Extranuclear Coactivator Signaling Confers Insensitivity to Tamoxifen
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Abstract

Purpose: Tamoxifen is one of many standard therapeutic options currently available for estrogen receptor-α-positive breast cancer patients. Emerging data have suggested that levels of estrogen receptor coregulatory proteins play a significant role in acquiring resistance to antiestrogen action. It has been suggested that high levels of estrogen receptor coactivators and its mislocalization may enhance the estrogen agonist activity of tamoxifen and contribute to tamoxifen resistance.

Experimental Design: In an effort to understand the impact of nongenomic signaling and its contribution to hormone resistance in a whole-animal setting, we generated a transgenic mouse expressing a cytoplasmic version of proline-, glutamic acid–, and leucine-rich protein–1 (PELP1) mutant defective in its nuclear translocation (PELP1-cyto) and implanted these mice with tamoxifen pellets to assess its responsiveness.

Results: We show that mammary glands from these mice developed widespread hyperplasia with increased cell proliferation and enhanced activation of mitogen-activated protein kinase and AKT as early as 12 weeks of age. Treatment with tamoxifen did not inhibit this hyperplasia; instead, such treatment exaggerated hyperplasia with an enhanced degree of alteration, indicative of hypersensitivity to tamoxifen. Analysis of molecular markers in the transgenic mammary glands from the tamoxifen-treated transgenic mice showed higher levels of proliferation markers proliferating cell nuclear antigen and activated mitogen-activated protein kinase than in untreated PELP1-cyto cell-derived mice. We also found that nude mice with MCF-7/PELP1-cyto cell-derived tumor xenografts did not respond to tamoxifen. Using immunohistochemical analysis, we found that 43% of human breast tumor samples had high levels of cytoplasmic PELP1, which shows a positive correlation between tumor grade and proliferation. Patients whose tumors had high levels of cytoplasmic PELP1 exhibited a tendency to respond poorly to tamoxifen compared with patients whose tumors had low levels of cytoplasmic PELP1.

Conclusions: These findings suggest that PELP1 localization could be used as a determinant of hormone sensitivity or vulnerability. The establishment of the PELP1-cyto transgenic mouse model is expected to facilitate the development of preclinical approaches for effective intervention of breast tumors using cytoplasmic coregulators and active nongenomic signaling.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Grants: NIH grant CA 90970 (R. Kumar).

Received 9/9/08; revised 3/12/09; accepted 3/12/09; published OnlineFirst 5/26/09.

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The steroid hormone 17-β estradiol is known to exert its cellular effects in tissues by binding to its major target, the estrogen receptor-α. Estrogen receptor, a ligand-dependent transcription factor, has been implicated in the progression of breast cancer, as evidenced by the fact that almost 70% of breast tumors are estrogen receptor–positive at the time of early diagnosis (1, 2). Currently, tamoxifen represent one of many available standard treatments for estrogen receptor–positive breast cancer patients.
Translational Relevance

Proline-, glutamic acid-, and leucine-rich protein–1 is widely expressed in breast cancer cells and its expression is deregulated in breast tumors. In this study, we show that mammary glands from a transgenic mouse expressing a cytoplasmic version of proline-, glutamic acid-, and leucine-rich protein–1 mutant defective in its nuclear translocation developed widespread hyperplasia and enhanced activation of extra nuclear signaling events. Treatment with tamoxifen did not inhibit this hyperplasia but rather exaggerated hyperplasia with an enhanced degree of alteration, indicative of hypersensitivity to tamoxifen. In the future, preclinical investigations using this animal model system that accurately mimic characteristics of estrogen independence would greatly assist in more efficient design of clinical trials by predicting hormonal effects and their mechanisms of action of developing resistance.

