Cytoplasmic PELP1 and ERRgamma Protect Human Mammary Epithelial Cells from Tam-Induced Cell Death

Brian J. Girard1, Tarah M. Regan Anderson1, Siya Lem Welch2, Julie Nicely2, Victoria L. Seewaldt2, Julie H. Ostrander1*

1 Department of Medicine, Masonic Cancer Center, University of Minnesota, Minneapolis, Minnesota, 55455, United States of America, 2 Department of Medicine, Duke University Medical Center, Durham, North Carolina, 27710, United States of America

* hans1354@umn.edu

Abstract

Tamoxifen (Tam) is the only FDA-approved chemoprevention agent for pre-menopausal women at high risk for developing breast cancer. While Tam reduces a woman's risk of developing estrogen receptor positive (ER+) breast cancer, the molecular mechanisms associated with risk reduction are poorly understood. Prior studies have shown that cytoplasmic proline, glutamic acid and leucine rich protein 1 (PELP1) promotes Tam resistance in breast cancer cell lines. Herein, we tested for PELP1 localization in breast epithelial cells from women at high risk for developing breast cancer and found that PELP1 was localized to the cytoplasm in 36% of samples. In vitro, immortalized HMECs expressing a nuclear localization signal (NLS) mutant of PELP1 (PELP1-cyto) were resistant to Tam-induced death. Furthermore, PELP1-cyto signaling through estrogen-related receptor gamma (ERRγ) promoted cell survival in the presence of Tam. Overexpression of ERRγ in immortalized HMECs protected cells from Tam-induced death, while knockdown of ERRγ sensitized PELP1-cyto expressing HMECs to Tam. Moreover, Tam-induced HMEC cell death was independent of apoptosis and involved accumulation of the autophagy marker LC3-II. Expression of PELP1-cyto and ERRγ reduced Tam-induced LC3-II accumulation, and knockdown of ERRγ increased LC3-II levels in response to Tam. Additionally, PELP1-cyto expression led to the upregulation of MMP-3 and MAOB, known PELP1 and ERRγ target genes, respectively. Our data indicate that cytoplasmic PELP1 induces signaling pathways that converge on ERRγ to promote cell survival in the presence of Tam. These data suggest that PELP1 localization and/or ERRγ activation could be developed as tissue biomarkers for Tam responsiveness.

Introduction

Progress in breast cancer prevention is currently limited by our lack of biological markers to identify which women will respond to prevention therapies. Tamoxifen (Tam), a selective
estrogen receptor modulator, is the most widely used treatment for estrogen receptor (ER)+ breast cancer. Tam treatment is approved for the prevention of breast cancer in pre-menopausal women, but it only reduces the risk of developing ER+ breast cancer by approximately 50% and does not prevent ER− breast cancer [1]. The increased risk of stroke, pulmonary emboli, cardiac events, endometrial cancer, and unwanted side effects (e.g., hot flashes, fatigue, depression, weight gain, and decreased libido) have decreased the acceptance of Tam among patients, particularly in the chemoprevention setting. Thus, there is a critical need to identify the women who are most likely to benefit from risk reducing strategies, and improve breast cancer prevention with novel prevention strategies. Inhibition of ER transcriptional activity is considered the predominate effect of Tam in invasive breast cancer; however, not all of Tam’s effects can be directly attributed to inhibition of ER. Tam is clinically effective in treatment of tumors that do not express ER [2]. Tam has a wide variety of ER-independent pharmacological activities including stimulation of transforming growth factor-beta, blockade of various chloride channels [3], inhibition of protein kinase C [4], and antagonism of calmodulin activity [5]. Additionally, Tam-binding sites independent of ER have been identified. Tam binds and regulates the G protein-coupled estrogen receptor (GPER) [6] and estrogen related receptors (ERRs) [7]. Furthermore, therapeutic concentrations of Tam are several orders of magnitude higher than the concentrations required to saturate ER [8]. On the basis of these observations, we hypothesized that ER-independent effects may play a role in Tam-induced cell death in normal or atypical breast tissue.

Members of the ERR subfamily of nuclear receptors (NRs) have been implicated in the ER-independent effects of Tam. ERR subfamily members include ERRα, ERRβ, and ERRγ. Although ERRs are considered orphan nuclear receptors with no known natural ligand, ERRβ and ERRγ have been shown to bind Tam [7,9,10]. ERRs are constitutively active transcription factors whose activity is predominately regulated through interactions with co-regulators. ERRs are primarily involved in the regulation of genes involved in cellular metabolism, energy homeostasis, and cancer [11]. While the role of ERRγ in breast cancer is relatively understudied, ERRγ expression has been associated with favorable breast cancer biomarkers, such as ER expression [12]. Conversely, ERRγ has been shown to promote Tam resistance in invasive ductal and lobular carcinoma cell culture models [13,14]. To date, a role for ERRγ in breast cancer initiation or response to Tam chemoprevention in mammary epithelial cell models has not been tested.

In addition to ERRγ, proline, glutamic acid and leucine-rich protein-1 (PELP1), a nuclear receptor co-activator protein, has been shown to promote Tam resistance in invasive breast cancer cell line models. Most co-activators function in the nucleus to enhance the transcriptional activation function of nuclear receptors (NRs), but PELP1 has been shown to regulate genomic and extra-nuclear (cytoplasmic) actions [15,16]. In vivo, PELP1 subcellular localization is primarily nuclear in normal breast tissue, but it is localized to the cytoplasm in about 40% of invasive breast tumors [17]. Targeting PELP1 to the cytoplasm by mutation of the nuclear localization signal (PELP-cyto) leads to activation of non-genomic signaling and Tam resistance in breast cancer cell line models [17]. Interestingly, in a mammary-specific transgenic mouse model, expression of PELP1-cyto induced mammary gland hyperplasia that was unaffected by Tam treatment [18]. Although PELP1 is localized to the cytoplasm in about 40% of invasive breast cancers, PELP1 localization in pre-malignant or high-risk tissues is not known. We therefore hypothesized that cytoplasmic PELP1 drives breast cancer initiation and epithelial cell survival though an ER-independent mechanism involving ERRγ. Upregulation of this pathway may explain resistance to Tam chemoprevention. Herein, we investigated the role of PELP1 and ERRγ in promoting cell survival in response to Tam in ER− models of early mammary carcinogenesis.
Materials and Methods
Subjects and Informed Consent
The study was approved by the Human Subjects Committee and Institutional Review Board at Duke University Medical Center in accordance with assurances filed with and approved by the Department of Health and Human Services. Subjects were recruited on entry to the Duke University High-Risk Clinic and were required to have one of the following: (a) a 5-year Gail model risk score $\geq 1.7\%$, (b) a prior biopsy exhibiting atypia, LCIS, or DCIS, or (c) known or suspected BRCA1/2 mutation. Women in the Duke High-Risk Clinic were initially approached by Dr. V.L. Seewaldt or her physician assistant for consent to random periaerolar fine needle aspiration (RPFNA). A study nurse or coordinator then obtained written consent by participants signing an IRB-approved consent form. The Duke High-Risk Research Cohort was established in 2003. Currently there are 1027 women participating in the Duke High-Risk Research Cohort (defined as all women participating in research studies). The protocol and consent has undergone yearly review and all investigators have completed full Duke human subjects, ethics, and compliance training.

