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Cytoplasmic ERα and NFκB Promote Cell Survival in Mouse Mammary Cancer Cell Lines

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Abstract

There is a desperate need in the field for mouse mammary tumors and cell lines that faithfully mimic estrogen receptor (ER) expression and activity found in human breast cancers. We found that several mouse mammary cancer cell lines express ER but fail to demonstrate classical estrogen-driven proliferation or transcriptional activity. We investigated whether these cell lines may be used to model tamoxifen resistance by using small molecule inhibitors to signaling pathways known to contribute to resistance. We found that the combination of NFκB inhibition and ER antagonists significantly reduced cell proliferation in vitro, as well as growth of syngeneic tumors. Surprisingly, we found that ER was localized to the cytoplasm, regardless of any type of treatment. Based on this, we probed extra-nuclear functions of ER and found that co-inhibition of ER and NFκB led to an increase in oxidative stress and apoptosis. Together, these findings suggest that cytoplasmic ER and NFκB may play redundant roles in protecting mammary cancer cells from oxidative stress and cell death. Although this study has not identified a mouse model with classical ER activity, cytoplasmic ER has been described in a small subset of human breast tumors, suggesting that these findings may be relevant for some breast cancer patients.

Keywords Estrogen receptor · Mouse · Mammary cancer · NFκB · Tamoxifen

Introduction

Breast cancer is the second most diagnosed cancer in females behind skin cancer and is expected to be responsible for more than 41,000 deaths in 2019. The majority of breast cancers, around 75%, express estrogen receptor alpha (ERα) and are diagnosed as estrogen receptor positive (ER+) [1]. The growth and survival of these tumors are stimulated by estrogen binding to ERα. Thus, treatments that disrupt or inhibit estrogen signaling, such as tamoxifen or aromatase inhibitors, are routinely used to treat women with ER+ breast tumors. Despite the success of these therapies, between 30 and 50% of ER+ breast cancers are expected to recur, frequently as more

aggressive, metastatic, and therapy-resistant disease [2]. Consequently, the majority of breast cancer deaths each year result from ER+ breast cancer. Clearly, new therapeutic targets and strategies are needed to combat recurrent ER+ disease.

Current approaches used to study hormone-dependent breast cancer are highly reliant on ER+ breast cancer cell lines grown in culture or as xenograft tumors in immunocompromised mice. Cell lines, such as MCF-7 and T47D, are the most widely used models to study ER+ breast cancer and have advanced our mechanistic understanding of estrogen and tamoxifen action tremendously. Cell line-derived xenografts are the most commonly used in vivo model to study breast cancer, due to their simplicity, low cost, and ease of cell line manipulation to study a protein/pathway of interest. However, these models fail to capture the complex tumor microenvironment and typical disease progression seen in human tumors. A more sophisticated adaptation of the xenograft model has been developed—mouse intraductal (MIND) models. Here, cells are injected into the ducts of immunocompromised mice rather than the fat pad, in order to mimic the normal epithelial environment. Brisken and colleagues showed that MCF-7 cells injected intraductally more closely resembled ER+ luminal disease characteristics than tumors injected into the fat pad

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and that tumors grew under physiological hormone levels without E2 supplementation [3].

Another approach to more closely model patient disease is patient-derived xenografts (PDX), where human tumor cells or pieces of tissue are engrafted into immunocompromised mice. This approach has the advantages of closely mimicking human breast cancer, inclusion of a stromal component, and retention of therapy response and histopathological features [4]. However, one major disadvantage is that the majority of PDX models are derived from more aggressive ER− phenotypes [5] so are often not useful for studying ER+ disease. One approach that can combine the advantages of cell lines and PDXs are patient-derived organoids (PDO). These have the versatility and amenability of cell lines, such as ability to be genetically manipulated and cultured indefinitely, with the clinical relevance PDX models, and show great promise for both research tools and modeling patient disease. Generation of ER+ PDOs has been more successful than with PDX. A 2017 study by Sachs et al. generated a biobank of organoids from patient breast tumors [6]. These organoids represented all molecular subtypes of breast cancer without bias and broadly matched the original tumor, suggesting minimal loss of oncogenic driver expression. ER+ organoids represented a large proportion of the organoids generated, showing much greater success rate than establishing ER+ PDX models. Importantly, when a response to tamoxifen was observed in patients, their organoids also responded. These models may prove extremely useful for dictating therapy and predicting response and may become as useful in the future as cell lines in a basic research setting.

Although cell lines, PDXs, and PDOs have their advantages, these models still cannot fully replicate human disease. They do not allow natural tumor initiation or progression in the correct in situ microenvironment nor are they heterogeneous like most human tumors. Importantly, they do not model a normal immune environment. To overcome these shortfalls, transgenic models have been developed. A number of genetically engineered mouse models (GEMM) have been shown to give rise to ER+ disease. These include ESR1 (estrogen receptor) [7], cyclin D1 [8, 9], Wnt1 (Wnt family member 1) [10], p53 (tumor protein p53) [11], Stat1 (signal transducer and activator of transcription 1) [12], TGFα (transforming growth factor alpha) [13], AIB1 (amplified in breast cancer 1) [14, 15], Espl1 (extra spindle pole bodies like 1) [16], PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) [17], and PyMT (polyoma middle T oncoprotein) [18, 19]. Crucial to modeling ER+ disease in GEMMs is to demonstrate reliance on E2 for growth and response to endocrine agents. However, only a few of these models demonstrate the expected hormonal responses. In Wnt1 transgenic mice that generated ER+ disease, tumors were refractory to both ovariectomy and tamoxifen treatment [10]. Similarity, tumors from ERα and cyclin D1 expressing transgenic mice developed despite tamoxifen treatment, suggesting inherent resistance [8]. Therefore, many of these models cannot lay claim to modeling ER+ breast cancer fully. Lack of response may be due to fast progression of these models to more aggressive phenotypes. For example in the PyMT model, ER+ tumors quickly lose expression and gain HER2 and cyclin D1 expression as tumors become more aggressive and metastatic [18]. Similarly, ER+ tumors in AIB1 transgenic mice have been shown to initially respond to tamoxifen treatment, but lose this response within days [20]. These studies indicate that early mouse mammary tumors may rely on estrogen activity; however, this is often lost, along with response to endocrine agents. Also, these studies highlight the need for further understanding and better models that do fully recapitulate human disease.