(3), although most patients who respond to tamoxifen eventually acquire tamoxifen resistance, allowing such tumor cells to metastasize to distant sites (4). Consequently, sustained tamoxifen resistance continues to be one of the major limiting factors in the management of advanced breast cancer. One of the major challenges for endocrine therapy of advanced breast cancer is to better understand the cellular basis of tamoxifen resistance and to develop novel approaches to overcoming estrogen resistance (5). Emerging data suggest that the status and functionality of estrogen receptor coregulatory proteins in the nucleus, as well as in the cytoplasm, and cross-talking between estrogen receptor coregulators and the components of growth factor receptors play important roles in acquiring resistance to antiestrogen action (6, 7).

Over the years, it has become apparent that the transcriptional effects of estrogen receptor are not limited to ligand-activated estrogen receptor in the nucleus but are also influenced by the participation of estrogen receptor in the cytoplasmic and membrane-mediated signaling (also known as nongenomic or extra-nuclear signaling) through complex interactions with coregulatory molecules (6, 9). However, the significance of cytoplasmic activators in nongenomic signaling is just beginning to be realized, and their role in resistance to selective estrogen receptor modulators remains poorly understood. One such molecule with a dual role of action in the nucleus and cytoplasm is proline-, glutamic acid-, and leucine-rich protein–1 (PELP1; refs. 10–12), a unique coactivator that plays an important role in the genomic (13) and nongenomic actions of estrogen receptor (14). PELP1 is widely expressed in breast and ovarian cancer cells, and its expression is deregulated in breast (15) and ovarian (16) tumors. PELP1 is an estrogen receptor–responsive gene, and its expression is differentially regulated by selective estrogen receptor modulators, depending on the cell line (17). Although PELP1 is predominantly localized in the nucleus in hormonally responsive tissues (18), a large body of evidence has shown that PELP1 is localized predominantly in the cytoplasm in a significant proportion of human breast (15), endometrial (19), and salivary gland (20) carcinomas.

Studies have also shown that differential compartmentalization of PELP1 may play a crucial role in modulating the status of nongenomic signaling using molecular mechanisms that remain poorly understood (15, 21).

Because, under physiologic conditions, PELP1 is localized in the cytoplasm, MCF-7 breast cancer cells that mimic PELP1 cytoplasmic localization in tumors (PELP1-cyto cells) have been developed as relevant model systems for studying nongenomic signaling (15). PELP1-cyto cells are hypersensitive to estrogen but resistant to tamoxifen (15) and form tumors in nude mice (15). In addition, relative to the parental MCF-7 cells, PELP1-cyto cells exhibit an increased association between PELP1 and Src, enhanced stimulation of mitogen-activated protein kinase (MAPK), and constitutive activation of the Akt pathway. The altered localization of PELP1 to the cytoplasm has been shown to be sufficient to trigger its interaction with the p85 subunit of phosphatidylinositol-3-kinase, leading to its activation (15).

In addition, PELP1 interacts with epidermal growth factor receptor and participates in growth factor–mediated estrogen receptor transactivation functions (22). Together, these findings suggest that PELP1 localization could potentially be used as a determinant of hormone sensitivity. However, our current understanding of the nongenomic action of PELP1 is derived from in vitro cell culture and xenograft models, and validation in a physiologically relevant whole-animal model awaits further investigation. In this context, we present a transgenic mouse model of PELP1-cyto nongenomic signaling and its inability to respond to the antiestrogenic action of tamoxifen.

Materials and Methods

Generation of PELP1-cyto transgenic mice. A mouse mammary tumor virus (MMTV)–human PELP1-cyto transgenic construct was created by subcloning T7-tagged PELP1-cyto cDNA using sites HindIII-XbaI (blunted) into HindIII-EcoRI (blunted) of the MMTV-SV40-BssK vector (23). The transgene was excised from plasmid DNA, and the 3.9 linear fragment–containing promoter sequences PELP1 cDNA and SV40 polyadenylation signals was injected to the pronuclei of a B6D2F1/J mouse embryos. Finder lines were identified by Southern blotting of tail DNA digested, which showed a band of 1.6-kb PELP1-cyto cDNA. Genotyping was done by using the PCR assay. The primers used were forward primer to the 7′-epitope encoding region (5′-CTGCAGACGGCTCAATTCTCGCTAC) and reverse primer (5′-CAAGAATTTCTCACCTCCCATGTGTCG) corresponding to PELP1-cyto cDNA. These primers only amplify T7-tagged PELP1-cyto and do not recognize endogenous mouse PELP1. As expected, these primers specifically amplified 420-bp band in PELP1-cyto transgene–positive founder lines.

