptotic Akt pathway precedes activation of the Fas/FasL apoptotic pathway; coordinately, these two pathways mediate tumor regression.

E2-Induced T47D:A18/PKCα Growth Inhibition Requires Contact with the Extracellular Matrix

We reported previously that E2 inhibits the growth of T47D:A18/PKCα cells in vivo but has no growth-inhibitory effect in vitro (31). To determine whether the extracellular matrix is sufficient to recapitulate the in vivo environment, the ability of T47D:A18/PKCα and T47D:A18/neo cells to form colonies in Matrigel was examined. T47D:A18/neo cells form statistically significantly more colonies in phenol red-containing Matrigel compared with T47D:A18/PKCα cells (Fig. 2A), suggesting that the presence of E2 in the Matrigel environment inhibits colony formation. When cells are plated in phenol red-free Matrigel (E2-depleted environment), T47D:A18/PKCα colony formation is inhibited by 50% in the presence of E2 compared with the absence of E2 (Fig. 2B). In the absence of E2, T47D:A18/neo cells form few colonies, whereas, in the presence of E2, numerous colonies are formed (Fig. 2B). These results suggest that a component provided by Matrigel is sufficient to recapitulate the E2-induced growth-inhibitory effects observed in vivo. To determine if growth factors present in Matrigel are required to produce E2-induced growth inhibition, colony formation was examined in growth factor-reduced Matrigel. E2 retains the ability to inhibit T47D:A18/PKCα colony formation in growth factor-reduced Matrigel, exhibiting similar results when cells are plated in either phenol red-containing or phenol red-free RPMI 1640 Matrigel (Supplementary data). Because cells grown two-dimensionally are also exposed to growth factors (31, 37), it is unlikely that growth factors present in Matrigel are responsible for mediating the E2 inhibitory effect. However, we cannot rule out this possibility because growth factors are not eliminated but simply reduced in growth factor-reduced Matrigel. It is likely that another Matrigel component is required for E2-induced growth inhibition. E2 does not inhibit colony formation when the cells are plated in soft agar (results not shown) nor have we been able to successfully establish T47D:A18 colonies on collagen- or laminin-coated plates.

E2 Induces Apoptosis and Increases Fas Expression in T47D:A18/PKCα Cells Grown in Matrigel

To determine whether the mechanism of E2-induced growth inhibition of T47D:A18/PKCα cells grown in Matrigel is similar to the E2 growth-inhibitory effects observed in vivo,
apoptosis and Fas/FasL protein expression was examined. The percentage of apoptotic cells in the T47D:A18/PKCα colonies increased with time following E2 treatment and was significantly increased compared with the untreated control colonies at 7 and 15 days post-E2 treatment (Fig. 3A).

Because both Fas and FasL protein expression exhibited a temporal increase with concomitant tumor regression (Fig. 1A and C), Fas/FasL expression was determined in T47D:A18/PKCα colonies at all three time points (Fig. 3B and C) and is consistent with Fas up-regulation observed in tumors (Fig. 1C). However, there was no difference in the expression of FasL in E2-treated colonies versus the untreated control (Fig. 3B and D). Neither Fas nor FasL expression is regulated in T47D:A18/PKCα cells growing two-dimensionally (results not shown), nor does E2 cause growth inhibition of cells on two-dimensional plastic, suggesting that the tumor microenvironment and Matrigel provide the context whereby E2 causes tumor regression or colony growth inhibition, respectively.

