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Extranuclear ER α is associated with regression of T47D PKC α -overexpressing, tamoxifen-resistant breast cancer

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Abstract

Background: Prior to the introduction of tamoxifen, high dose estradiol was used to treat breast cancer patients with similar efficacy as tamoxifen, albeit with some undesirable side effects. There is renewed interest to utilize estradiol to treat endocrine resistant breast cancers, especially since findings from several preclinical models and clinical trials indicate that estradiol may be a rational second-line therapy in patients exhibiting resistance to tamoxifen and/or aromatase inhibitors. We and others reported that breast cancer patients bearing protein kinase C α (PKC α)-expressing tumors exhibit endocrine resistance and tumor aggressiveness. Our T47D:A18/PKC α preclinical model is tamoxifen-resistant, hormone-independent, yet is inhibited by 17 β -estradiol (E2) *in vivo*. We previously reported that E2-induced T47D:A18/PKC α tumor regression requires extranuclear ER α and interaction with the extracellular matrix.

Methods: T47D:A18/PKC α cells were grown *in vitro* using two-dimensional (2D) cell culture, three-dimensional (3D) Matrigel and *in vivo* by establishing xenografts in athymic mice. Immunofluorescence confocal microscopy and co-localization were applied to determine estrogen receptor α (ER α) subcellular localization. Co-immunoprecipitation and western blot were used to examine interaction of ER α with caveolin-1.

Results: We report that although T47D:A18/PKC α cells are cross-resistant to raloxifene in cell culture and in Matrigel, raloxifene induces regression of tamoxifen-resistant tumors. ER α rapidly translocates to extranuclear sites during T47D:A18/PKC α tumor regression in response to both raloxifene and E2, whereas ER α is primarily localized in the nucleus in proliferating tumors. E2 treatment induced complete tumor regression whereas cessation of raloxifene treatment resulted in tumor regrowth accompanied by re-localization of ER α to the nucleus. T47D:A18/neo tumors that do not overexpress PKC α maintain ER α in the nucleus during tamoxifen-mediated regression. An association between ER α and caveolin-1 increases in tumors regressing in response to E2.

Conclusions: Extranuclear ER α plays a role in the regression of PKC α -overexpressing tamoxifen-resistant tumors. These studies underline the unique role of extranuclear ER α in E2- and raloxifene-induced tumor regression that may have implications for treatment of endocrine-resistant PKC α -expressing tumors encountered in the clinic.

Keywords: Breast cancer, PKC α , Extranuclear ER α , Tamoxifen, Raloxifene

Introduction

Patients with estrogen receptor α (ER α)-positive breast cancer are candidates for treatment with endocrine therapies such as the selective estrogen receptor modulator (SERM) tamoxifen (TAM), aromatase inhibitors (AIs) letrozole, anastrozole, or exemestane or the selective

estrogen receptor downregulator (SERD), fulvestrant. However, both *de novo* and acquired endocrine resistance represent a significant clinical problem. Mechanisms of endocrine resistance include activation of growth factor signaling and downstream pathway activation including phosphatidylinositol 3-kinase (PI3K) and mitogen activated protein kinase (MAPK) (reviewed in [1]). Numerous reports from our laboratory and others suggest that activation of protein kinase C (PKC) signaling, specifically PKC α , is associated with endocrine resistance in the clinic [2-4].

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We developed and previously described a preclinical TAM-resistant model where PKC α is stably overexpressed in the T47D:A18 breast cancer cell line [5]. Under two-dimensional (2D) culture conditions, T47D:A18/PKC α cells exhibit both TAM-resistance and hormone-independence characterized by proliferation in the presence and absence of 17 β -estradiol (E2). Paradoxically when T47D:A18/PKC α cells are grown *in vivo* as xenograft tumors, E2 administration inhibits tumor growth and induces complete tumor regression in established tumors [6,7]. Similarly, we previously reported that the MCF-7 TAM tumor model that exhibits the E2-inhibitory phenotype [8] also overexpresses PKC α [7]. Previous mechanistic studies in our laboratory determined that E2-induced T47D:A18/PKC α tumor regression is dependent upon ER α , increased Fas/FasL-mediated apoptosis and decreased AKT signaling [9]. Moreover, we showed that T47D:A18/PKC α cultured in three-dimensional (3D) MatrigelTM partially recapitulated the *in vivo* E2-inhibitory effects by inhibiting colony formation. Further, the membrane impermeable E2-BSA conjugate was shown to inhibit T47D:A18/PKC α colony formation in a manner similar to E2, suggesting the potential involvement of a plasma membrane localized ER α [9].

In addition to genomic signaling by nuclear ER α , examples of nongenomic rapid responses of extranuclear ER α in the presence of E2 are abundant in the literature [10-14]. Extranuclear ER α plays an important role in cell proliferation, cell cycle regulation and blockade of cell death by activating MAPK [15,16] and the AKT signaling pathways [17-19] in breast cancer cell lines. There is evidence that extranuclear ER α interacts with several growth factor receptors as a mechanism for endocrine-resistant breast cancer by promoting downstream proliferation and survival signals [20-22].

In the present study we determined that in 2D and 3D cell culture, TAM-resistant T47D:A18/PKC α cells exhibit cross-resistance to raloxifene (RAL). Similar to the paradoxical effects of E2 in this model, RAL induces T47D:A18/PKC α tumor regression. Based on our previous findings showing the dependence of ER α in tumor regression and the involvement of extranuclear ER α in colony inhibition, in this study we determined the subcellular localization of ER α in T47D:A18/PKC α tumors during regression (E2 and RAL) and during proliferation (absence or presence of TAM) using immunofluorescence (IF) confocal microscopy. Interestingly, ER α localizes to the nucleus in tumors proliferating in a hormone-independent manner or in mice treated with TAM, whereas ER α localizes to extranuclear sites in tumors undergoing regression with either E2 or RAL. Withdrawal of RAL treatment results in the resumption of T47D:A18/PKC α tumor growth accompanied by relocalization of ER α back into the nucleus. We further report an association of extranuclear ER α with caveolin-1 suggesting a mechanism whereby

ER α may influence growth factor signaling. These findings are in agreement with our previous report that E2-induced tumor regression is accompanied by downregulation of AKT signaling in this model [9]. To our knowledge this is the first study to report an association of extranuclear ER α with tumor regression, as opposed to the activation of growth factor receptor signaling. With the renewed interest in the use of E2 for treatment of endocrine resistant breast cancer [23,24], our model offers a potential inhibitory mechanism involving extranuclear ER α .

Results

RAL exerts opposite proliferative effects on T47D:A18/PKC α *in vitro* and *in vivo*

We previously reported that overexpression of PKC α in T47D:A18 cells (T47D:A18/PKC α) results in TAM-resistant and hormone-independent cell growth in 2D culture. When xenografts are established from these cells, tumors are growth-inhibited and completely regress in the presence of E2 [7]. To determine whether these cells also exhibit cross-resistance to RAL, a DNA assay in 2D culture was performed. Whereas the parental T47D:A18/neo cells are E2-dependent and growth inhibited by both 4-hydroxytamoxifen (4-OHT) and RAL (Figure 1A), the TAM-resistant T47D:A18/PKC α cells exhibit cross-resistance to RAL (Figure 1B). When cultured in 3D MatrigelTM, T47D:A18/PKC α colony formation is inhibited by E2 as previously reported [9] but grew in the presence of both 4-OHT and RAL (Figure 1C,D). Therefore T47D:A18/PKC α cells display similar cross-resistance to 4-OHT and RAL in 2D and 3D culture.