Mammary gland whole mounts, histology, and immunodetection and immunofluorescence. For whole-mount analysis, number 4 inguinal mammary glands were stained with carmine alun, as previously described (23). Briefly, the glands were fixed with acetic acid/ethanol (1:3) for 2 h and stained with 0.5% carmine/0.2% aluminum potassium sulfate for 16 h. After briefly being rinsed with distilled water, the mammary glands were dehydrated using graded ethanol, and lipids were removed with two changes of acetone. Finally, the glands were preserved in methyl salicylate. For histologic analysis, mammary-gland tissue was fixed in 10% neutral buffered formaldehyde and embedded in paraffin wax according to standard methods. Sections (4 μm) were stained with H&E. For immunostaining and immunofluorescence, the following primary antibodies were used: T7 tag were from Novagen; those against proliferating cell nuclear antigen were from Genetex; antibodies against AKT, phospho-AKT, and phospho-p44/42(Thr-202/ Tyr-204) were purchased from Cell Signaling; and extracellular signal-regulated kinase-1 and -2 were purchased from Santa Cruz Biotechnology. To detect

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bromodeoxyuridine (BrdUrd)–positive cells, a sterile solution of 5-bromo-2’-deoxyuridine (BrdUrd; 20mg/ml; Sigma-Aldrich) in PBS (pH 7.4) was administered to mice by i.p. injection (50 mg/kg). Mammary glands were harvested after 3 h, embedded in paraffin wax, and sectioned. BrdUrd incorporation was detected by immunohistochemistry using a mouse anti-BrdUrd monoclonal antibody.

Animal husbandry and treatment. Mice were housed at the animal care facility at the University of Texas MD Anderson Cancer Center. All procedures involving mice were conducted in accordance with NIH regulations about the use and care of experimental animals and were approved by the institutional review board. Mice were maintained on a mixed C57BL/6:DBA background. Line 5 of PELP1-cyto transgenic mice were used for further study. For tamoxifen pellet implantation, each group (intact and tamoxifen) included 10 mice. Age-matched wild-type (WT; most of them are littermates of the transgenic) were used as controls. Virgin mice were implanted with tamoxifen pellets (NE-361, Innovative Research of America) at 10 wk of age. Tissue sample were obtained at 19 wk of age for analysis.

Immunoblot and immunoprecipitation analysis. About 50 μg of total protein extract from cultured cells or mammary glands was used in the immunoblot assays. The lysates to immunoprecipitate T7-tagged proteins were immunoprecipitated for 2 h at 4°C using 50 μL of T7-agarose beads per milligram of protein.

Mouse xenograft studies. For the tumorigenesis studies, 4-wk-old, ovariectomized, female, athymic, nude mice (Charles River) were bilaterally injected with 5 × 10⁶ MCF-7 PELP1-cyto cells into the mammary fat pads, as previously described (15). Mice were divided in to two groups. One group received tamoxifen pellets, and the other group is the control. Tumors were allowed to grow for 5 wk, and tumor size was measured every 15 d. All animal procedures were done in compliance with the Institute Animal Care and Use Committee and the NIH Policy on Humane Care and Use of Laboratory Animals.

Results and Discussion

Recent studies using in vitro cell culture models have shown that cytoplasmic localization of PELP1 promotes resistance to tamoxifen (15). To validate these findings in an in vivo animal setting and to generate a mouse model of nongenomic signaling, we generated PELP1-cyto transgenic mice that express PELP1 in the cytoplasm by overexpression of a mutant of PELP1 (PELP1-cyto), in which the nuclear localization signal has been mutated from KKLK to EELE amino acids (Fig. 1A). As a means of targeting the expression of the PELP1-cyto transgene to the mammary gland, we placed PELP1-cyto cDNA under the control of the MMTV promoter, which directs transgene expression to the mammary gland by overexpression of a mutant of PELP1 (PELP1-cyto), in which the nuclear localization signal has been mutated from KKLK to EELE amino acids (Fig. 1A).