PELP1 localization in RPFNA samples
RPFNA samples were prepared for immunofluorescence at the Duke University Medical Center Cytology Laboratory by ThinPrep. Immunofluorescence staining on all 11 samples was performed together. Pan-cytokeratin staining was used to identify epithelial cells and total cell region. DAPI staining was used to define the nuclear region. PELP1 subcellular localization was quantified by using ImageJ software. Briefly, PELP1 fluorescence intensity of the cytoplasmic compartment was divided by the total PELP1 fluorescence intensity of each individual epithelial cell. Epithelial cells that contained at least 50% of total PELP1 in the cytoplasm were considered positive for cytoplasmic PELP1. Three independent cell clusters from each sample were analyzed for PELP1 localization on a cell-by-cell basis. PELP1 was considered to be predominately cytoplasmic if $\geq 50\%$ of total PELP1 expression was observed in at least 20% of analyzed cells.

Cell culture and reagents
MCF-10A and MCF-7 cells were purchased from American Type Culture Collection. HMEC-hTERT mammary epithelial cells were obtained from Lonza as primary cells and immortalized as previously described [19]. 240Lp16sMY mammary epithelial cells were a generous gift from Martha Stampher (Lawrence Berkeley National Laboratory) and have been described previously [20]. MCF-7 cells were cultured in minimal essential medium $\alpha$ (Invitrogen) supplemented with 5% fetal bovine serum, 10 mmol/L HEPES (Invitrogen), 1x nonessential amino acids (Invitrogen), 1x sodium pyruvate (Invitrogen), 1 $\mu$g/mL insulin (Invitrogen), 1 $\mu$g/mL hydrocortisone (Sigma Aldrich), and 10 ng/mL epidermal growth factor (EGF; Sigma Aldrich). HMEC-hTERT cells were cultured in HuMEC Ready Media (1X) (Life Technologies) supplemented with epidermal growth factor, hydrocortisone, isoproterenol, transferrin, insulin, and 25 mg of bovine pituitary extract. MCF-10A [21] and 240Lp16sMY [22] cells were cultured as described previously.

Generation of PELP1 and ERRγ Cell Lines
Full length PELP1 cDNA was generated by RT-PCR from RNA isolated from MCF-7 cells. The primers used to amplify PELP1 were 1) 5'-GAAGATGGCCAGCCGGTTACT-3' and 2) 5' - GTGGGGTGCAGAGATGGCT-3'. The amplified product was gel purified and ligated into
pCR-2.1. pCR-2.1-PELP1 cDNA was sequenced and compared to NCBI Reference Sequence NM_014389.2. Any base-pair changes were converted to match the reference sequence by site-directed mutagenesis. PELP1 encoding a mutation in the nuclear localization signal (KKLK → EELE), termed PELP1-cyto was made by site-directed mutagenesis of PELP1 wild-type (wt), as previously described [17]. PELP1-wt and PELP1-cyto were digested out of pCR2.1 and subcloned in to pLXSN for retrovirus production. HMEC-hTERT and 240Lp16sMY cells were transduced with retrovirus encoding pLXSN vector control, PELP1-wt, or PELP1-cyto. Cells were selected with 500 \( \mu \)g/mL of G418, and single-cell cloning was used on selected cells to generate clonal cell lines overexpressing the respective PELP1 cDNA. PELP1-wt and PELP1-cyto expression was confirmed by Western blot and immunofluorescence for PELP1.

We obtained 6 ERR\( \gamma \) shRNA target constructs (pLKO.1) from The RNAi Consortium shRNA Library. Lentivirus for all 6 shRNA was generated and knockdown tested in multiple cell lines after lentiviral infection, and stable pooled populations were selected with 0.5 ug/mL puromycin. ERR\( \gamma \) mRNA transcript levels were tested by qRT-PCR. The target shRNA sequence that achieved the best knockdown was 5’-CCTGTCAGGAAACTGTATGAT-3’. We used this shRNA for all subsequent knockdown experiments. To achieve knockdown in HMEC-hTERT cell lines, we subcloned the shRNA into pLKO.1-Hygro by annealing 50 base-pair oligos with suitable overhangs for inserting into this vector. Lentivirus was then produced, and cells were infected with the ERR\( \gamma \) targeting vector or shRNA control. Stable pooled populations were selected with 50 \( \mu \)g/mL hygromycin. To achieve overexpression of ERR\( \gamma \) in our cell lines, we purchased the lentiviral overexpression vector, pEZ-Lv152, containing a complete cDNA for ERR\( \gamma \) with control (GeneCopoeia, EX-E3315-Lv152). We validated the constructs by transfection into cells and measuring ERR\( \gamma \) mRNA transcript levels by qRT-PCR. Once validated, we generated lentivirus to make stable pools overexpressing ERR\( \gamma \), which were selected with 50 \( \mu \)g/mL of hygromycin and maintained in 20 \( \mu \)g/mL hygromycin.

**Western blotting and antibodies**

Whole-cell lysates were collected using RIPA buffer [10 nm sodium phosphate (pH 7.0), 150 mm NaCl, 2 mm NaCl, 2 mm EDTA, 1% (wt/vol) Nonidet P-40 (NP-40), 0.1% (wt/vol) SDS, 1% sodium deoxycholate, 0.1% (vol/vol) \( \beta \)-mercaptoethanol, supplemented with 20 \( \mu \)g/mL aprotinin, 0.1 m sodium fluoride, 1 mm sodium vanadate, 1 mm phenylmethylsulfonyl fluoride] and scraping on ice. Nuclear and cytoplasmic fractions were collected using the NE-PER Nuclear Protein Extraction Kit (Thermo Scientific). Lysates were centrifuged at 14,000 rpm at 4°C, and supernatant was collected. A BCA Assay (Thermo Scientific) was performed for protein concentration quantification, and lysates were resolved on an SDS-PAGE gel. Proteins were transferred onto a polyvinylidene difluoride membrane and blocked in 5% BSA in TBST for 1 hour. Membranes were subsequently incubated in primary antibody (1–2 hours at room temperature or overnight at 4°C), washed, and then incubated with an HRP-conjugated secondary antibody for 1 hour at room temperature. Antibody-protein interactions were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) or Immobilon Western Chemiluminescent HRP Substrate (Millipore) according to the manufacturer’s protocol. The following antibodies were used: PELP1 (A300–180A, Bethyl Laboratories, Inc.), MAP1LC3A/B (AHP2167, AbD Serotec), Actin-HRP, HDAC2, and p65 (sc-1616, sc-7899, sc-8008, Santa Cruz Biotechnology, Inc. respectively).

**Flow Cytometry**

For cell cycle analysis, cells were plated at a density of 100,000 cells per well into 6-well plates. Twenty four or 48 hours after treatment with Tam or nocodazole (positive control), cells were
harvested in StemPro Accutase Cell Dissociation Reagent (Life Technologies). Cells were rinsed with cold PBS and resuspended in 500 μL of staining solution as previously described [23]. Cells were incubated for 15 minutes and filtered through FACS tubes with a cell-strainer cap. Samples were then analyzed on a BD LSR II flow cytometer to measure propidium iodide incorporation. Data was analyzed in FlowJo and cell cycle analysis was carried out manually and using the Watson (Pragmatic) algorithm.

For Annexin V/7-AAD staining, cells were plated at a density of 100,000 cells per well into 6-well plates. Cells were treated the next day with Tam. Twenty four hours after treatment, cells were harvested using StemPro Accutase Cell Dissociation Reagent to preserve membrane integrity. Cells were then processed using PE Annexin V Apoptosis Detection Kit I (BD Pharmingen) and subsequently analyzed in a FACSCalibur flow cytometer to measure Annexin V and 7-AAD staining. Data was analyzed using FlowJo software.