To examine whether T47D:A18/PKC α cells are similarly resistant to RAL *in vivo*, we bilaterally injected T47D:A18/PKC α cells into the mammary fat pads of ovariectomized athymic mice and began treatment with TAM (1.5 mg/day), low dose RAL (0.5 mg/day) or high dose RAL (1.5 mg/day) (Figure 2A). As expected, T47D:A18/PKC α tumors are TAM-resistant as previously described [7] compared to the TAM and RAL-sensitive T47D:A18/neo tumors (Figure 2C). However, mice receiving the lower dose of RAL (0.5 mg/day), experienced tumor growth until week 5, followed by tumor stabilization and partial regression. Mice receiving the higher dose of RAL (1.5 mg/day) exhibited minimal tumor growth and achieved tumor stabilization by week 3 followed by tumor regression after 10 weeks of treatment (Figure 2A). These results indicate that (1) RAL is capable of inhibiting the growth of T47D:A18/PKC α TAM-resistant tumors and (2) RAL exerts contradictory *in vitro* and *in vivo* growth effects on T47D:A18/PKC α cells in a manner similar to E2. The distinction between E2 and RAL activity is that E2 but not RAL inhibits colony formation in 3D culture (Figure 1C, D) [9].

To more closely parallel the clinical situation where TAM is given to patients for 5 years, we created the

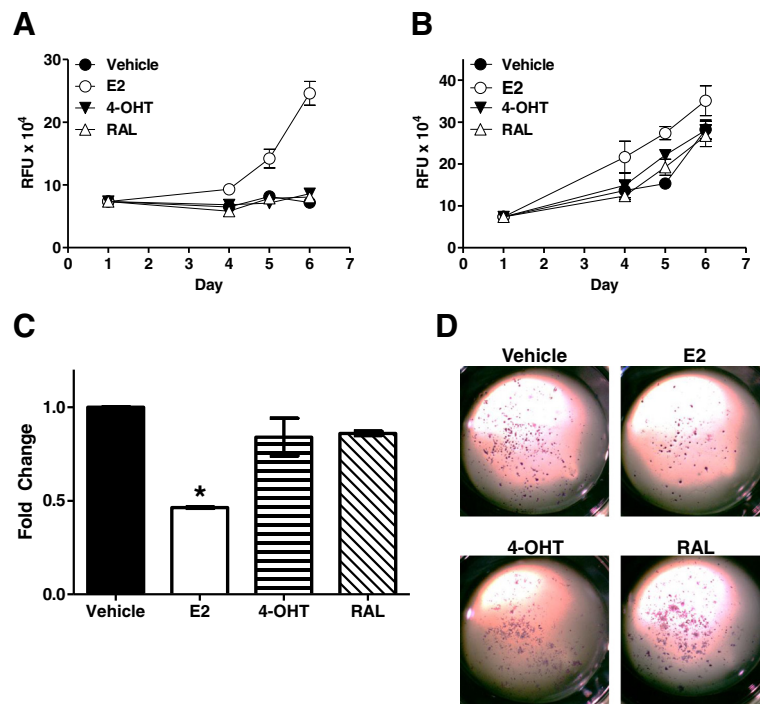


Figure 1 T47D:A18/PKCα cells are resistant to 4-OHT and RAL in 2D and 3D cell culture. DNA and Matrigel™ colony formation assays were performed as described in materials and methods. Cells were grown in the presence of vehicle (DMSO, 0.1%), E2 (10^{-9} M), 4-OHT (10^{-7} M) or RAL (10^{-7} M) with media changes every three days. **A.** T47D:A18/neo cells. **B.** T47D:A18/PKCα cells. RFU, relative fluorescence units. **C.** Quantification of T47D:A18/PKCα colonies. Graphs are representative of at least three independent experiments and error bars represent SEM. * $P < 0.05$ compared to vehicle; by one-way ANOVA followed by Bonferroni's post-test. **D.** Photographic representation of T47D:A18/PKCα colonies. Total magnification: 6X.

long-term TAM (LT-TAM) tumor model by serially passing T47D:A18/PKCα tumors in mice treated with 1.5 mg TAM 5 days/week for 5 years. We then asked whether RAL was capable of causing tumor regression in this LT-TAM tumor model. LT-TAM tumors were established and groups were treated with either 1.5 mg TAM or 1.5 mg RAL per day. During the first 7 weeks of treatment, both the TAM and RAL groups exhibited similar tumor growth. However between weeks 8–10, tumors in the RAL treated group began to regress (Figure 2B). These results suggest that RAL is a potential lead compound as an alternative to E2 for second-line treatment following tumor progression on TAM in those tumors that overexpress PKCα.

E2 and RAL induce ERα translocation from the nucleus to extranuclear sites *in vivo*

We previously reported that ERα and the extracellular matrix (ECM) are required for T47D:A18/PKCα tumor regression and that plasma membrane-associated ERα is likely to mediate the inhibitory effects of E2 [9]. To test our hypothesis that extranuclear ERα participates in E2-induced T47D:A18/PKCα tumor regression, we asked whether ERα localization differs in E2 and RAL-induced T47D:A18/PKCα regressing tumors compared with TAM-stimulated T47D:A18/PKCα tumors or E2-

stimulated T47D:A18/neo tumors. To address this question, we established T47D:A18/neo and T47D:A18/PKCα tumors in athymic mice (Figures 3A–3D) and as previously reported, T47D:A18/neo tumors are stimulated by E2 (Figure 3A) and are TAM and RAL-sensitive (Figure 2C), whereas T47D:A18/PKCα tumors are TAM-resistant and hormone-independent (Figure 3B) and regress following E2 treatment (Figures 3C and 3D) [7]. As we report here for the first time, RAL induces T47D:A18/PKCα tumor regression, although the degree of regression with RAL is not as complete as is seen with E2 (Figure 3C). Upon withdrawal of RAL, we observed re-growth of T47D:A18/PKCα tumors. In contrast, no resumption of tumor growth is seen upon discontinuation of E2 treatment for up to 31 weeks (Figure 3D). Since the E2 capsules maintain constant serum E2 levels for only 8–10 weeks, we are confident that the E2 capsule is depleted by week 20 and have confirmed no detectable serum E2 by mass spectrometry at 31 weeks (data not shown).