The lack of responsive ER+ models led us to fully characterize ER function in some of the available mouse cell lines of mammary cancer, with the goal of using these cells in a syngeneic immunocompetent model of ER+ disease. Our findings suggest that although many of these mouse cell lines do express ER, classical ER function and response to hormones and endocrine agents is lacking, most likely because ER is localized in the cytoplasm rather than nucleus. However, cytoplasmic ER appears to be functional and plays a role in promoting survival. This survival function requires NFκB activity since the combination of tamoxifen or other selective estrogen receptor modulators (SERMs) with NFκB inhibitors led to oxidative stress and apoptosis. Taken together, our findings suggest that mouse models of ER+ breast cancer fail to model human disease largely due to the lack of classical nuclear ER activity, but may be useful in understanding extranuclear functions of ER.

Materials and Methods

Reagents 17β-estradiol (E2), 4-hydroxy-tamoxifen (4OHT), Fulvestrant (ICI 182,780, referred to throughout as ICI), dimethyl fumarate (DMF), Raloxifene (RAL), and propidium iodide (PI) were purchased from Sigma. IKK7 was purchased from Selleck Chemicals. The antibodies for ERα (#8644) and TBP (#8515) were purchased from Cell Signaling. The antibody for GAPDH (10494-1-AP) was purchased from Proteintech. The anti-rabbit Alexa Fluor 488 antibody (ab150073) was purchased from Abcam. Goat anti-rabbit HRP-conjugated secondary antibody (#31460) was purchased from Invitrogen.

Cell Culture Mouse mammary cancer cell lines used in this study are detailed in Table 1. J110 cells were maintained in DMEM/F12 media and Py2T, E0771, 4T1, and Met1 were maintained in DMEM (1×) containing 4.5 g/L D-glucose, L-glutamine, and 25 mM HEPES. All media contained phenol

Table 1 Mouse mammary cancer cell lines

Cell line	Tumor origin	Description	Original Source	Published ERα Status
J110	GEMM	Derived from FVB-MMTV-AIB1 mouse tumor	Myles Brown; Dana-Farber Cancer Institute, Cambridge, USA [21]	+ [15]
Py2T	GEMM	Derived from FVB-MMTV-PyMT mouse tumor	Gerhard Christofori; Institute of Biochemistry and Genetics, Basel, Switzerland [19]	+ [19]
E0771	Spontaneous	Derived from a C57BL/6J mouse tumor	Sugiura K; Sloan-Kettering Institute, New York., USA [22]	+ (cyto) [23], + [24], − [25]
4T1	Spontaneous	Derived from BALB/cfC3H mouse tumor	Miller FR; Brown University, Providence, Rhode Island, USA [26]	− [25, 27]
Met-1 (fvb2)	GEMM	Derived from FVB/N-Tg(MMTV-PymT) mouse	Guy CT, McMaster University, Ontario, Canada [28]	− [25, 29]

red and 5% FBS and was therefore considered "estrogenized", as well as 1% penicillin-streptomycin antibiotics. Before treatment with E2, cells were cultured in phenol red free media supplemented with 5% charcoal-dextran stripped FBS (steroid depleted media) and 1% penicillin-streptomycin antibiotics for 3 days.

RNA Extraction and RT-qPCR Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. A total of 0.5 μg of RNA was used to generate cDNA at a total volume of 10 μl, using M-MLV Reverse Transcriptase reagents with the addition of 50 pmol random hexamer, and 1 mM deoxy-NTP per reaction (all Invitrogen). cDNA was diluted to a total volume of 100 μl with sterile ddH$_2$O. qPCR was performed using 2 μl of diluted cDNA, 1× SYBR Green PCR Master Mix (Applied Biosystems), and 125 nM forward and reverse primer (sequences available on request). Reactions were carried out on an ABI StepOne Real-time PCR system (Applied Biosystems) under the following conditions: 10 min at 95 C followed by 40 cycles of 95 C for 15 s then 60 C for 1 min. The fold change in expression of each gene was calculated using the $\Delta\Delta$ Ct method, with GAPDH as an internal control.

Protein Extraction and Western Blot Whole cell lysates were prepared using the M-PER reagent, and cytoplasmic and nuclear lysates were prepared using the NE-PER kit (Thermo Scientific), according to the manufacturer's protocol. Proteins were separated by SDS-PAGE (Bio-Rad Laboratories), transferred to nitrocellulose membranes (Thermo Scientific), blocked for 1 h in 5% nonfat dry milk (Lab Scientific) in 1× TBS-T, and incubated overnight with primary antibody. The following day, the membranes were rinsed with 1× TBS-T and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. Pierce Supersignal West Pico plus chemiluminescent substrate (Thermo Scientific) was applied to the membranes and the signal was visualized on a Molecular Imager ChemidocXRS (Bio-Rad Laboratories). Images were processed using Quantity One software (Bio-Rad Laboratories).

Cell Proliferation Cell confluency was determined using the Celigo Imaging Cytometer (Nexcelom Bioscience). DNA content was determined using Hoechst 33342 dye (Life Technologies). Fluorescence was read using Synergy HT microplate reader (BioTek). Cell numbers were extrapolated from standard curves.