**Knockdown of Fas by Short Hairpin RNA Partially Reverses E2-Induced Colony Formation in Matrigel**

To further clarify the role of the Fas/FasL pathway in E2-induced apoptosis in the T47D:A18/PKCα cell model, we applied RNA interference to stably knock down Fas. Lentiviral transduction particles containing shFas constructs were used to transfect T47D:A18/PKCα cells and selected clones were screened by Western blot to confirm decreased expression of Fas. Fas expression was reduced by 80% and 60% as determined by densitometry in two Fas stable T47D:A18/PKCα clones, 6-3 and 7-9, respectively (Fig. 4A). To determine whether reduced Fas expression was sufficient to abrogate Fas/FasL-mediated apoptosis, recombinant FasL peptide was used to initiate apoptosis in the shFas stable clones in two-dimensional tissue culture. Whereas apoptosis was increased by 2-fold in both the parental T47D:A18/PKCα and the negative control, T47D:A18/PKCα/NTS cells (stable transfectant with nontargeting sequence), the two shFas clones were not responsive to FasL (Fig. 4B). Therefore, we concluded that sufficient knockdown of Fas was achieved in the selected shFas clones and used to study the effect on the E2-inhibitory phenotype.

To study the effect of Fas knockdown on regulation of E2 inhibition in Matrigel, colony formation assays were done comparing the parental T47D:A18/PKCα and T47D:A18/PKCα/NTS with the shFas clones, 6-3 and 7-9. The E2-treated T47D:A18/PKCα cells formed 50% fewer colonies compared with the untreated control and the T47D:A18/PKCα/NTS cells formed 60% fewer colonies following E2 treatment (Fig. 4C). However, the shFas clones formed 30% to 35% fewer colonies compared with the untreated control groups. Therefore, Fas...
interference in these clones only partially reversed the E$_2$-inhibitory effects. To determine the role of the ER in E$_2$-induced tumor regression, the ability of the selective ER down-regulator fulvestrant to block E$_2$-induced tumor regression was tested. T47D:A18/PKC$_\alpha$ cells were injected into 50 ovariectomized athymic mice. Forty mice were not treated (NT group); 10 mice were implanted with a 1.0 cm E$_2$ capsule and given weekly injections with fulvestrant for all 13 weeks. After 7 weeks, 40 mice from the NT group were randomized to three treatment groups: continued NT (10 mice), E$_2$ capsule (10 mice), and E$_2$ capsule plus weekly injections with fulvestrant (20 mice; Fig. 5A). Fulvestrant prevented E$_2$-induced tumor growth inhibition and regression when given before or after tumor establishment, respectively. Because the mechanism of action of fulvestrant involves the destruction of the ER (49), these results suggest that the ER is required for E$_2$ to exert growth-inhibitory effects. This finding is in agreement with another study reporting participation of the ER in an MCF-7 tumor model exhibiting E$_2$-induced tumor regression (33).

To examine the mechanism whereby fulvestrant rescues E$_2$-mediated tumor regression, 40 mice were bilaterally injected with T47D:A18/PKC$_\alpha$ cells and tumors were left untreated for 5 weeks. At that time, mice were randomized into four treatment groups (10 mice per group): continued no treatment, E$_2$ capsule, fulvestrant, or E$_2$ + fulvestrant. Tumors were excised from all treatment groups 2, 4, and 8 days post-treatment for assessment of apoptosis by the TUNEL assay and expression of Fas/FasL proteins by Western blot. Tumor regression was observed in both E$_2$-treated and fulvestrant-treated mice, whereas tumors in the untreated and E$_2$ + fulvestrant-treated mice continued to grow (Fig. 5B). However, in contrast to the previous mouse experiment (Fig. 5A), tumors in the E$_2$ + fulvestrant group grew faster than the untreated control group in this experiment. TUNEL assay revealed that the level of apoptosis was greatest in the E$_2$-treated tumors following 4 and 8 days post-treatment, with a similar trend in the fulvestrant alone tumors, whereas the E$_2$ + fulvestrant group showed no increased apoptosis compared with the untreated control tumors (Fig. 5C). Tumors treated with either E$_2$ alone or fulvestrant alone exhibited up-regulation of both Fas and FasL expression; however, expression of Fas/FasL in the E$_2$-treated tumors occurred earlier (4 days) compared with the fulvestrant-treated tumors, where expression of Fas/FasL appeared later (8 days; Fig. 6A and B). This suggests that the mechanism whereby E$_2$ induces Fas/FasL protein expression is different than the mechanism initiated by fulvestrant treatment. Interestingly, treatment with a combination of E$_2$ + fulvestrant partially suppresses tumor Fas up-regulation on day 4 and completely reverses Fas expression on day 8. The E$_2$ + fulvestrant combination completely reverses FasL expression similar to levels observed in the untreated control tumors. Taken together, these results indicate that addition of the selective ER down-regulator fulvestrant can reverse E$_2$-induced apoptosis in T47D:A18/PKC$_\alpha$ tumors partially due to reversal of Fas/FasL expression and perhaps via a mechanism requiring the ER.