**MTT Assay**

Cells were plated at 5,000 cells per well into 1 mL of growth media in 24-well dishes. A treatment day reading was taken to ensure even cell plating density. Cells were treated with Tam or Tam plus inhibitors in HuMEC medium one day after plating. Cell viability was measured 3 days after treatment. Viability was assayed by the uptake of MTT as described previously [24].

**RNA isolation, cDNA synthesis, and Q-RT-PCR**

RNA extraction was carried out using TRI Reagent (Life Technologies) according to the manufacturer’s protocol. RNA concentration and purity were determined by using a Biotek Synergy 2 microplate reader. The 260/280 ratio of all samples used was ≥ 1.7. cDNA synthesis was carried out using 1 μg of RNA and qScript cDNA SuperMix (Quanta BioSciences) according to the manufacturer’s protocol. Real-time quantitative PCR analysis was performed on a Light Cycler (Roche Diagnostics) according to the manufacturer’s instructions. LightCycler 480 SYBR Green 1 Master (Roche) was used to detect double stranded DNA. Cumulative fluorescence was measured at the end of the extension phase of each cycle, and cycle numbers obtained at the log-linear phase of the reaction were plotted against a standard curve prepared with serially diluted samples for relative quantification of mRNA abundance. Results were normalized to either β-actin, GAPDH, or TBP-2, which served as housekeeping genes.

**Results**

**Cytoplasmic PELP1 localization in RPFNA samples from high-risk women**

To determine whether PELP1 localization is altered during early mammary carcinogenesis, we examined PELP1 localization in 11 randomly selected RPFNA samples from asymptomatic women at high risk for developing breast cancer. Needle aspirates were fixed as previously described [25] and prepared for immunofluorescence (IF) by ThinPrep. All samples were incubated with primary antibodies to PELP1 and pan-cytokeratin, followed by incubation with secondary antibodies and DAPI counterstaining. Epithelial clusters were imaged, and PELP1 localization was quantitated using ImageJ software (see Materials and Methods). We found that 4/11 (36%) RPFNA samples were positive for cytoplasmic PELP1 staining. Cytoplasmic PELP1 expression ranged from 5% to 70% of all epithelial cells (Table 1). Only samples that had at least 20% of the total population expressing cytoplasmic PELP1 were considered positive. These results indicate that PELP1 localization is altered in mammary epithelial cells.
from high-risk women and altered PELP1 localization may be an early event in breast cancer initiation.

**Immortalized HMECs are sensitive to Tam**

We and others have shown that ER-low or ER-negative cell lines are responsive to Tam at physiological concentrations [26–32]. Prior to testing the effect of PELP1-cyto expression on Tam responsiveness, we examined Tam sensitivity among a panel of HMECs: hTERT immortalized HMECs (HMEC-hTERT), immortalized 240Lp16sMY cells (p16 knockdown, c-myc overexpression), and spontaneously immortalized MCF-10A cells. All cell lines were plated in growth medium and treated the following day with human mammary epithelial cell growth medium (HuMEC, Life Technologies) containing Tam. MTT assays were performed three days following Tam treatment. We found that all cell lines tested were sensitive to Tam starting at 100 nM with increasing cell death from 250 nM to 2 μM (Fig. 1A). Of note, we did observe some experimental variability in the Tam dose needed to achieve 50% cell death; ranging from 250 nM to 750 nM. Therefore, all subsequent MTT experiments tested at least two concentrations of Tam.

To determine whether the effect of Tam in HMECs was independent of ER action, we compared the effects of Tam and 4-hydroxytamoxifen (4-OHT) in HMEC-hTERT (ER−) and MCF-7 (ER+) cells. Tam is taken orally as tamoxifen citrate and then metabolized in vivo to 4-OHT, which binds with higher specificity to ER [33]. Tam and 4-OHT treatment both resulted in significant cell death at a concentration of 0.5 μM in HMEC-hTERT cells (Fig. 1B). Interestingly, while MCF-7 cells were sensitive to Tam at the same concentrations, the effect in HMECs was more robust (Fig. 1B and 1C). This is likely because of ER-dependent growth arrest in MCF-7 cells [34] versus cytotoxic cell killing of HMECs. We also examined ESR1 mRNA expression in our HMEC lines and MCF-7 cells. As expected, very little mRNA for ESR1 was found in HMEC-hTERT, 240Lp16sMY, or MCF-10A cells, while MCF-7 cells expressed appreciable levels of ESR1 (Fig. 1D). To determine if ER-dependent gene expression was modulated in HMEC-hTERT cells, HMEC-hTERT and MCF-7 cells were treated with 17β-estradiol (E2), 4-OHT, and Tam for six hours, and cDNA was prepared from isolated RNA. qRT-PCR was then performed to examine levels of pS2 and Krt13. pS2 is a known E2-induced, ER-response gene, and Krt13 is an ER-dependent, 4-OHT-induced gene [35]. As expected, pS2 and Krt13 were induced by E2 and 4-OHT, respectively, in MCF-7 cells, but no regulation of these genes was observed in HMEC-hTERT cells (Fig. 1E and 1F). Together,
Fig 1. Human mammary epithelial cell lines are sensitive to Tam independent of ER expression. A, MTT assay of mammary epithelial cell lines treated with increasing concentrations of Tam for 3 days. MTT assay of HMEC-hTERT (B) and MCF-7 (C) cell lines treated with increasing concentrations of either Tam or 4-OH-Tam for 3 days. D, qRT-PCR of ESR1 and TATA-binding protein 2 (TBP2) (internal control) mRNA levels in three mammary epithelial cell lines and MCF-7 cells. HMEC-hTERT and MCF-7 cells were treated with ethanol (control), 10 nM E2, 10 nM 4-OH-Tam, or 0.5 μM Tam for 24 hours. ER target genes pS2 (E) and Krt13 (F) were measured by qRT-PCR and averaged over values for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (internal control). A student’s T-Test was performed to determine statistical significance for qRT-PCR data. P-values are given for the indicated comparisons. A-D) One-way ANOVA was performed to determine statistical significance of presented data. ** indicates p < 0.001.

doi:10.1371/journal.pone.0121206.g001
these data suggest that Tam-induced cell death of immortalized HMECs is independent of ER expression.

Expression of PELP1-cyto inhibits Tam-induced cell death

To determine whether PELP1 localization has an effect on Tam-induced cell death in immortalized HMECs, we established stable cell lines that express vector control (pLXSN), PELP1-wild-type (wt), or PELP1-cyto (NLS mutant). Cells were selected for stable integration of PELP1 with G418. Clonal cell populations were screened for PELP1 localization by immunofluorescence (Fig. 2A) and Western blotting of cytoplasmic and nuclear fractions (Fig. 2B and 2D). Clonal cell lines expressing PELP1-cyto showed increased PELP1 in the cytoplasm compared to PELP1-wt and vector control cell lines. Western blotting for p65 and HDAC2 was performed as controls for cytoplasmic and nuclear fractionation, respectively (Fig. 2B and 2D). Vector control, PELP1-wt, and PELP1-cyto cells were tested for response to Tam by MTT assay (as described in Materials and Methods). HMEC-hTERT and 240Lp16sMY clonal cell lines expressing PELP1-cyto, but not PELP1-wt or vector control, were more resistant to Tam-induced cell death (Fig. 2C and 2E).