IF confocal microscopy of T47D:A18/neo E2-stimulated tumors and TAM- and RAL-regressing tumors illustrates that ERα is mainly localized in the nucleus (Figure 4A). The T47D:A18/neo no treatment (NT) group is not available for comparison since T47D:A18/neo cells required E2 for tumor growth. Similarly, ERα is located within the

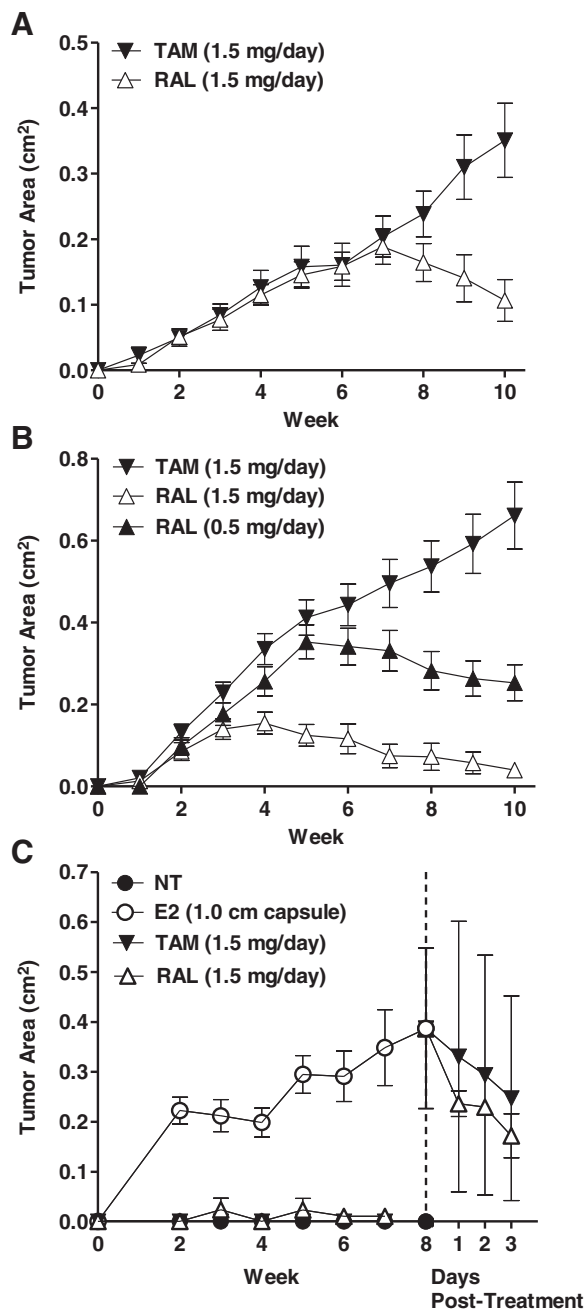


Figure 2 RAL inhibits TAM-resistant T47D:A18/PKC α xenograft tumors. Xenograft tumors were formed as described in materials and methods. Treatments for TAM and RAL were given by oral gavage 5 days/week. **A.** T47D:A18/PKC α tumors. Mice (10/group) were given TAM (1.5 mg/day) or RAL (0.5 mg/day or 1.5 mg/day). **B.** LT-TAM-treated T47D:A18/PKC α tumors. Mice (10/group) received TAM (1.5 mg/day) or RAL (1.5 mg/day). **C.** T47D:A18/neo tumors. Mice (6/group) were given no treatment, E2 capsule (1.0 cm), TAM (1.5 mg/day) or RAL (1.5 mg/day). The dotted line indicates initiation of TAM or RAL treatment following 8 weeks of E2 treatment. Error bars represent SEM.

nucleus in T47D:A18/PKC α NT and TAM treatment groups. However, ER α is almost completely localized to extranuclear sites in E2- and RAL-induced regressing T47D:A18/PKC α tumors. Interestingly, following withdrawal of RAL (RAL W/D) tumors resume growth and ER α re-localizes to the nucleus. Semi-quantitative analysis of ER α signals from tumor sections showed a significant re-localization from the nucleus to the cytoplasm in E2- and RAL-treated T47D:A18/PKC α tumors compared to NT, TAM or RAL W/D (Figure 4B). ER α translocation to extranuclear sites by E2 was verified with the 1D5 ER α antibody directed towards a different epitope of ER α (Additional file 1). ER α protein levels from each tumor group were also assessed by western blot (Figure 4C). As previously reported, ER α protein expression is elevated in T47D:A18/PKC α tumors even though ER function as determined by ERE-luciferase activity is decreased [5]. The abundance of ER α protein as assessed by western blot is in agreement with the IF image ER α signal intensity (Figures 4A,C). The observed downregulation of ER α protein by E2 and ER α stabilization by antiestrogens is considered classic ER α regulation as previously established [25-28].

Therefore TAM and RAL which oppositely regulate T47D:A18/PKC α tumor growth, induces differential ER α subcellular localization. Furthermore, T47D:A18/PKC α tumor regression induced by either E2 or RAL is associated with extranuclear ER α . The finding that ER α is localized to the nucleus during RAL and TAM-induced T47D:A18/neo tumor regression suggests that it is not simply regression that triggers ER α to exit from the nucleus, but localization may be influenced by PKC α overexpression.

Association of ER α with caveolin-1

ER α does not have a membrane localization sequence thus it does not behave like a transmembrane receptor [29]. Membrane ER α normally exists as a cytoplasmic pool and can be tied to the inner face of the plasma membrane bilayer through binding to the lipid raft protein caveolin-1 [30,31]. To determine whether there is a direct physical interaction between ER α and caveolin-1, we prepared total protein extract from tumors and performed co-immunoprecipitation (co-IP) using an ER α antibody followed by western blot analysis (Figure 5A). As expected, the level of total ER α was lower in tumors from the E2 treatment group. However, immunodetection with a caveolin-1 antibody showed a significant increase in complex formation between ER α and caveolin-1 in T47D:A18/PKC α tumors from the E2 treatment group compared with the T47D:A18/PKC α NT group and the T47D:A18/neo E2 group (Figure 5B). These results indicate that the abundance of the ER α /caveolin-1 complex is increased in response to E2, but not from treatment with TAM or RAL. We conclude that ER α /caveolin-1 complex