Both E$_2$ and fulvestrant are known to cause degradation of the ER; however, these ligands act via distinct pathways. Whereas E$_2$-mediated ER down-regulation is a result of transcriptional activation, coactivator recruitment, and subsequent proteosomal degradation (50-53), fulvestrant stimulates ER degradative proteasomal pathway (49, 54). Treatment
with either E₂ alone or fulvestrant alone for 2, 4, and 8 days results in ER-α protein down-regulation in T47D:A18/PKCα tumors (Fig. 7). This result indicates that although E₂ causes regression of these tumors, the down-regulation of ER-α in response to E₂ is as expected. Whereas fulvestrant treatment also results in ER-α down-regulation as predicted, the combination of E₂ + fulvestrant partially reverses ER-α down-regulation at all time points examined. Therefore, the differential regulation of ER-α may be responsible for the prevention of apoptosis and restoration of tumor growth.

Classic ER-α-Regulated Gene Expression

To investigate the role of ER-α in E₂-mediated tumor regression, expression of genes well known to be regulated by E₂ was determined in T47D:A18/PKCα tumors derived from treatment groups harvested on day 8 as shown in Fig. 5B. We chose to determine the expression of five classic estrogen-responsive element (ERE)-mediated E₂-responsive genes, C3 (complement component 3), PGR (progesterone receptor), CTSD (cathepsin D), TFF1 (trefoil factor 1 or pS2), and TGFα (transforming growth factor-α) by real-time reverse transcription-PCR (Fig. 8). Although E₂ treatment causes tumor regression and addition of fulvestrant prevents regression, four of the five classically estrogen-regulated genes including C3, PGR, CTSD, and TFF1 are up-regulated in both E₂-treated and E₂ + fulvestrant-treated tumors compared with the untreated control tumors. Despite the fact that treatment with either E₂ or fulvestrant causes tumor regression, these four genes exhibit opposite regulation. An exception to this is transforming growth factor-α, the expression of which appears to be up-regulated in tumors from all three treatment groups (E₂, fulvestrant, or E₂ + fulvestrant). These results suggest that although
E2 is causing complete regression of T47D:A18/PKCα tumors, E2 nonetheless can induce classic ERE-mediated gene expression in this tumor model. Therefore, perhaps E2-induced tumor regression is mediated by the ER via a nonclassic mechanism.

Plasma Membrane-Associated ER Is Implicated in the E2-Inhibitory Effect

To investigate the possibility that the E2-induced growth-inhibitory effects may be mediated by a plasma membrane-associated ER, the membrane-impermeable E2-BSA conjugate was used as a tool. To check whether free E2 was present in the E2-BSA conjugate, an ERE-luciferase construct was transfected into T47D:A18/neo cells. It was concluded that insignificant levels of free E2 were present in the E2-BSA conjugate because the E2-BSA-treated cells showed luciferase activity similar to the vehicle-treated control cells, whereas E2 treatment resulted in 17-fold induction of luciferase activity (Fig. 9A).