Prior studies in ER+ breast cancer cell line models have shown that PELP1-cyto promotes activation of ERK1/2 and Akt signaling through EGFR to promote resistance to Tam [17,18]. To determine if similar signaling pathways contributed to PELP1-cyto-induced cell survival in response to Tam in HMEC-hTERT cells we examined PELP1-cyto-induced effects on phosphorylation of Akt Ser473 and Erk1/2 Thr202/Tyr204 by Western blotting of whole cell extracts. Interestingly, we did not observe robust PELP1-cyto-, or PELP1-wt-, induced phosphorylation of Akt or Erk1/2 in HMEC-hTERT cells. We did observe increased phosphorylation of Erk1/2 in MCF-7 cells expressing PELP1-wt and PELP1-cyto compared to MCF-7 LXSN control cells. Phosphorylation of Akt was below our detection limits in MCF-7 cells (Fig. 3A). To determine whether inhibition of these pathways sensitized PELP1-cyto cells to Tam-induced death, HMECs were treated with increasing concentrations of the PI3K inhibitor LY294002 (LY) or the MEK1 inhibitor UO126 (UO) in the presence or absence of 0.5 μM Tam (Fig. 3B and 3C). Both LY and UO had a modest effect on the growth of HMEC-hTERT control and PELP1-cyto HMECs in the absence of Tam. Neither drug, however, sensitized PELP1-cyto expressing HMECs to Tam-induced cell death when compared to vehicle (DMSO) treated cells. These data suggest that the mechanism of PELP1-cyto-induced cell survival in response to Tam is largely independent of Akt and Erk1/2 signaling in HMECs.

ERRγ expression modulates response to Tam in HMECs

Tam modulates intracellular signaling in cell line models through a variety of pathways (reviewed in [36]). Tam binds ERRγ [37], and ERRγ promotes Tam resistance in ER+ breast cancer cell line models [13,14,38]. To determine whether ERRγ expression affects response to Tam, we first knocked down ERRγ in MCF10A cells. MCF10A cells were infected with lentivirus encoding control shRNA (pLKO.1 shGFP) and ERRγ shRNA. Cells were selected for stable incorporation of the shRNA with puromycin. Pooled cells were tested for ERRγ expression by qRT-PCR to confirm ERRγ knockdown (Fig. 4A). MCF10A cells positive for ERRγ knockdown were more sensitive to 0.5 μM Tam compared to shRNA control cells (Fig. 4B, p = 0.001). Next, we knocked down ERRγ in vector control and PELP1-cyto HMEC-hTERT cells. After lentiviral transduction with virus encoding control shRNA or ERRγ shRNA, infected cells were selected for stable incorporation of the shRNA with hygromycin. Pooled populations were tested for ERRγ knockdown (Fig. 4C) and response to Tam by MTT. Similar to what we observed in MCF-10A cells, control HMEC-hTERTs expressing ERRγ shRNA were sensitized to Tam at
Additionally, resistance to Tam treatment was lost in HMEC-hTERT-Cyto cells expressing ERRγ shRNA at 0.25 and 0.5 μM Tam (Fig. 4D, p < 0.0001).

To complement our ERRγ knockdown results, ERRγ was overexpressed in HMEC-hTERT and MCF10A cells. Parental HMEC-hTERT and MCF10A cells were infected with lentivirus encoding vector control or ERRγ. Cells were selected for stable integration of ERRγ with
Fig 3. PELP1-cyto does not activate Akt and Erk1/2 to promote cell survival of HMECs in the presence of Tam. A, Western blotting for phospho-Akt and phospho-Erk1/2 in HMEC-hTERT and MCF-7 cells. Actin served as a loading control. B and C, MTT assay of HMECs expressing pLXSN or PELP1-cyto were treated with increasing concentrations of the Akt inhibitor LY294002 (LY) (B) or the MAPK inhibitor UO126 (UO)(C). The vehicle for LY and UO was DMSO.

doi:10.1371/journal.pone.0121206.g003
Fig 4. Knockdown and overexpression of ERRγ alters response to Tam in HMECs. A, qRT-PCR for ERRγ mRNA to verify knockdown in shControl and shERRγ stable cell lines. B, MTT assay of MCF-10A cell lines expressing either shRNA control (shControl) or shRNA against ERRγ (shERRγ). Cells were treated with 0.5 or 1.0 μM Tam for 3 days. C, qRT-PCR for ERRγ mRNA to verify knockdown in HMEC-LXSN and HMEC-Cyto cell lines stably expressing PELP1 Protects HMECs from Tam-Induced Death.
hygromycin. As expected, ERRγ overexpression protected both HMEC-hTERT and MCF-10A cells from Tam-induced cell death compared to vector control expressing cells, particularly at 0.5 and 1.0 μM Tam (Fig. 4E, p = 0.001) in MCF-10A cells, and 0.25 and 0.5 μM Tam in HMEC-hTERT cells (Fig. 4F, p<0.001 and p = 0.001). Overexpression of ERRγ mRNA in pooled populations was confirmed by qRT-PCR (Fig. 4G and 4H). Of note, we relied on mRNA expression of ERRγ to confirm knockdown and overexpression because all ERRγ antibodies that we tested did not specifically recognize ERRγ (an HA-tagged ERRγ construct was utilized to test all antibodies and then confirm ERRγ expression with an HA antibody). These data indicate that cytoplasmic PELP1-mediated resistance to Tam-induced cell death is dependent on ERRγ expression.

PELP1-cyto signaling and ERRγ regulated gene expression

To determine whether PELP1-cyto signaling modulates ERRγ-dependent gene expression, we examined expression of MAOB and MMP-3, genes previously identified to be regulated by ERRγ or PELP1, respectively. RNA was isolated from HMEC-hTERTs (control and PELP1-cyto) expressing either control shRNA or ERRγ shRNA. We found that MAOB, a classical ERRγ target gene [39], was upregulated in PELP1-cyto expressing HMECs. Furthermore, shRNA knockdown of ERRγ inhibited PELP1-cyto-induced upregulation of MAOB (Fig. 5A). We found that MMP-3 expression, which is regulated by PELP1 in MDA-231 breast cancer cells [40], was upregulated in PELP1-cyto expressing HMECs. Similar to MAOB, knockdown of ERRγ inhibited PELP1-cyto-induced MMP-3 expression (Fig. 5B). These data suggest that PELP1-cyto mediated signaling events modulate ERRγ transcriptional activity.

PELP1-cyto and ERRγ protect HMECs from Tam-induced autophagy-associated cell death