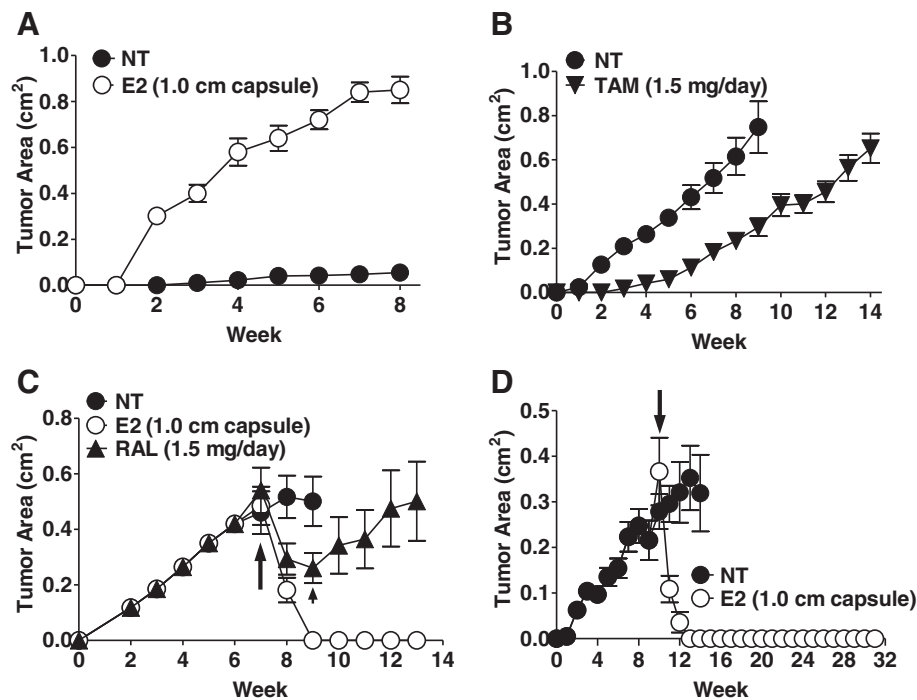


Figure 3 Growth of T47D:A18/neo and T47D:A18/PKCα xenograft tumors. Xenograft tumors were formed as described in materials and methods. **A.** T47D:A18/neo tumors (NT, 15 mice/group and E2, 3 mice/group). **B.** T47D:A18/PKCα tumors (10 mice/group). **C.** T47D:A18/PKCα tumors. Tumors were grown to an average size of 0.5 cm². Mice were then randomized into NT, RAL or E2 groups (large arrow, 9 mice/group). Two weeks later RAL treatment was stopped (small arrow). **D.** T47D:A18/PKCα tumors (5 mice/group). Tumors were grown to an average size of 0.3 cm². Mice were then randomized into NT or E2 groups (arrow).

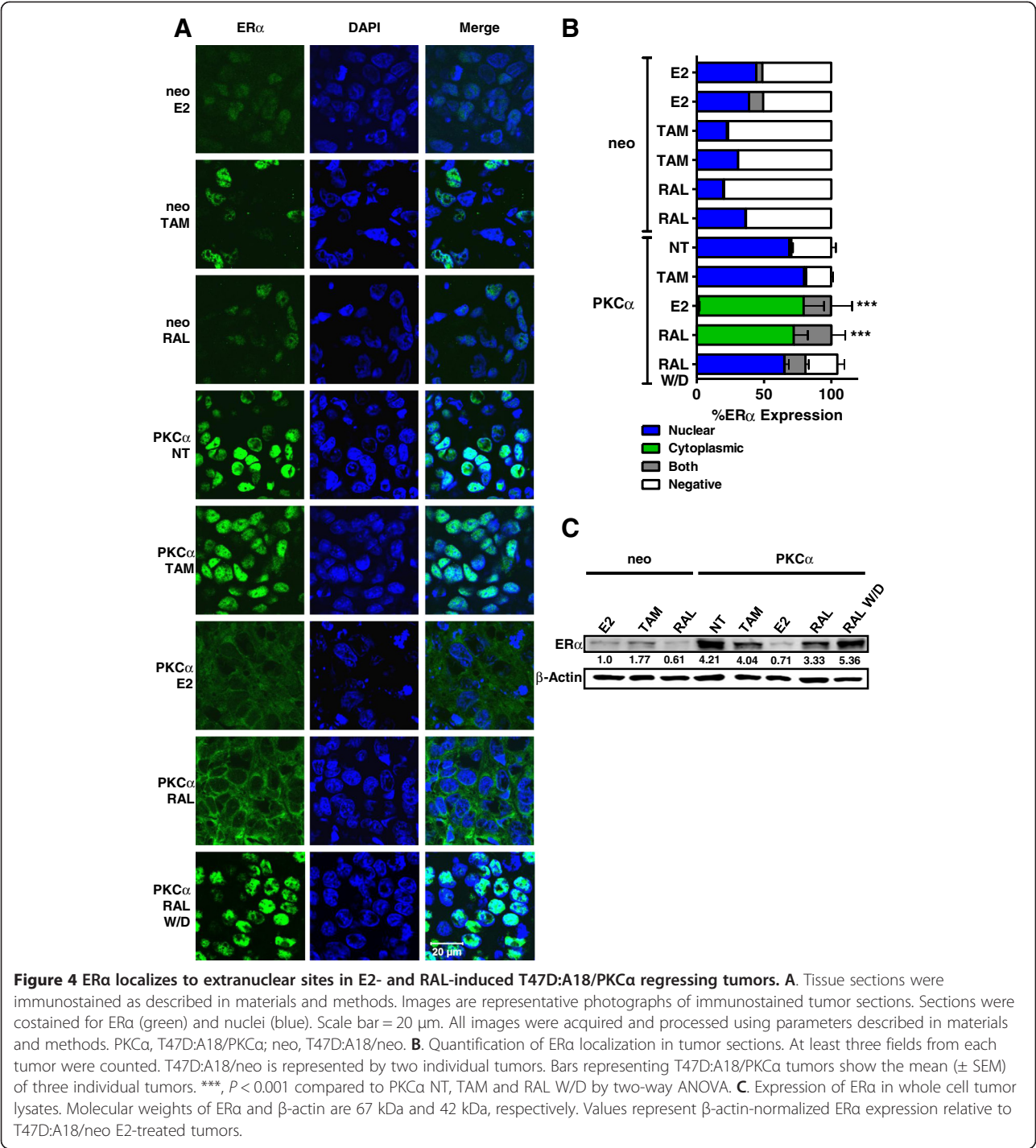
formation correlates with durable tumor regression produced with E2, but not with transient tumor regression as observed with RAL, nor with proliferating T47D:A18/PKCα tumors (NT, TAM, RAL W/D). This result is consistent with the hypothesis that E2-induced tumor regression is accompanied by ERα exit from the nucleus and association at the plasma membrane, perhaps via caveolin-1.

ERα localization in the 2D and 3D microenvironment

As previously described [9], the ECM is required for the growth inhibitory effect of E2 on T47D:A18/PKCα cells; E2 stimulates T47D:A18/PKCα cells proliferation on 2D cell culture, yet E2 inhibits colony formation in 3D MatrigelTM. However we report here that T47D:A18/PKCα cells are resistant to RAL both on 2D and 3D (Figures 1B, C), yet RAL inhibits tumor growth (Figure 2). Therefore we wanted to determine whether extranuclear ERα correlates with inhibition of growth (on 2D and 3D) and/or colony regression. Inhibition of colony formation by E2 in 3D culture is analogous to the *in vivo* phenotype whereby E2 prevents tumor establishment [7]. However, unlike the *in vivo* phenotype, E2 is incapable of initiating regression of an established T47D:A18/PKCα colony in MatrigelTM. To determine whether extranuclear ERα is a