The ability of the membrane-impermeable E2-BSA conjugate to inhibit T47D:A18/PKCα colony formation was examined in Matrigel. Treatment with the E2-BSA conjugate resulted in slightly more T47D:A18/neo colonies compared with the untreated control group when grown in Matrigel, suggesting that E2-BSA can stimulate modest colony formation. However, 3.5-fold more colonies were present in the E2-treated group compared with the E2-BSA group (Fig. 9B). Both E2-BSA alone and E2 alone inhibited T47D:A18/PKCα colony formation compared with vehicle-treated control cells (Fig. 9C). These results imply that perhaps the E2-inhibitory effects may be mediated via an ER associated with the plasma membrane.

Discussion

The possibility of using an E2-like compound in patients that exhibit tamoxifen-resistant breast cancer is a concept that is reemerging (55-58), and the ability to predict a priori patients that would benefit from such an approach is very attractive. Our laboratory reported the correlation of PKCα...
overexpression with disease recurrence following tamoxifen treatment (38). We have explored the signaling events leading to E2-induced tumor regression in our unique T47D:A18/PKCα tumor model (31, 37). This is a relevant model of autonomous growth, tamoxifen resistance, and E2-induced tumor regression, characteristics often encountered in the clinical setting. In this study, we report the requirement of the tumor microenvironment, specifically the extracellular matrix, for E2 to elicit inhibitory effects. The ER is likely required for E2-induced tumor regression because the selective ER down-regulator fulvestrant prevents these effects. We provide evidence that up-regulation of the Fas/FasL apoptotic pathway occurs concurrent with E2-induced tumor regression and this pathway appears to be modulated only in vivo or in Matrigel but not in two-dimensional tissue culture. These results suggest that communication between the extracellular matrix and the tumor plays an important role in eliciting the E2-induced growth-inhibitory effects. Tumor regression is also accompanied by down-regulation of the Akt prosurvival pathway. ERE-mediated induction of five known E2-regulated genes is not altered in this model indicative of intact classic ER signaling. The membrane-impermeable E2-BSA conjugate elicits growth-inhibitory effects; therefore, a plasma membrane form of the ER is likely involved.

PKC isozyme expression has been studied in the MCF-7 cell lines by several investigators (44, 45, 47). To our knowledge, our T47D:A18/PKCα cell and tumor model is the only reported breast cancer model other than MCF-7 examining the effect of PKC isozyme expression on antiestrogen resistance. In this model, although we reported cross-up-regulation of PKCβ and PKCδ (37), we have determined that coordinate overexpression of PKCδ and PKCβ is not sufficient to impart autonomous, tamoxifen-resistant, and E2-inhibitory growth (59).