Syngeneic Tumor Study Mouse experiments were carried out at the animal facility at the University of Illinois at Chicago in accordance with the institutional guidelines and procedures, with approval from the Institutional Animal Care and Use Committee. FVB/N mice at 4 weeks of age were purchased from Taconic. A total of 1000 J110 cells were orthotopically injected into both left and right thoracic mammary fat pads and allowed to form tumors of 0.2 cm^2 in area. Animals were then randomized into four groups and treated daily (5 days/week) as follows: vehicle control, tamoxifen (100 μg in corn oil, by intraperitoneal injection), DMF (30 mg/kg in 0.8% methyl cellulose, by oral gavage), or the combination of tamoxifen and DMF. Tumor size was measured using an electronic caliper and volume (V = (length × width^2 × π)/2) calculated.

Immunofluorescence Cells were seeded onto glass coverslips and allowed to attach. Cells were fixed with 4% paraformaldehyde for 10 min followed by permeabilization with 0.2% triton X-100, blocking with casein, and then incubation with the primary ERα antibody for 1 h. After washing with TBS, cells were incubated with the secondary Alexa Fluor 488 antibody and further washed with TBS. Coverslips were mounted onto glass slides with ProLong™ Gold Antifade Mountant with DAPI reagent (Life Technologies). Images were acquired on a Leica DMi8 microscope at 63× magnification. J110, Py2T, and Met1 images were acquired using the same microscope settings. MCF-7 cells, which served as a positive control, were imaged with reduced exposure in the FITC channel due to high expression of ERα.

H$_2$O$_2$ Assay H$_2$O$_2$ was measured using ROS-Glo™ H$_2$O$_2$ Assay (Promega) in accordance with the manufacturer's

instructions. Luminescence was read on a Synergy HT microplate reader (BioTek). Cells were then counterstained with Hoechst 33342 dye for 30 min, and the fluorescence reading taken, which was used to normalize H_2O_2 values for cell number.

ATP Assay ATP was extracted from treated cells using the method described by Yang et al. [30], with some modifications. Briefly, cell pellets were re-suspended in 500 μl of boiling ddH$_2$0 and heated at 100 C for 10 min, vortexing frequently. Samples were centrifuged at 1500×g and supernatant removed and put on ice. The concentration of ATP was quantified using the ATP determination kit (Invitrogen), as per the manufacturer's protocol and the concentration of ATP in each sample was determined using a standard curve.

Cell Death Assays Cell viability was measured using 1 μg/ml PI to stain dead/dying cells along with Hoechst 33342 dye to stain all cells for 30 min. Total and dead cells were detected using the Celigo Imaging Cytometer (Nexcelom Bioscience), and percentage of total cells stained with PI calculated. To assess apoptosis, levels of activated caspase 3/7 were measured. Briefly, cells were washed with PBS and then incubated for 30 min at 37 C with Hoechst 33342 dye and CellEvent Caspase-3/7 Green Detection Reagent (Invitrogen). The number of cells with positive green fluorescent signal was measured in the green fluorescence channel and the total number of cells was counted in blue fluorescence channel, both using Celigo's colony count application. The percentage of apoptotic cells was calculated as the number of cells with positive green fluorescent signal over the total number of cells per well.

Statistical Analysis Data are presented as mean ± SD or SEM of two or more biological replicates, representative of two or more experimental replicates. Statistical analysis was performed using GraphPad 8 software using linear regression analysis or one-way/two-way ANOVA (followed by Dunnett's/Tukey posttest), as appropriate.

Results

ERα Is Expressed but Lacks Classical Activity in a Panel of Mouse Mammary Cancer Cell Lines

A panel of cell lines generated from mouse mammary tumors (see Table 1 for details), many of which have been reported to be ERα positive in the literature, were assessed for the presence of ERα. We found that J110, Py2T, E0771, and 4T1 cells expressed ERα mRNA and protein, albeit at a lower level than in mouse uterus and MCF-7 cells (Fig. 1). The majority of uterine ER protein was around 55–60 KDa in size, possibly representing a truncated/modified form of the protein,

whereas the mammary cell lines expressed 66 kDa ERα, which was similar in size to that seen in MCF-7 cells. In contrast, Met1 cells, which are widely considered to be ERα negative [25, 29], showed minimal ERα mRNA and undetectable ERα protein. It is important to note that ERβ mRNA was not detected in any of the mouse cell lines (data not shown).

We next examined ERα activity based on hormone-dependent proliferation and target gene expression in two of these cell lines: J110 and Py2T. In steroid-depleted culture media, we found that neither cell line proliferated in response to E2 (Fig. 2a). Furthermore, in estrogenized media, SERMs (4OHT and RAL) and a selective estrogen receptor degrader (SERD; ICI) had only a minimal effect on cell growth (Fig. 2b), indicating that cells may proliferate independently of ER ligand. To further characterize ER activity, we assessed a number of classical ER target genes based on the literature [31, 32]. We found that none of these genes were significantly regulated in response to E2 and/or 4OHT (Fig. 2c). This was true for up to 8 h of treatment, where still no response was observed (data not shown). The lack of ER transcriptional activity or ligand-dependent proliferation indicates that neither cell line exhibits classical ER function.