Our data indicate that PELP1-cyto and ERRγ are involved in an ER-independent molecular mechanism of Tam resistance. Tam has been shown to induce cell cycle arrest, apoptosis, and autophagy. To understand the mechanism of Tam-induced death in our in vitro HMEC models, we examined the biological effects of Tam on HMEC-control and HMEC-cyto cells. Cell cycle arrest and apoptosis was tested by flow cytometry of propidium iodide stained cells after 24 hours of 1.0 μM Tam treatment. We found that the G1/G0 population increased 12.7 ±0.9% and 11.4±1.7% in the HMEC-LXSN and HMEC-cyto cells, respectively (Fig. 6A). One-way ANOVA was performed and no significant difference was found between HMEC-LXSN and HMEC-cyto cells. Additionally, no increase in the sub-G1 population was observed in HMEC-LXSN or HMEC-cyto cells in response to Tam (data not shown). We used several additional approaches to determine whether Tam induced apoptosis in our HMEC models. First, we tested for caspase 3/7 activation using a fluorometric caspase activity assay. While we observed increased caspase activity in response to taxol, we did not observe increased caspase activity in HMECs treated with Tam for 24 hours (data not shown). Furthermore, the addition of the pan-caspase inhibitor Z-VAD-FMK did not inhibit Tam-induced cell death in HMECs when
tested by MTT (Fig. 6B). To further rule out the possibility that Tam induced apoptosis in HMECs, we performed annexin V (AnV)/7-AAD staining on HMEC-control and HMEC-cyto cells. We found that Tam treatment for 24 hours resulted in a statistically significant decreases in viable cells (AnV-/7-AAD-) in both HMEC-LXSN and HMEC-cyto cells. A statistically significant difference in the Tam-treated AnV-/7-AAD- populations was observed between HMEC-LXSN and HMEC-cyto cells, with HMEC-cyto cells having more viable cells compared to HMEC-LXSN following Tam treatment. An increase in the AnV+/7-AAD+ (late apoptotic or necrotic) population was observed in both HMEC-LXSN and HMEC-cyto cells in response to Tam. Again, a statistically significant difference between HMEC-LXSN and HMEC-cyto Tam treated cells was observed (Fig. 6C). Fewer HMEC-cyto cells were AnV+/7-AAD+. A significant increase in the AnV+/7-AAD+ population, indicative of early apoptosis, was not observed in either cell line at 24 hours or at an earlier 6 hour time point (data not shown). Taken together, the data presented in Fig. 6 suggests Tam induces apoptosis-independent cell death, which likely involves necrosis, and is inhibited by expression of PELP1-cyto.
Fig 6. Cytoplasmic PELP1 and ERRγ protect cells from Tam-induced autophagy. A, Cells in G1/G0 from cell cycle analysis of propidium iodide stained HMEC-LXSN and HMEC-Cyto cells treated with vehicle control or 1.0 μM Tam for 24 hours. A student’s T-Test was performed to determine statistical significance for changes in G1/G0 cell populations in response to Tam. P-values are given for the indicated comparisons. B, MTT assay of HMEC-hTERT cells treated with 0.5 or 1.0 μM Tam and ethanol control or 25 μM Z-VAD-FMK. A student’s T-Test was performed to determine statistical significance between control and Z-VAD-FMK treated cells in the presence of Tam. “ns” indicates no statistically significant differences were observed. C, Annexin V/7-AAD staining of HMEC-LXSN and HMEC-cyto cells treated with 1.0 μM Tam for 24 hours. Student’s T-test was performed to determine statistical significance between Tam treated HMEC-LXSN and HMEC-cyto cells; p-values are shown for the indicated comparisons. D, Western blotting for LC3-I and LC3-II from HMEC-LXSN, HMEC-WT-1/WT-2, and HMEC-cyto lysates treated with ethanol control, 1 μM Tam, or 2 μM Tam for 48 hours. E, Western blot for LC3-II of whole cell lysates from HMECs expressing control or ERRγ overexpression vector, treated with control or 0.5, 1, or 2 μM Tam for 24 hours. F, Western blot for LC3 from whole cell lysates of HMEC-LXSN and HMEC-cyto cells expressing either shControl or shERRγ, treated with ethanol control or 1 μM Tam for 24 hours.

doi:10.1371/journal.pone.0121206.g006
Tam is known to induce autophagy [32,41,42]. Processing of LC3-I to LC3-II and subsequent accumulation of LC3-II is a marker of autophagy. To investigate the possibility that Tam treatment induced autophagy-associated necrosis in HMECs, we examined LC3-II levels in response to Tam in HMEC-LXSN, HMEC-PELP1-wt, and HMEC-PELP1-cyto cells by Western blot. Cell cultures were treated with increasing concentrations of Tam (1.0 and 2.0 μM) for 24 hours. Protein lysates were collected and separated by SDS-PAGE. Western blotting for LC3 revealed that Tam induced LC3-II accumulation in HMEC-LXSN and HMEC-PELP1-wt, but LC3-II accumulation was reduced in HMEC-PELP1-cyto cells compared to control cells (Fig. 6D).

To determine whether ERRγ expression has an effect on Tam-induced autophagy in HMECs, we examined LC3-II levels in response to Tam in MCF10A cells stably expressing vector control or ERRγ. MCF10A cells stably expressing ERRγ were more resistant to Tam-induced cell death (Fig. 4E). In cell cultures treated with increasing concentrations of Tam for 24 hours, we found that overexpression of ERRγ reduced Tam-induced LC3-II accumulation (Fig. 6E). We also examined Tam-induced LC3-II accumulation in HMEC-hTERT's expressing either shRNA control or ERRγ shRNA. Tam treatment of HMEC-LXSN cells expressing ERRγ shRNA resulted in a significant increase in LC3-II accumulation compared to HMEC-LXSN control cells. Similarly, ERRγ knockdown in HMEC-cyto cells also resulted in a significant increase in LC3-II accumulation in response to Tam (Fig. 6F). Together, the ERRγ overexpression and knockdown MTT data presented in Fig. 4 and the LC3 data presented in Fig. 6 indicate that PELP1-cyto and ERRγ expression suppresses Tam-induced LC3-II accumulation and autophagy-associated necrosis.

Discussion

Herein, our study demonstrates a novel link between cytoplasmic PELP1 signaling, ERRγ, and cell survival in the presence of Tam. Our in vivo and in vitro findings suggest that PELP1 may be a useful biomarker of cell survival in response to Tam. PELP1 was localized to the cytoplasm in 36% of RPFNA samples from women at high-risk for developing breast cancer, and our in vitro findings show that cytoplasmic PELP1 localization promotes HMEC survival in response to Tam. Furthermore, we show that PELP1-cyto expression enhances ERRγ-dependent gene expression and that ERRγ expression promotes Tam resistance, in part, by blocking autophagy-associated necrosis. These data suggest an alternate mechanism of HMEC cell survival in the presence of Tam that could decrease the effectiveness of Tam chemoprevention.

PELP1 and ERRγ as biomarkers of Tam resistance

The normal mammary gland is comprised of a heterogeneous population of cells, including luminal epithelial cells, basal myoepithelial cells, stromal cells, and immune cells. Relative to ER+ invasive breast cancer, which expresses high levels of ER, approximately 90% of normal mammary epithelial cells do not express ER and only 5–10% express moderate levels of ER [43]. Importantly, while the normal mammary gland expresses low ER levels compared to ER+ breast cancer, the normal mammary gland is clearly responsive to estrogens and Tam. Previously, we showed ER positivity in RPFNA samples from high-risk women predicts response to Tam chemoprevention [25]. Surprisingly, women with ER− RPFNA samples also responded to Tam chemoprevention. Thus, it is important to understand how both ER+ and ER− cells in high-risk breast tissue respond to Tam chemoprevention. Our data suggest that the presence of cytoplasmic PELP1 in high-risk epithelial cells could be an ER-independent biomarker that predicts survival of abnormal epithelial cells in the presence of Tam chemoprevention. There are limitations to the PELP1 IF results from RPFNA samples. The sample size is very small and currently
none of the women whose RPFNA samples were tested have developed breast cancer in the five years in since the samples were tested. To test the hypothesis that cytoplasmic PELP1 promotes breast cancer initiation we would need to examine PELP1 localization in 300 samples to obtain 85% power to detect a difference in the incidence rate for a new breast cancer of 0.60 vs 0.44, assuming a one-sided alpha of 0.05. This calculation assumes that we will observe cytoplasmic PELP1 staining about 36% of the samples. Additionally, these women would need to be followed for up to 8 years to have 85% power to detect a difference in 3-year incidence rate for a new breast cancer of 26% vs 17%, assuming a one-sided alpha of 0.05. Further evidence supporting a role for PELP1 as a biomarker of breast cancer initiation would provide a strong rationale for these larger and longer-term studies.