Several reports of MCF-7 tumor models describe E2-induced growth inhibition and regression. A cyclical response to E2 in MCF-7 tumors was first reported by Yao et al. (27), whereby short-term exposure to tamoxifen (1 year) yielded
tamoxifen-resistant and E2-responsive tumors, whereas long-term tamoxifen exposure (5 years) tumors remained tamoxifen-resistant but also acquired an inhibitory response to E2 (27). We found elevated PKCα expression in both tumor types, suggesting a correlation of PKCα overexpression with the hormoneresponsive phenotype (31). Shim et al. (32) showed that long-term estrogen-deprived cells form tumors that can be inhibited by E2 via activation of the apoptotic Fas pathway (28). The Fas pathway also mediates the E2-induced apoptosis in MCF-7 TAMLT tumors (33) and the MCF-7/Ral tumor model (34). However, T47D:A18/PKCα tumor regression is likely mediated by both proapoptotic signals through Fas/FasL and inhibition of prosurvival signals by down-regulation of the Akt pathway. Another example of E2-induced tumor regression was established by the stable transfection of MCF-7 cells with Akt-3 (60). Tumors derived from this cell line are hormone-independent tamoxifen-stimulated and growth inhibited by E2. Whereas all of the MCF-7 tumor models show E2-induced cell growth inhibition both in vitro and in vivo, our T47D:A18/PKCα cell model is not inhibited by E2 under two-dimensional culture conditions; E2 only elicits growth inhibition either in vivo (31) or in Matrigel. Matrigel is a solubilized basement membrane preparation derived from the Engelbreth-Holm-Swarm mouse sarcoma and the major components include laminin, collagen IV, heparan sulfate proteoglycans, entactin, and nidogen. The advantage of using three-dimensional models for breast cancer research is well-described and shown to better mimic the tumor microenvironment (61). T47D:A18/PKCα colony formation is inhibited in Matrigel in either medium-containing serum and phenol red (Fig. 2A) or with the addition of exogenous E2 (Fig. 2B). The ability of E2 to inhibit colony formation is not impaired when T47D:A18/PKCα cells are grown in growth factor-reduced Matrigel (Supplementary Data), suggesting that a component in Matrigel other than E2-induced cell growth factors is responsible for mediating the inhibitory effect. However, the inhibition of colony formation by E2 does not completely mimic tumor regression in vivo. Both Fas and FasL are up-regulated by E2 in tumors (Fig. 1C), whereas Fas alone but not FasL is up-regulated in Matrigel (Fig. 3). Another distinction from the in vivo model is the inability of fulvestrant to reverse E2-mediated inhibition of colony formation (results not shown). Taken together, these results suggest that Matrigel cannot completely recapitulate the tumor microenvironment. Stromal cells, which are obviously absent from Matrigel, may contribute to E2-induced tumor regression and apoptosis. One possibility is that E2 induces antiangiogenic signals, although no changes in vascular endothelial growth factor was observed in tumors following E2 treatment (results not shown). There is abundant evidence in the literature that PKC interacts with integrins (62-64), specifically a direct interaction between PKCα and β1 integrins was reported (65). Integrin expression following E2 treatment both in tumors and colonies growing in Matrigel was not altered (results not shown), but this does not rule out the possibility that enhanced PKCα/integrin signaling may lead to secretion of an antiangiogenic factor that mediates tumor regression in vivo. Another potential mediator of extracellular matrix interactions is syndecan-4, a transmembrane proteoglycan known to physically interact with PKCα (66, 67). Our laboratory is currently investigating this potential pathway.

Our results indicate that E2-induced tumor regression may also require the participation of the ER itself. We find that E2-induced tumor regression can be prevented by coadministration of fulvestrant (Fig. 5A and B), a selective ER downregulator currently approved as a second-line endocrine therapy that binds to and causes complete destruction of the ER (68).
ER-α expression is down-regulated in T47D:A18/PKCα tumors in response to E2 or fulvestrant alone but is retained in tumors treated with the E2 + fulvestrant combination, most evident on day 8 (Fig. 7). E2 is reported to cause rapid down-regulation of the ER that is dependent on coactivator recruitment and new protein synthesis, whereas destruction of the ER by fulvestrant is independent of these processes (69). In our T47D: A18/PKCα tumor model, ER-α regulation by the combination of E2 + fulvestrant appears to be dissimilar from regulation by either E2 or fulvestrant treatment alone. Perhaps this differential ER-α regulation is vital in the reversal of the growth-inhibitory effects observed with E2. Similar to our model, MCF-7 TAMLT E2-induced tumor regression is also abrogated by fulvestrant, implicating the participation of the ER (33).

We reported previously that ER function is reduced 10-fold in T47D:A18/PKCα cells as assessed by ERE-luciferase reporter assays (37). This suggested to us that classic ERE-mediated signaling may not be the mechanism whereby the ER mediates E2-induced tumor regression. However, we find that five classically ERE-regulated genes, p52, PR, transforming growth factor-α, C3, and cathepsin D, are up-regulated in tumors from E2-treated mice (Fig. 8). Except for transforming growth factor-α, transcription of the other genes is not induced by fulvestrant. Interestingly, all five genes are induced by the combination of E2 + fulvestrant to a similar level as E2 treatment alone. We conclude that transcriptional induction of these genes is independent of tumor response to E2; therefore, classic signaling is not likely to mediate tumor growth inhibition. Because interaction of T47D:A18/PKCα cells with the extracellular matrix appears to be crucial for E2 to exhibit growth-inhibitory effects, an attractive scenario is that tumor growth inhibition is mediated by the plasma membrane ER (70). The plasma membrane ER interacts with G proteins affecting downstream signaling cascades including extracellular signal-regulated kinase, phosphatidylinositol 3-kinase, and PKC (71-73). There is evidence that E2 can directly bind and activate PKCα at the plasma membrane through nongenomic effects in endometrial cancer cells and rat colon (74, 75). The fact that the membrane-impermeable E2-BSA conjugate can inhibit colony formation in Matrigel equally well as E2 (Fig. 9) suggests that the plasma membrane ER is more likely to mediate the E2 growth-inhibitory effect. Alternatively, we do not rule out the genomic nonclassic ER signaling with other transcription factors such as activator protein-1 or Sp1 (76, 77). We are currently pursuing these possibilities.