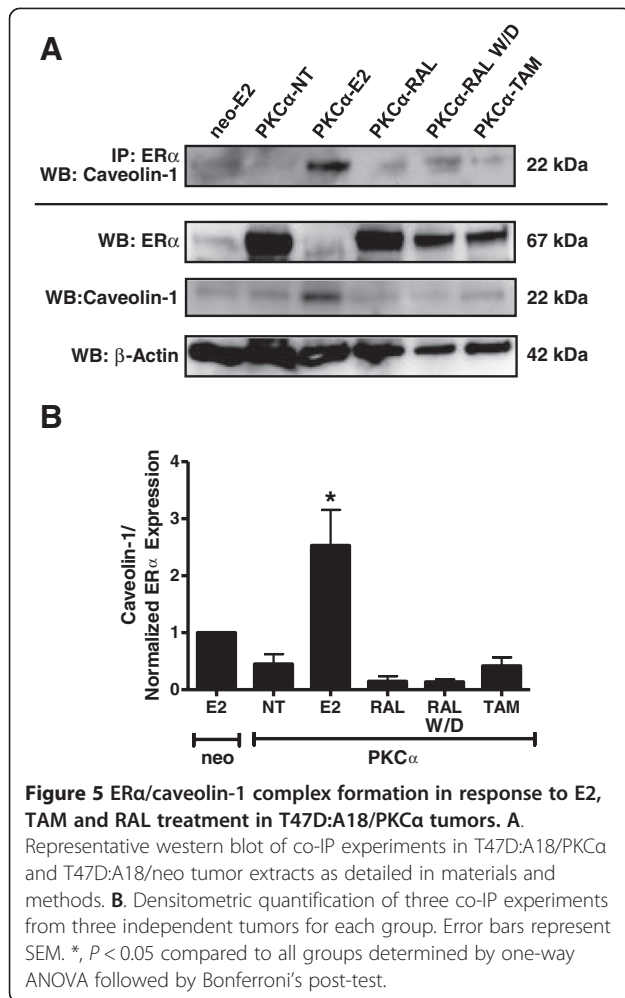
response to E2 and RAL treatment in 3D culture or whether ERα translocation occurs only during regression in tumors, we compared ERα subcellular localization in T47D:A18/neo and T47D:A18/PKCα cells grown in 2D and 3D culture. In 2D culture ERα is both nuclear and cytoplasmic in T47D:A18/neo cells, whereas ERα is mainly nuclear in T47D:A18/PKCα cells following 1 h exposure to E2, 4-OHT or RAL (Additional file 2). These results indicate that ERα localization does not change in T47D:A18/neo and T47D:A18/PKCα following 1 h treatment in 2D culture.

To address ERα localization in 3D culture, T47D:A18/neo and T47D:A18/PKCα cells were plated in MatrigelTM under two treatment paradigms. The first paradigm is known to inhibit colony formation in the presence of E2 where cells are plated (as shown in Figure 1C, D) and given continuous treatment for 6 days with media changes every third day. Under these conditions, T47D:A18/neo cells in colonies showed nuclear ERα expression in the E2 treatment group and no expression in vehicle control, 4-OHT or RAL groups and T47D:A18/PKCα colonies had cells with nuclear ERα expression in all groups (Additional file 3). These results indicate that ERα subcellular localization does not change as a result of continuous treatments in 3D culture (Additional file 3).



The second paradigm was designed to mimic tumor regression. Colonies were allowed to establish for 10 days when treatments were initiated and continued for either 24 h or 10 days with E2, 4-OHT or RAL. In contrast to E2-induced tumor regression seen *in vivo*, treating colonies does not cause a decrease in colony number or size (data not shown). Following 24 h treatment of established T47D:A18/neo colonies, there was no ER α expression in

the vehicle and E2 treatment groups and sparse staining in the 4-OHT and RAL groups (Additional file 4). Examination of T47D:A18/PKC α colonies under the same conditions, shows strong ER α nuclear staining in the vehicle, 4-OHT and RAL treated groups. However, in the 24 h E2 treatment group, some colonies showed nuclear staining while other colonies showed membrane and/or cytoplasmic staining (Additional file 4). To determine if treating



established colonies for a longer period would lead to the complete translocation of ERα from the nucleus to the cytoplasm, we extended treatment for 10 days with media changes every three days before IF staining. Under these conditions, ERα is localized to the nucleus in all groups of T47D:A18/neo colonies as well as T47D:A18/PKCa vehicle control, 4-OHT and RAL groups (Figure 6). However, ERα is completely extranuclear in all cells growing in response to E2. Taken together these findings suggest that ERα localization does not correlate with proliferative response in 2D cell culture nor with inhibition of colony formation in 3D Matrigel™. However, under conditions that mimic tumor regression, T47D:A18/PKCa colonies exhibit complete ERα translocation out of the nucleus in response to E2 after 10 days and this effect is seen as early as 24 h. While E2 administration to established colonies in Matrigel™ induces ERα translocation to extranuclear sites, ERα translocation alone is not sufficient to induce regression likely due to the requirement of additional factors found in the tumor microenvironment, but not in

Matrigel™. We also find E2 and RAL exert opposite effects on ERα localization in T47D:A18/PKCa cells plated in 3D Matrigel™, but similar localization *in vivo*.

Discussion

In this paper we have shown by IF confocal microscopy that ERα translocates from the nucleus to the extranuclear space upon E2 and RAL-induced tumor regression in our T47D:A18/PKCa preclinical TAM-resistant model. This model is clinically relevant as evidenced by the reported success of E2 in the clinic [23,24]. We initially associated PKCa expression with TAM resistance [2], and others further identified PKCa as a marker of endocrine resistance and breast cancer aggressiveness [3,4]. Extranuclear ERα was previously reported to play a role in endocrine-resistant breast cancers specifically by interacting with growth factor receptors to activate proliferative and pro-survival signals [20-22]. However we demonstrate here that ERα translocation is associated with tumor regression only in PKCa overexpressing tumors in response to E2 and RAL. Our findings imply that a specific subset of endocrine-resistant breast cancers that express PKCa may be uniquely susceptible to E2 therapy. Although the literature is conflicting regarding the level of PKCa expression in breast cancers compared to the normal breast [32-36], variability in PKCa expression amongst breast cancers and the link to endocrine resistance and tumor aggressiveness is clear. Based on three reports in the literature, the prevalence of PKCa expression in all breast cancers ranges between 28% to as high as 70% [3,4,37]. Even if the lowest estimate of 28% prevalence is the most accurate, this still represents a significant number of patients that may benefit from E2 treatment.

There are numerous reports of nongenomic signaling by estrogen in breast cancer cell lines [38,39] and there is evidence that this pathway is upregulated in endocrine resistant breast cancers. Translocation of nuclear ERα to extranuclear sites is reported to be involved in cytoskeletal remodeling, migration and invasion [40] and recently shown to play an important role in breast cancer cell motility and metastasis [41]. High expression of the MTA1 protein is reported to sequester ERα in the cytoplasm and activate MAPK signaling [42], and the same group reported that overexpression of Her-2 causes ERα nuclear to cytoplasmic translocation [43]. Fan *et al.* [44] showed that long term exposure to TAM causes translocation of ERα from the nucleus to the cytoplasm and enhances the interaction between ERα and EGFR. All of these examples in the literature describe the activation of signaling pathways by extranuclear ERα leading to cancer cell proliferation and survival. However in our study, we present a novel finding that translocation of ERα from the nucleus to extranuclear sites occurs following E2- and RAL-induced T47D:A18/PKCa tumor regression.