The Combination of ER and NFKB Inhibitors Suppress J110 and Py2T Cell and Tumor Growth

As J110 and Py2T express ER but had little response to ER ligands, we postulated that these cells could represent models
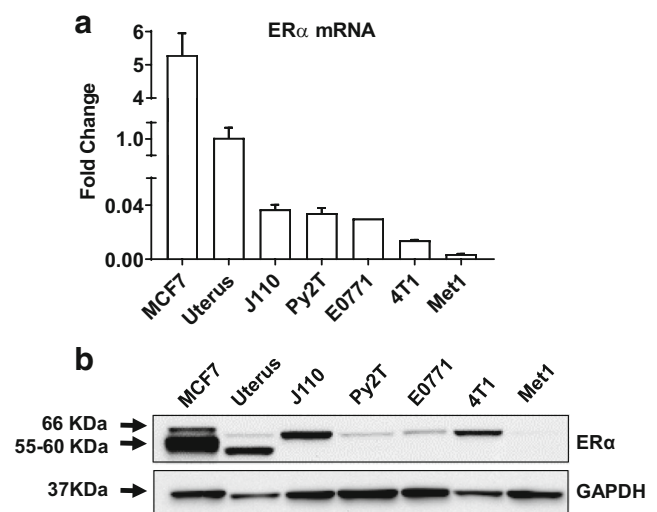

Fig. 1 Estrogen receptor α is expressed in mouse mammary cancer cell lines. **a** ERα mRNA levels were assessed by qPCR in mouse cell lines J110, Py2T, E0771, 4T1, and Met1, and normalized to GAPDH. Fold change relative to ERα in mouse uterine tissue (positive control) is indicated. MCF-7 cells served as a positive human control. **b** Whole cell extracts from J110, Py2T, E0771, 4T1, and Met1, along with mouse uterine tissue and MCF-7 cells (positive controls), were assessed for ERα protein expression by Western blot. Five times less uterine and MCF-7 protein was loaded compared to mouse cell lines. GAPDH was used as a loading control
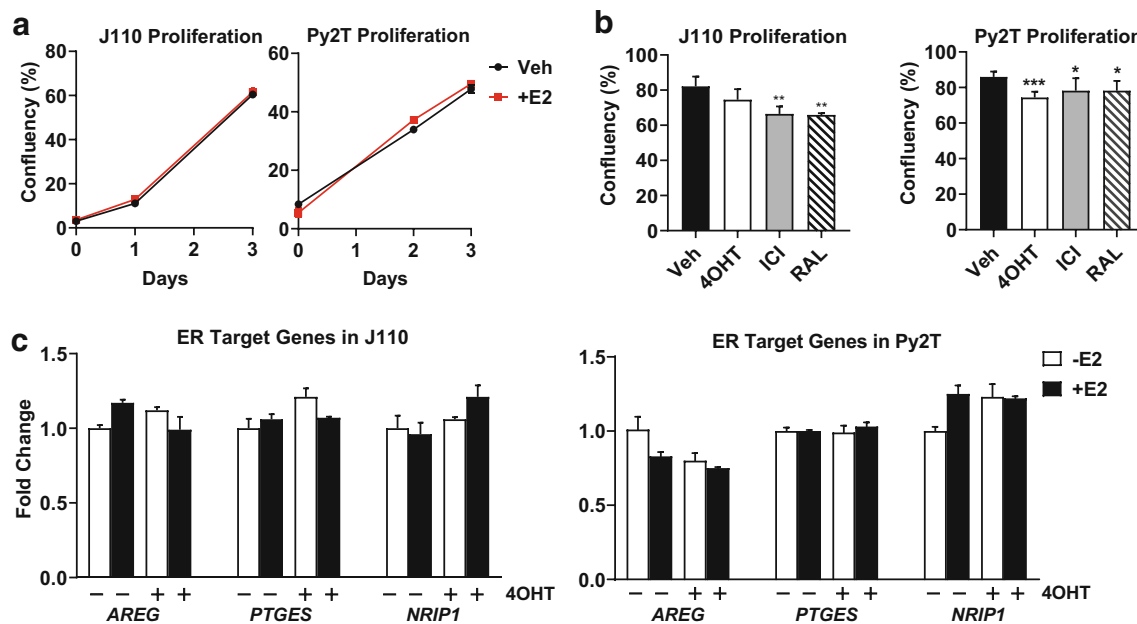
Fig. 2 J110 and Py2T cells do not respond to ER ligands. **a** J110 and Py2T cells were cultured in steroid-depleted media and treated for 3 days with E2 (10 nM) or vehicle (Veh, 0.001% EtOH). Cell confluency measured using a Nexcelom Celigo Imaging Cytometer. **b** J110 and Py2T cells were cultured in estrogenized media and then treated with 4OHT (1 μM), ICI (1 μM), or Ral (1 μM) for 3 days. Cell confluency measured using a Nexcelom Celigo Imaging Cytometer. **c** Cells were pretreated with vehicle or 4OHT for 2 h, and then, vehicle or E2 was added for an additional 2 h. RNA was extracted and qPCR was performed for the ER target genes *AREG*, *PTGES*, and *NRIP1*. Fold change for each gene was calculated relative to vehicle control. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ relative to vehicle treatment

of endocrine therapy-resistant breast cancer. In patients with ER+ breast cancer, endocrine therapy resistance develops, in part, due to activation of other signaling pathways such as MAPK, AKT, EGFR/HER2 [33–35]. Therefore, inhibitors to several of these pathways were tested. Few of the inhibitors suppressed cell growth alone. However, in combination with 4OHT, several were found to significantly suppress cell growth (Supplemental Fig. 1).

Of particular note, two NFκB inhibitors IKK7 and DMF, in combination with 4OHT, suppressed growth cells to the greatest extent. IKK7 is an inhibitor of IKKα/β, a key complex in the NFκB signaling pathway [36, 37]. DMF covalently modifies the NFκB transcription factor p65 and blocks its nuclear translocation and DNA binding ability, thus suppressing NFκB transcriptional activity [38]. As shown in Fig. 3, the combination of NFκB inhibitors with either SERMs or a SERD resulted in a significant suppression of cell growth, compared to either compound alone, except DMF and ICI, which showed a similar trend but did not reach significance. In contrast, the ER-negative Met1 cell line showed no further suppression of growth with combination treatment compared to the NFκB inhibitor alone (Supplemental Fig. 2).