We described here the characterization of a tamoxifen-resistant T47D:A18/PKCα tumor model that has several similarities and distinctions with the MCF-7-derived tumor models. This T47D:A18/PKCα tumor model is a unique tool to further study potential therapeutic targets for tamoxifen-resistant breast cancer, especially because we and others have evidence that PKCα overexpression may be a predictive marker of tamoxifen resistance (38, 46). It may be possible to identify patients that would benefit from treatment with E2 or E2-like compounds simply by screening for PKCα expression. Perhaps the success rate of the PKCα antisense oligonucleotide Affinitak (LY900003/ISIS 3521; ref. 78) can be improved by identification of patients with PKCα-overexpressing tumors. Alternatively, the plasma membrane ER may be a potential novel therapeutic target. Finally, our findings have important implications for the application of fulvestrant and aromatase inhibitors in patients that harbor PKCα-overexpressing tumors. Based on our findings, the efficacy of fulvestrant may potentially be compromised in patients with circulating estrogens. Furthermore, E2 deprivation created by aromatase inhibitors may cause tumor growth. We are currently testing the latter hypothesis in a preclinical tumor model.

**Materials and Methods**

**Cell Culture Conditions**

Human breast cancer cell line T47D:A18 is a hormone-responsive clone that has been described previously (79) and was maintained in phenol red-containing RPMI 1640 supplemented with 10% fetal bovine serum. Stable transfectant cell lines T47D:A18/neo and T47D:A18/PKCα (37) were maintained in RPMI 1640 (phenol red) supplemented with 10% fetal bovine serum-containing G418 (500 μg/mL). When indicated, before treatment with E2 or fulvestrant for protein isolation, Western blot, or Matrigel experiments, cell lines were placed in phenol red-free RPMI 1640 supplemented with 10% of 3× dextran-coated charcoal-treated fetal bovine serum (E2-depleted medium) for 3 days.

**Growth of Cells in Matrigel**

T47D:A18/neo and T47D:A18/PKCα cells were maintained either in phenol red-free RPMI 1640 (E2-depleted) for 3 days or in phenol red-containing RPMI 1640 supplemented with 10% fetal bovine serum. Matrigel (Becton Dickinson) was thawed overnight at 4°C and E2 (10−9 mol/L), E2-BSA (10−7 mol/L), or vehicle (ethanol) was added to the Matrigel (phenol red-free, phenol red-containing, or growth factor-reduced Matrigel). Six-well plates were coated with 800 μL Matrigel/well and incubated at 37°C for 30 min. Cells were suspended at 5 × 106/mL in 1.3 mL of either phenol red-containing or phenol red-free RPMI 1640 with E2 or E2-BSA (10−7 mol/L) supplemented with 500 μg/mL G418 and spread on pre-gelled Matrigel. Plates were incubated at 37°C for 3 weeks; medium was replaced to the top of the Matrigel every 3 days. Five 1.0 cm² areas were counted under ×5 power, and the average number of colonies ± SD was determined.