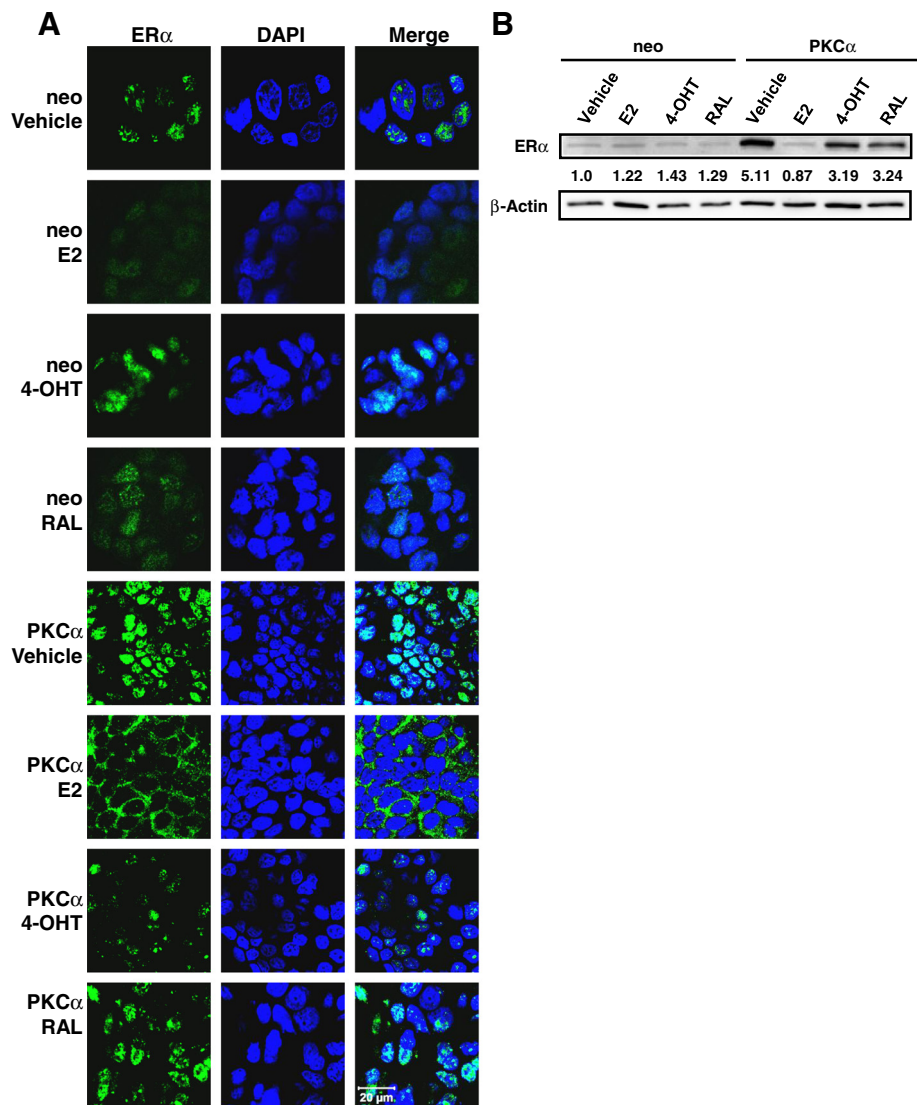


Figure 6 E2 induces complete relocation of ER α in established T47D:A18/PKC α colonies after 10 days. **A.** T47D:A18/neo colonies (neo) and T47D:A18/PKC α colonies (PKC α) colonies were immunostained for ER α (green) and nuclei (blue). All images were acquired and processed using parameters described in materials and methods. Colonies were grown for 10 days then treated for 10 days with vehicle (EtOH, 0.1%), E2 (10^{-9} M), 4-OHT (10^{-7} M) or RAL (10^{-7} M). Scale bar = 20 μ m. **B.** Expression of ER α in whole cell colony lysates. Molecular weights of ER α and β -actin are 67 kDa and 42 kDa, respectively. Values represent β -actin-normalized ER α expression relative to T47D:A18/neo E2-treated colonies.

We previously reported that E2-induced regression is accompanied by apoptosis mediated in part by Fas/FasL and downregulation of the AKT pathway [9]. An additional novel finding is that TAM and RAL elicit opposite growth effects in our T47D:A18/PKC α tumor model. We hypothesize that PKC α , a cytoplasmic protein that translocates to the plasma membrane when activated [45], may physically interact with other growth factor receptors and signaling pathways [46]. A recent publication by Gutierrez *et al.* shows that translocation of ER α to the plasma membrane in response to E2 results in activation of PKC α /ERK 1/2 signaling in anterior pituitary cells, yet PKC α is not responsible for mediating the physical translocation of ER α

to the plasma membrane [47]. Src kinase is one of the important molecules of the signalosome complex which plays a critical role in E2-mediated nongenomic signaling [48]. It has been reported in the literature that Her-2 upregulates and activates PKC α through src kinase in Her-2 mediated cancer cell invasion [49]. Longo *et al.* has shown that a PKC α -src kinase-ER α interaction is critical in the modulation of estrogen responsiveness and the differentiation process in osteoblasts [50]. However, we were unable to detect a physical interaction between PKC α and ER α , Her2 or src in our tumor model.

We detected a physical interaction between ER α and caveolin-1 by co-IP (Figures 5A-B). These results suggest

that caveolin-1 may be responsible for transporting ER α to the plasma membrane during E2-induced tumor regression. Palmitoylation of ER α is known to be necessary for the physical association with caveolin-1 and in particular palmitoylation of the E domain of ER α at C447 along with nine flanking amino acids are required for association with caveolin-1 [30,31,51,52]. The ER α -caveolin-1 complex in turn facilitates the translocation of the caveolae rafts to the plasma membrane. Caveolin-1 serves as a scaffold protein at the membrane in the recruitment of signaling molecules to form a signalosome complex that can include ER α . Taken together these results suggest that perhaps PKC α is capable of modifying the interaction of ER α and caveolin-1, potentially at the membrane via the proposed signalosome to effect tumor regression. It is interesting to note that ER α /caveolin-1 complex formation correlates with durable tumor regression produced with E2, but not with transient tumor regression as observed with RAL, nor with proliferating T47D:A18/PKC α tumors (NT, TAM, RAL W/D). Although ER α translocation to extranuclear sites does occur in MatrigelTM in response to E2 (Figure 6), colony regression is not initiated perhaps because a component in the tumor microenvironment is also required to initiate the regression signal. As shown in Figures 3C-D, E2-induced tumor regression occurs rapidly and tumors are gone within 2–3 weeks. MatrigelTM results reveal that the translocation of ER α may be an early event as ER α was seen in the membrane and cytoplasm in some colonies at 24 h further illustrating a rapid response to E2 treatment. Our results regarding ER α translocation in the MatrigelTM environment compared with *in vivo* tumors highlight the importance of the ECM in triggering tumor regression.