To explore whether combination therapy targeting ER and NFκB would inhibit tumor growth in vivo, J110 cells were injected into the mammary fat pad of immunocompetent FVB/Ntac mice and syngeneic tumors were allowed to form. After initiation of the primary tumor, tamoxifen, DMF, or both were administered to mice and tumor volume observed over

time. By the end of the study, only the combination treatment resulted in a significant reduction in tumor size and weight compared to tumors in untreated mice (Fig. 4). Together, these data indicate that co-inhibition of NFκB and ER pathways results in a tumor suppressive response in mouse cell lines and tumors that are unresponsive to endocrine agents alone.

Cytoplasmic ER and NFκB Protect Cells from Oxidative Stress and Apoptosis

Our results thus far suggest that activation of NFκB may be a contributing factor in suppressing classical ER activity, which has been previously described in the literature [39–41]. There is some evidence that inhibition of NFκB in endocrine-resistant cells restores ER activity, resulting in cells that are responsive to endocrine agents [42, 43] . Therefore, we tested whether combination therapy with ER and NFκB inhibitory agents affected ER's transcriptional function. However, neither IKK7 nor DMF alone or in combination with 4OHT had any effect on the expression of the ER target gene AREG, whereas the combination caused a minimal increase in PTGES expression (Fig. 5a). This increase was unexpected given that we have previously shown that ER and NFκB stimulate PTGES expression [44]. We also examined whether NFκB inhibitors affected ER expression or localization. Surprisingly, ER was exclusively cytoplasmic, regardless of treatment (Fig. 5b). ER localization was also confirmed by immunofluorescence where ER exhibited a distinct punctate

Fig. 3 The combination of ER and NFκB inhibitors reduced J110 and Py2T cell number in vitro. Cells were cultured in estrogenized media and then treated with **a** 4OHT (1 μM), **b** ICI (1 μM), or **c** Ral (1 μM) in the presence of IKK7 (1 μM, left panels) or DMF (30 μM, right panels) for 48 h. DNA was quantified as an indication of cell number using Hoechst 33342 dye. ns, not significant, *P < 0.05; **P < 0.01; ***P < 0.001 relative to IKK7 or DMF treatment alone
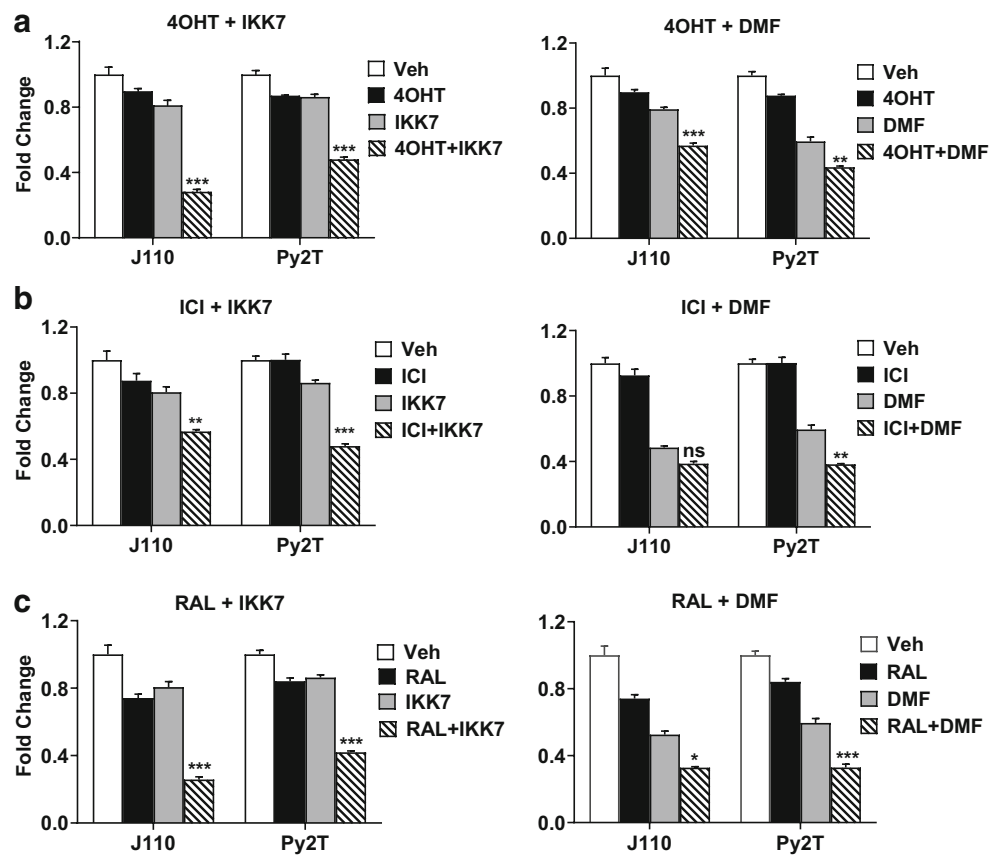
staining pattern in the cytoplasm (Fig. 5c, Supplemental Fig. 3). This was in contrast to MCF-7 cells, which display typical nuclear ER localization, and Met1 cells, which lack ER altogether (Supplemental Fig. 3). Together, these data suggest that restoration of ER's transcriptional activity does not occur upon NFκB inhibition, as it is not localized to the nucleus. Moreover, the cytoplasmic location of ER in these cells may suggest alternative extra-nuclear mechanisms contributing to growth suppression observed with ER and NFκB inhibition.