PELP1 and ERRγ both function to promote Tam-resistance

PELP1 is a large multi-domain protein that modulates many cell signaling pathways and biological processes. PELP1 was first identified as an ER co-activator protein and has since been shown to function as a transcriptional co-activator and co-repressor for several nuclear receptors and other transcription factors. PELP1 is frequently overexpressed in breast cancer [44], and altered localization to the cytoplasm has been described in approximately 50% of PELP1-positive breast tumors [17,18]. Vadlamudi and colleagues were the first to demonstrate altered PELP1 localization in human tumors and show that cytoplasmic PELP1 signaling promotes Tam resistance in ER+ breast cancer cell line models [17,18,45]. MCF-7 cells stably expressing PELP1-cyto had increased activation of Akt and Erk1/2 signaling, which promoted phosphorylation of ER at serines 167 and 118, respectively. While PELP1-induced signaling to Erk1/2 was dependent on the presence of ER, Akt activation was independent of ER expression [17]. PELP1-cyto signaling to Akt and Erk1/2 was confirmed in the MMTV-PELP1-cyto transgenic mouse model [18]. These mice formed widespread epithelial hyperplasia, which was resistant to Tam treatment. Interestingly, in primary HMECs Tam inhibits Akt serine 473 phosphorylation, which promotes Tam-induced cell death [27]. Overexpression of constitutively active Akt was shown to block Tam-induced cell death in this model. Thus, we hypothesized that PELP1-cyto-induced Akt activation, independent of ER, would inhibit Tam-induced cell death in our immortalized HMEC models. We found that PELP1-cyto does in fact protect HMECs from Tam-induced cell death, but independent of Akt activation. As Erk1/2 activation also plays a role in Tam-resistance in ER+ breast cancer, we also tested our models for Erk1/2 activation. Similar to our Akt studies, we did not observe a significant increase in Erk1/2 activation in our cell line models. The modest effect of Akt and MEK inhibitors on Tam-induced cell death suggests that other signaling mechanisms are likely to be more important.

Similar to PELP1, ERRγ promotes breast cancer progression both in vivo and in vitro. In vivo, ERRγ expression is correlated with ER expression [12]. While this was initially interpreted as ERRγ being a favorable prognostic indicator, subsequent studies have found ERRγ expression to be associated with positive lymph node status [46] and an increased risk of recurrence in ER+ breast cancer [14,38]. In vitro studies support the in vivo observation, where it has been found that ERRγ promotes Tam resistance in ER+ breast cancer cell line models [13,14]. Tam-resistant SUM44 invasive lobular carcinoma cells (LCCTam) were found to upregulate ERRγ expression. ERRγ knockdown in LCCTam cells resulted in enhanced sensitivity to Tam, while ERRγ overexpression promoted Tam resistance in the parental SUM44 cell line [13]. Riggins and colleagues went on to show that Erk signaling stabilizes ERRγ protein levels via phosphorylation of specific serine residues, which contributes to ERRγ-induced Tam resistance in MCF-7 and SUM44 cell lines [14]. Our studies in HMECs compliment these previous reports. Overexpression of ERRγ enhanced cell survival, while ERRγ knockdown sensitized
PELP1-cyto-expressing HMECs to Tam-induced cell death (Fig. 4). Because our cell line models do not express ER, our studies suggest that mechanistically ERRγ contributes to Tam-resistance independent of ER signaling. Additionally, our data suggest that inhibition of ERRγ will sensitize ER-low pre-malignant or atypical mammary epithelial cells expressing cytoplasmic PELP1 to Tam chemoprevention.

**PELP1 and ERRγ signaling mediate resistance to Tam-induced autophagy associated necrosis**

Tam’s actions have primarily been studied in ER+ breast cancer cell line models *in vitro* and *in vivo*. In ER+ breast cancer cell line models, Tam promotes cell cycle arrest, apoptosis, and autophagy [34,36]. Early studies found that treatment of cells with Tam, 4-OH-Tam, and other anti-estrogens resulted in ER-dependent G1 cell cycle arrest (reviewed in [34]). Interestingly, Tam’s ER-independent effects have been observed in both ER+ and ER− cell lines. In most of the studies describing ER-independent effects, Tam induced apoptosis and/or autophagy at 5–10 μM concentrations of Tam. In our immortalized HMEC lines, we observed Tam-induced autophagy starting at 0.25 μM and complete toxicity at 2 μM, well below therapeutic tissue concentrations achieved *in vivo*. Similar to other reports in ER− cells lines [32], our results suggest that Tam-induced cell death is dependent on autophagy-associated necrosis.

While PELP1 was previously shown to localize to autophagosomes [47,48], the work presented here is the first to show a definitive role for PELP1 and ERRγ in autophagy. Prior reports suggest that the PELP1-interacting protein, HRS, promotes PELP1 autophagosome localization in response to resveratrol. The authors suggest that resveratrol-induced autophagy selectively results in PELP1 degradation to promote the inhibitory effects of resveratrol, but knockdown or overexpression of PELP1 was not performed to determine whether PELP1 expression affects cell proliferation or survival in the presence of resveratrol. Interestingly, we did not observe changes in PELP1 protein levels after Tam treatment or co-localization of PELP1 and LC3 following Tam treatment; this suggests that Tam-induced autophagy does not target PELP1 for degradation (data not shown). The results presented here indicate that PELP1-cyto, but not wild-type PELP1, promotes cell survival in the presence of an autophagy-inducing stimulus. Further studies are needed to determine whether enhanced cell survival in the presence of PELP1-cyto is specific to Tam or can be expanded to other autophagy inducing compounds.

The predominate transcriptional targets of ERRs are involved in metabolism and mitochondrial biogenesis (reviewed in [11]). It is therefore surprising—given the intimate connection between nutrient conditions, cell metabolism, and autophagy [49]—that a connection between ERRs and autophagy has not been described. Our data clearly show that overexpression of ERRγ inhibits LC3-II accumulation in response to Tam, while ERRγ knockdown enhances Tam-induced LC3-II accumulation (Fig. 6). While we have not identified the ERRγ-dependent molecular mechanisms or gene targets associated with the autophagy response, it is likely that future global gene expression studies will help unravel these questions. Interestingly, indirect evidence for a link between ERRγ and autophagy does exist. Hypoxia induces autophagy in the tumor microenvironment to promote cell survival and tumorigenesis [50]. Interestingly, hypoxia also induces ERRγ expression and ERRγ-dependent gene expression [51]. This study did not examine the effect of ERRγ overexpression or knockdown in response to hypoxia, so it is unknown how ERRγ expression affects hypoxia-induced cell death or if this is associated with enhanced autophagy.
Future Directions and Conclusion

Our study is the first to report a link between PELP1-cyto signaling and ERRγ. Indeed, PELP1 has been shown to interact with, and act as a transcription co-regulator for many nuclear receptors including ERRα (reviewed in [52] and [53]). Although possible, our data does not suggest a direct interaction between PELP1 and ERRγ. Instead, we propose that PELP1-induced signaling in the cytoplasm promotes activation of ERRγ and specific ERRγ-dependent gene expression to promote cell survival in response to Tam. We show that PELP1-cyto induces expression of MAOB, a known ERRγ-dependent gene, and that ERRγ regulates a known PELP1-regulated gene, MMP-3. As inhibitors of MAOB (rasagiline mesylate) and MMP-3 (UK-356618) are available, we tested these compounds in MTT assays to determine whether they sensitize PELP1-cyto cells to Tam. Unfortunately, neither compound sensitized PELP1-cyto expressing cells to Tam (data not shown). Therefore, it is likely that other PELP-cyto-induced, ERRγ-dependent genes exist and play a role in response to Tam chemoprevention. Global gene expression analysis of PELP1-cyto/ERRγ knockdown cells will likely reveal novel gene targets important for response to autophagy-inducing compounds.