Since we and others have reported that PKC α expression can be a predictive marker of TAM resistance [2-4] our T47D:A18/PKC α model suggests that detection of extranuclear ER α can be used to monitor therapeutic response in TAM-resistant, PKC α -expressing breast cancers. Unfortunately, extranuclear ER α is not currently measured clinically and although pathologists may observe such staining, it is not reported. A recent report by Welsh *et al.* [53] with the purpose of testing a panel of ER α -specific antibodies to detect non-nuclear ER α in clinical specimens found the average incidence to be only 1.5%. In an accompanying commentary, Levin points out that while it is possible that the number of breast tumors that express extranuclear ER α may indeed be small, it is also possible that more sensitive techniques are required to detect the very small ER α pools located outside of the nucleus [54]. We offer the possibility that extranuclear ER α may be detected more frequently in PKC α -expressing tumors that are regressing possibly indicating a response to treatment. It remains to be seen whether other techniques will be developed

that may improve the detection of extranuclear ER α in clinical specimens.

We have previously suggested that PKC α may be used as predictive biomarker for the use of E2 or an E2-like compound to effect tumor regression [9], and in fact the utility of using E2 was demonstrated [23]. We report here that not only E2, but RAL is capable of eliciting T47D:A18/PKC α tumor regression, despite the fact that these tumors are TAM-resistant. Further we have shown that following 5 years of TAM treatment, these tumors are still sensitive to RAL-induced tumor regression (Figure 2B). Although RAL may be considered as a potential treatment for patients with PKC α -expressing breast cancers, RAL is not as durable as E2 to elicit complete tumor regression (Figure 3D). Since RAL has poor bioavailability, we are currently testing a series of benzothiophene analogues in our T47D:A18/PKC α preclinical model for improved tumor inhibitory activity.

Conclusions

In summary, we report for the first time the involvement of extranuclear ER α in an endocrine resistant-tumor model to be associated with tumor regression and not growth stimulation. Key to this phenomenon may be expression of PKC α , frequently associated with endocrine resistance and a potential biomarker for the use of E2 or RAL-like compounds for the treatment of endocrine-resistant breast cancer.

Methods

Reagents

For *in vitro* experiments dimethylsulfoxide (DMSO), ethanol, E2, 4-OHT and RAL were obtained from Sigma-Aldrich (St. Louis, MO USA). For *in vivo* experiments E2 and TAM were obtained from Sigma. RAL (Evista[®], Eli Lilly and Company, Indianapolis, IN USA) was purchased from the University of Illinois at Chicago Hospital Pharmacy. Cell culture reagents were obtained from Life Technologies (Carlsbad, CA USA). Tissue cultureware was purchased from Becton-Dickinson (Franklin Lakes, NJ USA). The following antibodies were used: rabbit monoclonal ER α (for tissue and cells, SP1, Lab Vision, Thermo Scientific, Kalamazoo, MI USA), mouse monoclonal ER α (alternative epitope to confirm specificity for tissue, 1D5, N-terminal epitope, Abcam, Cambridge, MA USA), rabbit polyclonal ER α (for colonies, HC20, Santa Cruz Biotechnology, Santa Cruz, CA USA), and mouse monoclonal caveolin-1 (Clone2234, BD Transduction Laboratories, Franklin Lakes, NJ USA). Secondary antibodies included: anti-rabbit Alexa Fluor 488 (Life Technologies, Carlsbad, CA USA), anti-mouse Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA USA) and HRP-cojugated anti-rabbit and anti-mouse (GE Healthcare UK Limited, Buckinghamshire, UK).

Cell culture conditions

T47D:A18/neo and T47D:A18/PKC α [5] cells were maintained in RPMI 1640 with phenol red supplemented with 10% fetal bovine serum (FBS) and G418 (500 μ g/ml) at 37°C, 5% CO₂. Prior to experiments cell lines were placed in phenol red-free RPMI 1640 supplemented with 10% stripped FBS (E2-depleted media) for 3 days and maintained in the same manner for the duration of experiments. Cell lines were tested for Mycoplasma contamination on a regular basis (MycoAlert™ Mycoplasma Detection Kit, Lonza Ltd., Rockland, ME, USA). Cell lines were not authenticated by the authors.

DNA growth assay

Cells were plated at a density of 15,000 cells/well in 24-well plates. Treatment media (vehicle, DMSO [0.1%], E2 [10⁻⁹M], 4-OHT [10⁻⁷M] or RAL [10⁻⁷M]) was added the following day (Day 1) and changed every three days. Growth was determined by incubating cells with Hoechst 33342 cell permeable dye (Life Technologies, Carlsbad, CA USA) for 1 h at 37°C and reading fluorescence at excitation 355 nm/emission 460 nm on a Perkin Elmer Victor³ V (Waltham, MA USA) plate reader.

Matrigel™ colony formation assay

Treatments (ethanol [0.1%], E2 [10⁻⁹M], 4-OHT [10⁻⁷M] or RAL [10⁻⁷M]) were added to liquefied phenol-red free Matrigel™ matrix (BD Biosciences, Franklin Lakes, NJ USA) and used to coat 6-well plates and solidified at 37°C for 30 min. Cells (5000) were seeded in E2-depleted media containing treatments on top of pre-gelled Matrigel™ and incubated at 37°C with 5% CO₂. Treatment media were changed every three days. Colonies were stained with 0.25% crystal violet (Sigma-Aldrich, St. Louis, MO USA) solution for 30 min and then destained with 0.9% saline for 20 min at room temperature. Colony number was determined by counting five 1.0 cm² areas.

Xenograft tumor establishment

All procedures involving animals were approved by the Animal Care and Use Committee of the University of Illinois at Chicago according to institutional and national guidelines. T47D:A18/neo and T47D:A18/PKC α tumors were established in 4–6 week old ovariectomized athymic nude mice (Harlan Laboratories) as previously described [7]. LT-TAM tumors were derived by *in vivo* serial transplantation in the presence of TAM for 5 years. Where indicated, mice were given the following treatments as previously described: E2 (1.0 cm silastic capsule, s.c.), TAM (1.5 mg/day, p.o.), RAL (0.5 mg/day, p.o.), or RAL (1.5 mg/day, p.o.) [55]. Tumor cross-sectional area was determined at least weekly and sometimes daily using digital calipers and calculated using the

formula: length/2 \times width/2 \times π . Mice were euthanized by CO₂ inhalation and cervical dislocation. Tumors were immediately excised and either fixed in 10% buffered formalin for paraffin block preparation or snap frozen in liquid nitrogen and stored at -80°C for co-immunoprecipitation and western blot analysis.