Previous work from the Levin group demonstrated that extra-nuclear ER was associated with protection from oxidative stress, and subsequent apoptosis, by reprogramming their cell metabolism [45]. Other studies have confirmed a protective role for extra-nuclear ER, specifically in ischemic heart injury [46], by attenuating oxidative stress. Likewise, NFκB is a well-established inhibitor of oxidative stress [47, 48]. We therefore investigated oxidative damage as a mechanism for the growth suppressive effects we observed in ER+ mouse cell lines following ER and NFκB inhibition. Indeed, we found an increase in H_2O_2 production upon ER and NFκB co-inhibition, indicative of oxidative stress, compared to the NFκB inhibitors alone (Fig. 6a). This finding was further
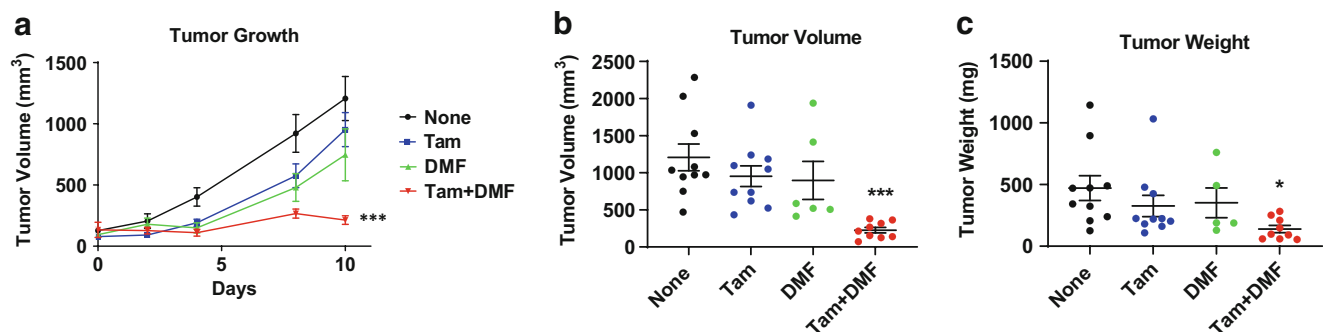

Fig. 4 The combination of ER and NFκB inhibitors reduced J110 tumor growth in vivo. J110 cells were injected into mammary fat pads of FVB/Ntac mice. Once tumors reached 0.2 mm², vehicle, tamoxifen (100 μg/mouse), DMF (30 mg/kg), or both were administered daily for 10 days. **a** Tumor volume was measured every 2 days and plotted over time. **b**, **c** Final tumor volume and tumor wet weight were measured post-mortem. *P < 0.05; ***P < 0.001 relative to none

Fig. 5 Inhibition of ER and NFκB does not alter ER's transcriptional function nor cytoplasmic localization. J110 cells were treated with vehicle or 4OHT (1 μM) in the presence or absence of IKK7 (1 μM) or DMF (30 μM) for 48 h. **a** RNA was extracted and mRNA levels of the ER target genes PTGES and AREG were assessed. **b** Nuclear and cytoplasmic protein fractions from J110 cells were assessed for ER protein level Western blot. TBP was used as a nuclear and GAPDH as a cytoplasmic loading control. **c** ERα protein localization (green) in J110 cells was assessed using immunofluorescence. Punctate extra-nuclear staining indicated by white arrows. Cell nuclei were counterstained with DAPI (blue). *$P =$ <0.05, **$P < 0.01$ relative to IKK7 or DMF treatment alone
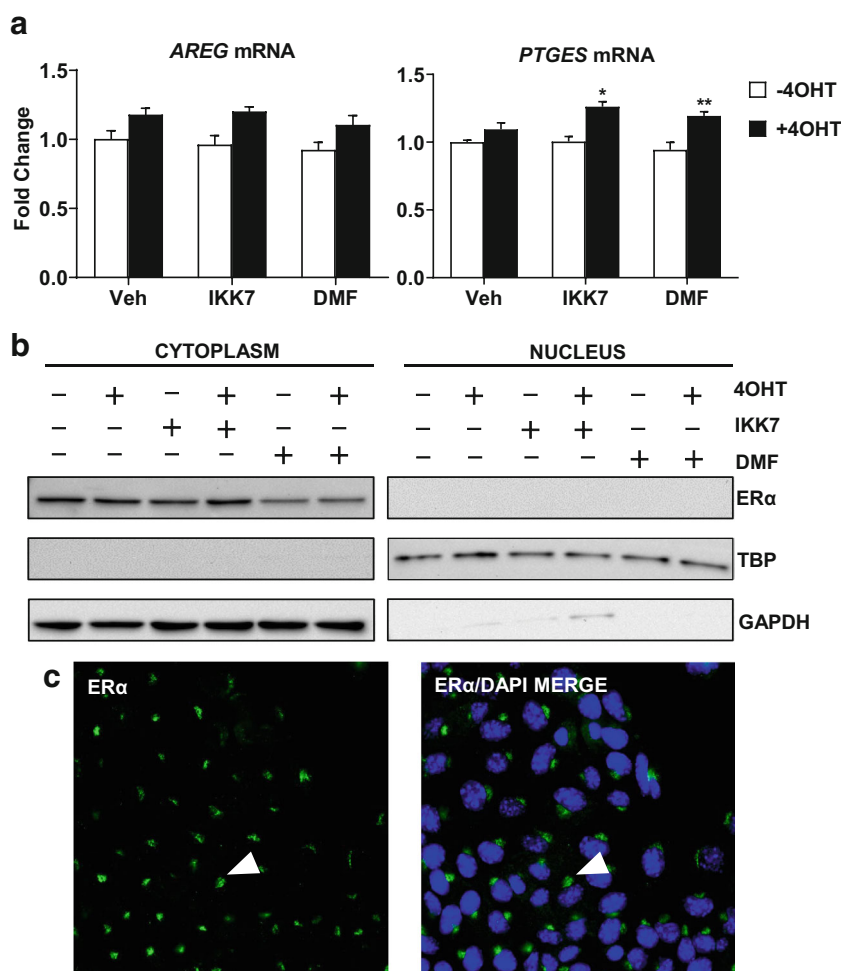
supported by the upregulation of oxidative stress-associated genes, HMOX1, NRF2, and GPX1, with combination treatment (Fig. 6b). Moreover, when cells undergo oxidative stress, the levels of ATP in the cell are significantly attenuated [49] and apoptosis can occur [50]. We observed both significantly reduced ATP and increased cell death/apoptosis in J110 cells co-treated with ER and NFκB inhibitors compared to IKK7 or DMF alone (Fig. 6c–e). As mitochondrial ER has been associated with oxidative stress by others [51–53], we investigated whether cytoplasmic ER is localized to the mitochondria. However, ER and mitochondrial staining patterns were distinctly different, suggesting no colocalization (Supplemental Fig. 4). These data suggest that cytoplasmic but not mitochondrial ER, in addition to NFκB, protect mouse mammary cancer cells against oxidative stress.