In conclusion, our data support a model in which altered PELP1 localization to the cytoplasm during early mammary carcinogenesis has the potential to alter response to Tam chemoprevention. The molecular mechanism involves cytoplasmic PELP1 signaling that promotes ERRγ-dependent gene expression, which functions to promote cell survival in the presence of Tam chemoprevention. We hypothesize that targeting PELP1, PELP1 downstream signaling, or ERRγ would promote Tam-induced cell death in atypical mammary epithelial cells. Thus, PELP1 localization, and/or ERRγ activation could be developed as a biomarker for response to Tam chemoprevention and could be targeted to enhance the efficacy of Tam chemoprevention.

Acknowledgments

We are grateful to Martha Stampfer (Lawrence Berkley National Laboratory) for the gift of the 240Lp16sMY human mammary epithelial cell line, Gloria Broadwater (Duke University) for statistical consultation, and Michael Franklin (University of Minnesota) for editing of the manuscript. This work was supported in part by NIH P30 CA77598 for utilizing the Masonic Cancer Center, University of Minnesota Flow cytometry shared resource.

Author Contributions

Conceived and designed the experiments: BJG VLS JHO. Performed the experiments: BJG TMR-A SLW JN JHO. Analyzed the data: BJG TMR-A SLW JN JHO. Contributed reagents/materials/analysis tools: SLW VLS JHO. Wrote the paper: BJG JN VLS JHO.

References

1. Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, et al. (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 90: 1371–1388. PMID:9747868
2. Tan CK, Chow PK, Findlay M, Wong C, Machin D (2000) Use of tamoxifen in hepatocellular carcinoma: a review and paradigm shift. J Gastroenterol Hepatol 15: 725–729. PMID:10937676
3. Benson JR, Wakefield LM, Baum M, Colletta AA (1996) Synthesis and secretion of transforming growth factor beta isoforms by primary cultures of human breast tumour fibroblasts in vitro and their modulation by tamoxifen. Br J Cancer 74: 352–358. PMID:8695348
4. O’Brian CA, Liskamp RM, Solomon DH, Weinstein IB (1985) Inhibition of protein kinase C by tamoxifen. Cancer Res 45: 2462–2465. PMID:3157445
5. Lopes MC, Vale MG, Carvalho AP (1990) Ca2(+)-dependent binding of tamoxifen to calmodulin isolated from bovine brain. Cancer Res 50: 2753–2758. PMID: 2139630
6. Thomas P, Pang Y, Filardo EJ, Dong J (2005) Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology 146: 624–632. PMID: 15539556
7. Tremblay GB, Bergeron D, Giguere V (2001) 4-Hydroxytamoxifen is an isoform-specific inhibitor of orphan estrogen-receptor-related (ERR) nuclear receptors beta and gamma. Endocrinology 142: 4572–4575. PMID: 11564725
8. Friedman ZY (1998) Recent advances in understanding the molecular mechanisms of tamoxifen action. Cancer Invest 16: 391–396. PMID: 9679530
9. Greschik H, Wurtz JM, Sanglier S, Bourguet W, van Dorsselaer A, et al. (2002) Structural and functional evidence for ligand-independent transcriptional activation by the estrogen-related receptor 3. Mol Cell 9: 303–313. PMID: 11864064
10. Lu X, Peng L, Lv M, Ding K (2012) Recent advance in the design of small molecular modulators of estrogen-related receptors. Curr Pharm Des 18: 3421–3431. PMID: 22632982
11. Deblois G, Giguere V (2011) Functional and physiological genomics of estrogen-related receptors (ERRs) in health and disease. Biochim Biophys Acta 1812: 1032–1040. doi: 10.1016/j.bbadis.2010.12.009 PMID: 21172432
12. Ariazi EA, Clark GM, Mertz JE (2002) Estrogen-related receptor alpha and estrogen-related receptor gamma associate with unfavorable and favorable biomarkers, respectively, in human breast cancer. Cancer Res 62: 6510–6518. PMID: 12438245
13. Riggins RB, Lan JP, Zhu Y, Klimach U, Zwart A, et al. (2008) ERRgamma mediates tamoxifen resistance in novel models of invasive lobular breast cancer. Cancer Res 68: 8908–8917. doi: 10.1158/0008-5472.CAN-08-2669 PMID: 18974135
14. Heckler MM, Thakor H, Schafer CC, Riggins RB (2014) ERK/MAPK regulates ERRgamma expression, transcriptional activity and receptor-mediated tamoxifen resistance in ER+ breast cancer. FEBS J 281: 2431–2442. doi: 10.1111/febs.12468
15. Vadlamudi RK, Manavathi B, Balasenthil S, Nair SS, Yang Z, et al. (2005) Functional implications of altered subcellular localization of PELP1 in breast cancer cells. Cancer Res 65: 7724–7732. PMID: 16140940
16. Vadlamudi RK, Wang RA, Mazumdar A, Kim Y, Shin J, et al. (2001) Molecular cloning and characterization of PELP1, a novel human coregulator of estrogen receptor alpha. J Biol Chem 276: 38272–38279. PMID: 11481323
17. Vadlamudi RK, Manavathi B, Balasenthil S, Nair SS, Yang Z, et al. (2005) Functional implications of altered subcellular localization of PELP1 in breast cancer cells. Cancer research 65: 7724–7732. PMID: 16140940
18. Kumar R, Zhang H, Holm C, Vadlamudi RK, Landberg G, et al. (2009) Extranuclear coactivator signaling confers insensitivity to tamoxifen. Clinical cancer research: an official journal of the American Association for Cancer Research 15: 4123–4130. doi: 10.1158/1078-0432.CCR-08-2347 PMID: 19470742
19. Ostrander JH, McMahon CM, Lem S, Millon SR, Brown JQ, et al. (2010) Optical redox ratio differentiates breast cancer cell lines based on estrogen receptor status. Cancer Res 70: 4759–4766. doi: 10.1158/0008-5472.CAN-09-2572 PMID: 20460512
20. Garbe JC, Yrba L, Sputova K, Fuchs L, Novak P, et al. (2014) Immortalization of normal human mammary epithelial cells in two steps by direct targeting of senescence barriers does not require gross genomic alterations. Cell Cycle 13: 3423–3435. doi: 10.4161/cc.2014.954456 PMID: 25485586
21. Debnath J, Muthuswamy SK, Bruges J (2003) Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods 30: 256–268. PMID: 12798140
22. Garbe JC, Bhattacharya S, Merchant B, Bassett E, Swisschelm K, et al. (2009) Molecular distinctions between stasis and telomere attrition senescence barriers shown by long-term culture of normal human mammary epithelial cells. Cancer Res 69: 7557–7568. doi: 10.1158/0008-5472.CAN-09-0270 PMID: 19773443
23. Krishnam A (1975) Rapid flow cytometric analysis of mammalian cell cycle by propidium iodide staining. J Cell Biol 66: 188–193. PMID: 49354
24. Twentyman PR, Luscombe M (1987) A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br J Cancer 56: 279–285. PMID: 3663476
25. Baker JC Jr., Ostrander JH, Lem S, Broadwater G, Bean GR, et al. (2008) ESR1 promoter hypermethylation does not predict atypia in RPFNA nor persistent atypia after 12 months tamoxifen chemoprevention. Cancer Epidemiol Biomarkers Prev 17: 1884–1890. doi: 10.1158/1055-9965.EPI-07-2696 PMID: 18708376
26. Dietze EC, Caldwell LE, Grupin SL, Mancini M, Seewaldt VL (2001) Tamoxifen but not 4-hydroxytamoxifen initiates apoptosis in p53(-) normal human mammary epithelial cells by inducing mitochondrial depolarization. J Biol Chem 276: 5384–5394. PMID: 11094056