**Growth of T47D:A18/PKCα Tumors In vivo**

T47D:A18/PKCα cells were injected bilaterally (1 × 10⁶ per site) into the axillary mammaary fat pads of ovariectomized nude 4- to 6-week-old athymic mice (Harlan-Sprague-Dawley). Mice were randomized into treatment groups consisting of at least 10 mice per group. E2 was administered via silastic capsules (1.0 cm) implanted subcutaneously between the scapulae. The 1.0 cm³ capsules produce a mean serum E2 level of 379.5 pg/mL (80) and were replaced every 8 weeks. Fulvestrant, ICI 182,780 (a generous gift from AstraZeneca Pharmaceuticals), was injected subcutaneously at a dose of 5 mg (0.1 mL peanut oil) per animal once per week. Tumor cross-sectional area was determined weekly using Vernier calipers and calculated using the formula: length / 2 × width / 2 × π. Mean tumor area was plotted against time in weeks to monitor tumor growth. The mice were sacrified by CO2 inhalation and cervical dislocation, and tumors were excised and either immediately...
fixed in 10% buffered formalin for TUNEL assay, stored in RNAlater buffer (Ambion), or snap frozen in liquid nitrogen and stored at −80°C. The Animal Care and Use Committee of the University of Illinois at Chicago approved all of the procedures involving animals.

**Tumor and Cell Protein Isolation and Western Blot**

Tumors were ground in liquid nitrogen into a fine powder and resuspended in cell lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na₂EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na₃VO₄, 1 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, and 2% glycerol] and homogenized. The tumor lysate was centrifuged at 12,000 rpm for 15 min at 4°C. Protein concentration was determined by the BCA method (Bio-Rad Laboratories) and stored at −80°C. Equal amounts of protein were run in a standard Western blot protocol using the following antibodies: Fas (B-10; Santa Cruz Biotechnology), FasL (G2474; BD Pharmingen), p473-Akt and T-Akt (Cell Signaling Technology), and ER-α (G-20; Santa Cruz Biotechnology). All the antibodies were diluted in TBST [20 mmol/L Tris (pH 7.6), 137 mmol/L NaCl, 0.1% Tween 20] containing 5% dry milk.

Either the Enhanced Chemiluminescent Detection system (Amersham) or the SuperSignal West Dura Western detection system (Pierce) was used to visualize the target band. Equal loading of total protein per lane was assessed by blotting with β-actin antibody (Sigma-Aldrich). Chemiluminescent signal was captured using a Chemi Doc Gel Documentation System (Bio-Rad Laboratories).

**Assessment of Apoptosis**

The identification of apoptotic cells in situ was determined using the ApopTag TUNEL Apoptosis Detection kit (Chemicon International). This method detects DNA fragments by end-labeling 3' hydroxyl DNA with digoxigenin using terminal deoxynucleotidyl transferase. An anti-digoxigenin antibody conjugated with peroxidase is bound and visualized with peroxidase substrate. Tissue sections of T47D:A18/PKCα were 5 μm thick and spread on silanized slides. Paraffin-embedded tissue sections were deparaffinized and progressively rehydrated. Sections were then pretreated with proteinase K from Sigma-Aldrich (20 μg/mL for 20 min at room temperature). Endogenous peroxidase was blocked with 0.3% H₂O₂ in PBS. ApopTag Plus Peroxidase In situ Apoptosis Detection kit (Chemicon) was then used according to the manufacturer’s instructions. Staining of nuclei was expressed as the percentage of TUNEL-positive apoptotic cells in each tumor. TUNEL-positive cells were counted in 10 different high-power fields (×40) from six sections representing at least three independent tumors of each group. Repeated measures were obtained at 8, 12, and 15 days post-E₂ capsule implantation.