Discussion

We observed ER expression in multiple mouse mammary cancer cell lines, derived from either GEMM or spontaneous tumor models, and yet these cells were unresponsive to E2 and only minimally responsive to SERMs or SERDs. Interestingly, ER was found to be predominantly localized to the cytoplasm. Despite an apparent lack of classical nuclear activity, ER still appeared to be functionally important as co-inhibition of ER and NFκB resulted in an increase in apoptosis and oxidative damage. Thus, this study provides new insights into why many ER+ mouse mammary cancer models lack E2-dependence and response to tamoxifen that is typically observed in human ER+ breast tumors and cell lines.

One hypothesis suggested by our findings is that the cytoplasmic localization of ER may be a consequence of disease progression. Tumors that form in AIB1 mice are reported to be ER+ and tumor formation is reliant on ER, as ovariectomy prevented the development of invasive mammary tumors. However, once developed, they subsequently lose their response to estrogen [21]. In ovariectomized mice, injection of cells derived from AIB1 tumors resulted in a similar growth rate compared to untreated mice. However, it was interesting that the ovariectomized mice had fewer metastasis, suggesting that despite their lack of response, E2 may provide protection from tumor progression [21]. Another study, also using cells derived from the AIB1 transgenic model, showed that tumors
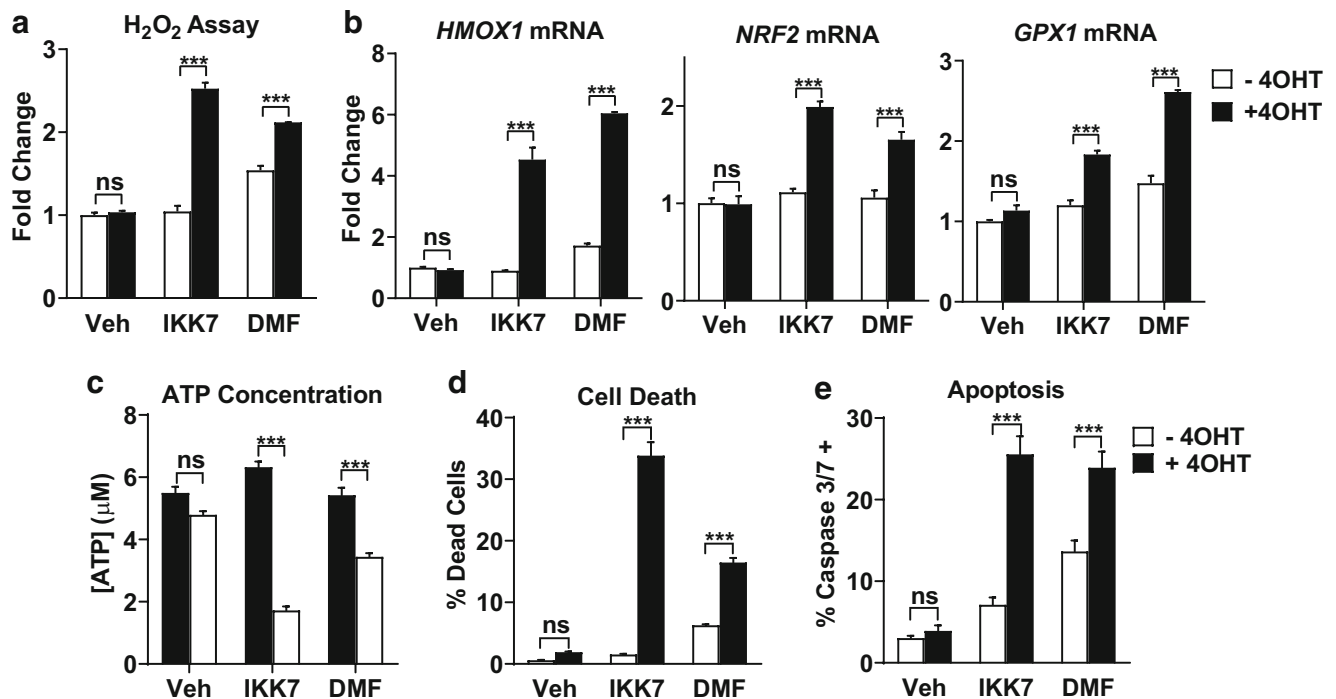
Fig. 6 The combination of ER and NFκB inhibitors promoted cell death via an increase in oxidative stress apoptosis. J110 cells were treated with vehicle (0.001% EtOH) or 4OHT (1 μM) in the presence or absence of IKK7 (1 μM) or DMF (30 μM) for 48 h. **a** Oxidative stress was measured by quantification of H_2O_2 levels in cells after treatments. Fold change for each treatment is indicated relative to vehicle. **b** RNA was extracted and qPCR performed to assess mRNA levels of the oxidative stress-associated genes HMOX1, NRF2, and GPX1. **c** ATP concentration (μM) was measured in cells after treatments. **d** The percentage of dead cells was measured using propidium iodide stain to stain dead cells and DAPI to stain all cells. **e** Activated caspase 3/7, as an indication of apoptosis, was measured using a CellEvent™ Caspase-3/7 Green Detection Reagent. The percentage of apoptotic (green) cells is indicated for each treatment condition. ns = not significant, ***$P < 0.001$, relative to IKK7 or DMF treatment alone