27. Dietze EC, Troch MM, Bean GR, Heffner JB, Bowie ML, et al. (2004) Tamoxifen and tamoxifen ethyl bromide induce apoptosis in acutely damaged mammary epithelial cells through modulation of AKT activity. Oncogene 23: 3851–3862. PMID: 14990993

28. Seewaldt VL, Mrozek K, Dietze EC, Parker M, Caldwell LE (2001) Human papillomavirus type 16 E6 inactivation of p53 in normal human mammary epithelial cells promotes tamoxifen-mediated apoptosis. Cancer Res 61: 616–624. PMID: 11212259

29. Charlier C, Chariot A, Antoine N, Merville MP, Gielen J, et al. (1995) Tamoxifen and its active metabolite inhibit growth of estrogen receptor-negative MDA-MB-435 cells. Biochem Pharmacol 49: 351–358. PMID: 7853222

30. Grenman SE, Van Dyke DL, Worsham MJ, England B, McClatchey KD, et al. (1990) Phenotypic characterization, karyotype analysis and in vitro tamoxifen sensitivity of new ER-negative vulvar carcinoma cell lines, UM-SCV-1A and UM-SCV-1B. Int J Cancer 45: 920–927. PMID: 2335395

31. Grenman SE, Worsham MJ, Van Dyke DL, England B, McClatchey KD, et al. (1990) Establishment and characterization of UM-EC-2, a tamoxifen-sensitive, estrogen receptor-negative human endometrial carcinoma cell line. Gynecol Oncol 37: 188–199. PMID: 2344964

32. Cho KS, Yoon YH, Choi JA, Lee SJ, Koh JY (2012) Induction of autophagy and cell death by tamoxifen in cultured retinal pigment epithelial and photoreceptor cells. Invest Ophthalmol Vis Sci 53: 5344–5353. doi: 10.1167/iovs.12-9827 PMID: 22786900

33. Robertson DW, Katzenellenbogen JA, Long DJ, Rorke EA, Katzenellenbogen BS (1982) Tamoxifen antiestrogens. A comparison of the activity, pharmacokinetics, and metabolic activation of the cis and trans isomers of tamoxifen. J Steroid Biochem 16: 1–13. PMID: 7062732

34. MacGregor JI, Jordan VC (1998) Basic guide to the mechanisms of antiestrogen action. Pharmacol Rev 50: 151–196. PMID: 9647865

35. Frasor J, Chang EC, Komm B, Lin CY, Vega VB, et al. (2006) Gene expression preferentially regulated by tamoxifen in breast cancer cells and correlations with clinical outcome. Cancer Res 66: 7334–7340. PMID: 16849584

36. Mandlekar S, Kong AN (2001) Mechanisms of tamoxifen-induced apoptosis. Apoptosis 6: 469–477. PMID: 11595837

37. Liu X, Matsushima A, Okada H, Shimohigashi Y (2010) Distinction of the binding modes for human nuclear receptor ERRgamma between bisphenol A and 4-hydroxytamoxifen. J Biochem 148: 247–254. doi: 10.1093/jb/mvq056 PMID: 20542892

38. Riggins RB, Mazzotta MM, Maniya OZ, Clarke R (2010) Orphan nuclear receptors in breast cancer pathogenesis and therapeutic response. Endocr Relat Cancer 17: R213–231. doi: 10.1677/ERC-10-0058 PMID: 20576803

39. Ren Y, Jiang H, Ma D, Nakaso K, Feng J (2011) Parkin degrades estrogen-related receptors to limit the expression of monoamine oxidases. Hum Mol Genet 20: 1074–1083. doi: 10.1093/hmg/ddq550 PMID: 21177257

40. Roy S, Chakravarty D, Cortez V, De Mukhopadhyay K, Bandypadhyay A, et al. (2012) Significance of PELP1 in ER-negative breast cancer metastasis. Mol Cancer Res 10: 25–33. doi: 10.1158/1541-7786.MCR-11-0456 PMID: 22086908

41. Bursch W, Karwan A, Mayer M, Dornetshuber J, Frohwein U, et al. (2008) Cell death and autophagy: cytokines, drugs, and nutritional factors. Toxicology 254: 147–157. doi: 10.1016/j.tox.2008.07.048 PMID: 18694801

42. Kohli L, Kaza N, Coric T, Byer SJ, Brossier NM, et al. (2013) 4-Hydroxytamoxifen induces apoptotic death through K-Ras degradation. Cancer Res 73: 4395–4405. doi: 10.1158/0008-5472.CAN-12-3765 PMID: 23722551

43. Anderson E, Clarke RB, Howell A (1998) Estrogen responsiveness and control of normal human breast proliferation. J Mammary Gland Biol Neoplasia 3: 23–35. PMID: 10819502

44. Habashy HO, Powe DG, Rakha EA, Ball G, Macmillan RD, et al. (2010) The prognostic significance of PELP1 expression in invasive breast cancer with emphasis on the ER-positive luminal-like subtype. Breast cancer research and treatment 120: 603–612. doi: 10.1007/s10549-009-0419-9 PMID: 19495959

45. Vallabhaneni S, Nair BC, Cortez V, Challa R, Chakravarty D, et al. (2010) Significance of ER-Src axis in hormonal therapy resistance. Breast cancer research and treatment.
46. Ijichi N, Shigekawa T, Ikeda K, Horie-Inoue K, Fujimura T, et al. (2011) Estrogen-related receptor gamma modulates cell proliferation and estrogen signaling in breast cancer. J Steroid Biochem Mol Biol 123: 1–7. doi: 10.1016/j.jsbmb.2010.09.002 PMID: 20883782

47. Ohshiro K, Rayala SK, El-Naggar AK, Kumar R (2008) Delivery of cytoplasmic proteins to autophagosomes. Autophagy 4: 104–106. PMID: 18000392

48. Ohshiro K, Rayala SK, Kondo S, Gaur A, Vadlamudi RK, et al. (2007) Identifying the estrogen receptor coactivator PELP1 in autophagosomes. Cancer research 67: 8164–8171. PMID: 17804729

49. Ryter SW, Cloonan SM, Choi AM (2013) Autophagy: a critical regulator of cellular metabolism and homeostasis. Mol Cells 36: 7–16. doi: 10.1007/s10059-013-0140-8 PMID: 23708729

50. Koritzinsky M, Wouters BG (2013) The roles of reactive oxygen species and autophagy in mediating the tolerance of tumor cells to cycling hypoxia. Semin Radiat Oncol 23: 252–261. doi: 10.1016/j.semradonc.2013.05.006 PMID: 24012339

51. Lee JH, Kim EJ, Kim DK, Lee JM, Park SB, et al. (2012) Hypoxia induces PDK4 gene expression through induction of the orphan nuclear receptor ERRgamma. PLoS One 7: e46324. doi: 10.1371/journal.pone.0046324 PMID: 23050013

52. Girard BJ, Daniel AR, Lange CA, Ostrander JH (2014) PELP1: A review of PELP1 interactions, signaling, and biology. Mol Cell Endocrinol 382: 642–651. doi: 10.1016/j.mce.2013.07.031 PMID: 23933151

53. Vadlamudi RK, Rajhans R, Chakravarty D, Nair BC, Nair SS, et al. (2010) Regulation of aromatase induction by nuclear receptor coregulator PELP1. The Journal of steroid biochemistry and molecular biology 118: 211–218. doi: 10.1016/j.jsbmb.2009.09.009 PMID: 19800002