**Generation of T47D:A18/PKCα/Fas Short Hairpin RNA Stable Transfectant**

T47D:A18/PKCα cells were transfected with MISSION short hairpin RNA lentiviral particles for Fas (Sigma-Aldrich) following the manufacturer’s instructions. T47D:A18/PKCα cells were suspended in culture medium (2 × 10⁴/mL medium) and seeded in each well of a 12-well plate. After 30 h incubation, medium was removed and 1 mL fresh medium with hexadimethrine bromide (8 μg/mL; Sigma-Aldrich) was added. Hexadimethrine bromide was used to enhance transduction according to the manufacturer’s instructions. Lentiviral transduction particles for Fas were provided in a set of four clones. For each clone, 4 μL particles (4 × 10⁴ transducing units) were added into the wells. Medium containing the lentiviral particles was removed and fresh medium was added after 18 h incubation at 37°C in a humidified atmosphere containing 5% CO₂. Drug selections with puromycin (InvivoGen) at 1 μg/mL and G418 (500 μg/mL) were started on the following day. T47D:A18/PKCα cells were transfected with MISSION nontarget short hairpin RNA control transduction particles (Sigma-Aldrich) and selected in the same way to serve as a negative control. Individual colonies were picked following 3 weeks of selection and screened for Fas expression by Western blot.

**Real-time PCR Analysis of Estrogen-Responsive Genes**

For each treatment and control, three independent tumor samples were available. RNA was extracted using the RNeasy Mini kit (Qiagen) and reverse transcribed with the RevertAid kit (Ambion). TaqMan Gene Expression Assays were purchased from Applied Biosystems. The following assays were used: HPRT1, C3, PGR, CTSD, TFF1, and TGFα. PCRs were run in duplicate to check for reproducibility, and the Ct values were averaged. PCR was conducted in an Applied Biosystems 7900HT Real-time PCR System. The 20 μL reaction mix consisted of 1× Taqman Universal PCR master mix, 1× gene expression assay, and 10 ng cDNA template. PCR conditions were 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For data analysis, the comparative Ct method was used, with HPRT1 serving as housekeeping gene.

**Transient Transfection and Luciferase Assays**

Before transient transfection, T47D:A18/neo and T47D:A18/PKCα cells were maintained for 3 days in phenol red-free, E₂-depleted RPMI 1640 supplemented with G418. After stripping, cells were transiently transfected by electroporation as described previously (37). Briefly, 8 × 10⁶ cells were harvested and resuspended in 0.5 mL serum-free, phenol red-free RPMI 1640. ERE-tk-Luc plasmid containing the luciferase reporter gene controlled by the ERE (ref. 81; 5 μg) and β-galactosidase (β-gal; 1 μg) expressing plasmid pCMVβ were added to the cell suspension and incubated for 5 min at room temperature. The cells were pulsed at 250 V at 950 μF, resuspended in whole culture medium, and incubated at 37°C in a humidified CO₂ incubator overnight. On the following day, medium containing E₂ (10⁻⁹ mol/L), E₂-BSA conjugate (10⁻⁹ mol/L), Sigma-Aldrich), or vehicle (ethanol) was added.

Luciferase activities were measured using Luciferase Reporter Gene Assay System from Applied Biosystems. β-gal signals were measured with Galac-to-Light Plus assay systems (Applied Biosystems). After 18 to 20 h incubation, cells were washed with ice-cold PBS and lysed in the lysis buffer provided. The cell lysates were cleared by centrifugation and luciferase activity and β-gal signals were determined after adding corresponding substrates and read by a Monolight.
2010 luminometer (Analytical Luminescence Laboratory). β-gal signals were applied to normalize the luciferase activity.

Statistical Analysis

Differences in mean tumor area between groups of three or more were measured using ANOVA test followed by Bonferroni Multiple Comparisons Test. Differences in three or more groups were measured using ANOVA followed by Tukey-Kramer Multiple Comparisons Post-test. Unpaired Student’s t test was used to determine statistical significance between two groups and was used to analyze data obtained from the colony formation assays, TUNEL stain, and Western blots. The GraphPad InStat version 3.06 statistical software package was used (GraphPad Software) and SPSS version 13 (SPSS). All statistical tests were two-sided.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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Extracellular Matrix Requires the Estrogen Receptor and Interaction with the Extracellular Matrix

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