initially responded to tamoxifen in vivo but stopped responding over time, and no difference in size between control and tamoxifen-treated tumors was observed at the end of the study [20]. Similarly, ER+ tumors arising from the PyMT models express ER early on but this is lost as tumors progress. These tumors appear to be estrogen-independent since ovariectomized mice or tamoxifen-treated mice still developed mammary tumors [54]. Cell lines derived from this model express ER but also express the basal keratin KRT14, suggesting that progression and transformation to a more aggressive phenotype may have begun [55]. Loss of ligand response has also been observed the mouse mammary cancer cell line E0771, originally from a spontaneous mammary tumor in a female C57BL/6 mouse [23]. Not only were the cells minimally responsive to tamoxifen in vivo and unresponsive in vitro, they also displayed diffuse cytoplasmic ER staining patterns. Clearly, loss of ER activity in ER+ mouse models is common and progression to this state is rapid. Thus, it is possible that cytoplasmic ER and lack of response in J110 and Py2T cells may result from disease progression to an ER-independent state.

Several studies have described interacting partners of ER that can drive its cytoplasmic location and subsequent extranuclear role. For example, the proto-oncogene tyrosine-protein kinase Src is the most widely studied ER interacting protein that is responsible for sequestering ER in the cytoplasm, and its inhibition has been shown to increase nuclear shuttling of ER [56–58]. The protein PELP1 (proline, glutamate and leucine-rich protein 1) can act as a scaffolding protein between ER and Src and aids sequestration of ER in the cytoplasm [57, 59]. Similarly, MEMO (mediator of ERBB2-driven cell motility) can also enhance interaction between Src and ER, resulting in cytoplasmic retention, by inhibiting nuclear transport of this complex [60]. In addition, MTA1 (metastasis-associated 1) has the ability to sequester ER to the cytoplasm due to its ER binding motif [61]. Finally, methylation of ER by PRMT1 (protein arginine N-methyltransferase 1) at R260 can promote cytoplasmic location of ER [62]. Whether any of these mechanisms may explain why ER is sequestered in the cytoplasm of mouse cell lines remains to be investigated.

Although nuclear ER has been well studied to promote cell survival through suppression of apoptotic genes or upregulation of anti-apoptotic genes, e.g., BCL-2 [63], several studies suggest that cytoplasmic ER is also linked to survival through various signaling pathways, such as PI3K/AKT [59], ERK1/2 [64], and MAPK [57, 65], as well as oxidative stress. For example, in ischemic neuronal injury, ER was found to protect against oxidative damage by attenuating NADPH oxidase

activation and superoxide production [66]. Another study found that extra-nuclear ER was able to mediate protection from cardiac ischemia-reperfusion in mice, due to an increase in myocardial protein S-nitrosylation, which protects from oxidative stress [67]. Extra-nuclear ER has been shown to protect from apoptosis in pancreatic β-cells. In these cells, ER is predominantly cytoplasmic, and after acute H_2O_2 exposure to mimic oxidative injury, stimulation of ER by E2 protected cells from apoptosis [68]. These studies provide compelling evidence of a role for extra-nuclear ER in cellular protection against oxidative stress and may be applicable to the mouse mammary cancer models used in these studies.

It is interesting to ask if cytoplasmic ER promotes survival, then why do not SERMs or SERDs have discernable effects on J110 and Py2T cell survival? The finding that co-inhibition of ER and NFκB resulted in a significant increase in oxidative damage, apoptosis, and cell death is suggestive of a synthetic lethality paradigm. Synthetic lethality is a type of genetic interaction where suppression of two genes results in cell death whereas suppression of either one alone leaves cells viable [69, 70]. This concept can also be applied to suppression of proteins or pathways. It is hypothesized that synthetically lethal interactions occur because redundant mechanisms are in place to maintain homeostasis when cells are subjected to various deficiencies, such as environmental, genetic, or mutational [71]. In the case of J110 and Py2T cells, when ER and NFκB, two pro-survival pathways, are inhibited by chemical compounds, we observe oxidative damage leading to increased cell death, suggesting a compensatory or buffering relationship between ER and NFκB. This relationship could be the result of "between-pathway" or "within-pathway" interactions [72], either of which could be possible as ER and NFκB crosstalk has been addressed heavily in the literature [41]. Although this work has predominantly described transcriptional crosstalk, cytoplasmic crosstalk can also occur. For example, activation of PI3K, which is mediated by extra-nuclear ER, results in sequestration of NFκB in the cytoplasm [73]. Therefore, the survival mechanisms in J110 and Py2T cells may involve a complex convergence and compensatory function between cytoplasmic ER and NFκB.

Of utmost importance is whether the cytoplasmic function of ER and redundancy with the NFκB pathway is relevant to human disease. Although activation of NFκB is observed clinically during tumor progression and has been linked to endocrine resistance [39, 40, 43, 74, 75], cytoplasmic ER is less well described. One study by Welsh et al. assessed ER expression in nearly 3200 patient cases and found the average incidence of cytoplasmic ER was around 1.5% [76]. However, this study examined ER expression in primary tumors. We hypothesize the context in which cytoplasmic ER may be more applicable is in advanced disease, e.g., having progressed on or after therapy. These samples may be less well represented in studies of cytoplasmic ER due to difficulty in obtaining samples after therapeutic failure or progression to aggressive disease. Taken together, our studies suggest that analysis of ER localization as a consequence of disease progression may represent a promising new direction to develop novel therapeutic strategies.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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