Tumor IF confocal microscopy and co-localization analysis

Tumors sections (4 μ m) were prepared from paraffin blocks for IF staining by deparaffinization and rehydration. Antigen retrieval was performed by incubating slides in Tris-EDTA (pH = 9.0) buffer at 90°C and allowed to cool at room temperature for 45 min. Slides were blocked with antibody diluent (DAKO, Carpinteria, CA USA) for 20 min followed by primary antibody at 1:100 in antibody diluent for 1 h at room temperature. Slides were incubated with fluorescence-conjugated secondary antibodies at 1:100 in antibody diluent for 45 min at room temperature followed by 4',6-diamidino-2-phenylindole (DAPI) (1 μ g/mL, DAKO, Carpinteria, CA USA) for 15 min and mounted with Vectashield mounting media (Vector Laboratories, Burlingame, CA USA). Confocal microscopy was performed with a Zeiss LSM 510 microscope (Carl Zeiss, Incorporated, North America, Thornwood, NY USA). The objective used was a C-Apochromat 63X with a numerical aperture of 1.2. Image acquisition scaling was X: 0.14 μ m and Y: 0.14 μ m and stack size was X: 142.86 and Y: 142.86, these two parameters were kept constant across samples. Pinholes and laser intensities were kept constant for each wavelength (green: λ = 488 nm, laser = 15%, pinhole = 228 μ m and blue: λ = 405 nm, laser = 5%, pinhole 194 μ m) across all samples. Images were modified following acquisition using the Zeiss LSM Image Browser by similarly enlarging images 2X and increasing the brightness and contrast by 10%.

Co-IP and western blot

Tumors were ground into a fine powder in liquid nitrogen and resuspended in cell lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton X-100, with protease [Sigma, St. Louis, MO] and phosphatase [Calbiochem, Bilerica, MA] inhibitor cocktails) and homogenized using a Polytron hand-held homogenizer (Fisher Scientific, Pittsburgh, PA USA). Protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA USA). Equal amounts of total tumor extract (500 μ g) were immunoprecipitated by rotating for 2 hr at 4°C with antibody followed by overnight rotation with protein-A Dynabeads (Life Technologies, Carlsbad, CA), at 4°C. Samples were washed and boiled for 10 min then eluted from beads with sample buffer containing 2-mercaptoethanol (Sigma, St. Louis, MO USA). Samples were subjected to 8% SDS-PAGE, followed by western blot with respective primary

and secondary antibodies. Proteins were detected by chemiluminescence using a Chemi Doc Gel Documentation System (Bio-Rad Laboratories, Hercules, CA USA).

Cell IF microscopy

Cells were seeded in phenol red-containing media onto Lab-Tek II 4-well chamber slides (Millipore, Billerica, MA) at a density of 3×10^4 cells/well. The following day cells were placed in E2-depleted media for 3 days then given treatment media (DMSO [0.1%], E2 [10^{-9} M], 4-OHT [10^{-7} M] or RAL [10^{-7} M]). For IF, cells were fixed in 100% methanol overnight at -20°C and stained as described above for tissue sections. Cells were imaged using Zeiss Axiovision Observer D1 microscope (Carl Zeiss, LLC, Thornwood, NY USA).

Colony IF microscopy

Colonies were formed by seeding cells in Matrigel™ as described above and treated with DMSO (0.1%), E2 (10^{-9} M), 4-OHT (10^{-7} M) or RAL (10^{-7} M). Colonies were extracted from the Matrigel™ by adding ice-cold PBS-EDTA to the rinsed and aspirated wells. Gel was lifted from the bottom of the well with a cell scraper and plates were shaken gently on ice. Colonies were then transferred to a conical tube and shaken on ice for an additional 30 min until Matrigel™ was completely dissolved, collected by centrifugation at 115g for 2 min and pipetted onto a slide. Slides were then fixed in ice cold methanol and stored at -80°C until staining (as described above). Confocal microscopy was performed with a Zeiss LSM 510 microscope. The objective used was a C-Apochromat 63X with a numerical aperture of 1.2. Image acquisition scaling was X: 0.14 μm and Y: 0.14 μm and stack size was X:142.86 and Y: 142.86, these two parameters were kept constant across samples. Pinholes and laser intensities were kept constant for each wavelength (green: $\lambda = 488$ nm, laser = 10%, pinhole = 200 μm and blue: $\lambda = 405$ nm, laser = 13%, pinhole 92 μm) across all samples. Images were modified following acquisition using the Zeiss LSM Image Browser by similarly enlarging images 2X and increasing the brightness and contrast by 10%.

Statistical analysis

The specific statistical test applied to the data is described in the figure legends. All of the statistics on the data were performed using GraphPad Prism 5.02 Software (La Jolla, CA USA).

Additional files

Additional file 1: ER α is localized to extranuclear sites in E2-regressing tumors with an antibody directed to an alternative epitope. Tissue sections were immunostained as described in materials and methods. Images are representative photographs of immunostained

tumor sections. Sections were costained for ER α (green) and nuclei (blue). Scale bar = 20 μm .

Additional file 2: ER α localization does not change in cells grown in 2D culture. T47D:A18/neo (neo) and T47D:A18/PKCa (PKCa) cells were immunostained for ER α (green) and nuclei (blue) as detailed in materials and methods. Cells were treated (Vehicle [EtOH 0.1%], E2 [10^{-9} M], 4-OHT [10^{-7} M] or RAL [10^{-7} M]) for 1 h. Scale bar = 50 μm .

Additional file 3: Continuous E2 treatment inhibits colony formation but does not induce extranuclear ER α in T47D:A18/PKCa cells. T47D:A18/neo colonies (neo) and T47D:A18/PKCa colonies (PKCa) colonies were immunostained for ER α (green) and nuclei (blue) as detailed in materials and methods. Colonies were given treatment upon plating with vehicle (EtOH, 0.1%), E2 (10^{-9} M), 4-OHT (10^{-7} M) or RAL (10^{-7} M) and were treated continuously for 6 days. Scale bar = 20 μm .

Additional file 4: E2 treatment in established T47D:A18/PKCa colonies induces partial extranuclear ER α following 24 h treatment. T47D:A18/neo colonies (neo) and T47D:A18/PKCa (PKCa) colonies were immunostained for ER α (green) and nuclei (blue) as detailed in materials and methods. Colonies were grown for 10 days then treated for 24 h with vehicle (EtOH, 0.1%), E2 (10^{-9} M), 4-OHT (10^{-7} M) or RAL (10^{-7} M). N: nuclear, M/C: membrane/cytoplasmic. Scale bar = 20 μm .

Abbreviations

4-OHT: 4-Hydroxytamoxifen; AI: Aromatase inhibitor; co-IP: Co-immunoprecipitation; DAPI: 4', 6-Diamidino-2-phenylindole; DMSO: Dimethylsulfoxide; E2: 17 β -Estradiol; ER α : Estrogen receptor alpha; ECM: Extracellular matrix; IF: Immunofluorescence; LT-TAM: Long-term TAM; MAPK: Mitogen activated protein kinase; PI3K: Phosphatidylinositol 3'kinase; PKCa: Protein kinase C alpha; RAL: Raloxifene; SERM: Selective estrogen receptor modulator; SERD: Selective estrogen receptor downregulator; TAM: Tamoxifen.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

BPW and MEM contributed equally to this study and contributed to writing portions of the manuscript. BPW made figures and designed layout. All authors contributed to xenograft experiments, HZ and YZ developed the LT-TAM tumor model, HZ performed IF staining and microscopy on cell lines, BPW and MEM performed IF and confocal microscopy on tumor sections and colonies. DAT conceived of the study and wrote the manuscript. All authors read and approved the final manuscript.

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