A means of targeting the expression of the PELP1-cyto transgene to the mammary gland, we placed PELP1-cyto cDNA under the control of the MMTV promoter, which directs transgene expression to the mammary gland in the early stages of puberty and is hormonally regulated by progesterone during estrus and pregnancy (23). Founders with integration of the transgene constructs into the genome were screened using PCR (Fig. 1B, bottom), and results were confirmed by Southern blot analysis (Fig. 1B, top). Founder line 5 of PELP1-cyto transgenic mice was used for the study. The protein product of the transgene

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**Fig. 1.** Generation of PELP1-cyto transgenic mice. A, schematic representation of the MMTV–T7–PELP1-cyto transgenic construct. B, Southern blot detection of the transgene in mouse tail genomic DNA (top). PCR (bottom) for genotype of transgenic mice using genomic DNA from the tail of different lines of transgenic and WT mice. P, plasmid as positive control; M, marker; C, control. C, transgene protein expression in the mammary glands, as detected by T7 immunoprecipitation, followed by Western blotting with T7 antibody. D, immunofluorescence analysis showing T7-tagged mutant PELP1 (red) in the cytoplasm of mammary epithelial cells and 4’,6-diamidino-2-phenylindole-stained nuclei (blue) in WT (top) and transgenic (bottom) mice.
was detected by immunoprecipitation using anti-T7 agarose beads, followed by immunoblotting with anti-T7 polyclonal antibody (Fig. 1C). Furthermore, to assess that the levels overexpressed cytoplasmic PELP1 in PELP1-cyto transgenic mice are higher than the endogenous nuclear PELP1 level, we did Western blot analysis with an anti-PELP1 antibody. We found that the amount of overexpressed cytoplasmic PELP1 is comparatively much higher than the endogenous nuclear PELP1 level (Supplementary Fig. 1A), and thus, the phenotype reported here will be predominantly under the influence of the cytoplasmic PELP1. In addition, immunofluorescence staining of paraffin section of the mammary gland with an anti-T7 antibody revealed PELP1-cyto transgene localization exclusively in the cytoplasm (Fig. 1D).

To investigate the effect of PELP1-cyto transgene on the development of mammary glands, we examined whole-mount preparations of mammary glands from littermates with matching estrous cycles at different developmental stages. We found that about 46% of transgenic females developed atypical hyperplasia at 12 weeks of age, and this became more abundant as mice aged, whereas WT littermates did not exhibit such phenotypes at the same ages (Fig. 2A). To determine whether the observed phenotypic alterations were the result of increased cell proliferation, DNA synthesis was measured in an in vivo BrdUrd incorporation assay. In mammary glands of transgenic mice, the proliferation index, calculated as the percentage of BrdUrd-positive cells among total epithelial cells, was significantly higher in transgenic than in WT mice (Fig. 2B). Consistent with our previous in vitro data (15), phosphorylated MAPK levels were significantly higher in transgenic than WT mice, as assayed by immunohistochemistry (Fig. 2C) and immunoblotting (Supplementary Fig. 2) with phospho-MAPK-specific antibody. Also consistent with an active nongenomic signaling, we found increased levels of phospho-AKT and phospho-estrogen receptor–Ser-118 in PELP1-cyto transgenic mice compared with levels in WT mice (Supplementary Fig. 2). To our knowledge, this is the first in vivo evidence that altered localization of estrogen receptor coactivator PELP1 promotes increased AKT and MAPK activation, leading to enhanced cell proliferation and thereby promoting mammary hyperplasia. However, none of the transgenic mice developed mammary gland tumors even after 2 years. This could be because we used the transgenic mice expressing MMTV–PELP1-cyto under the influence of hormones or because of the mixed genetic background of these animals, which is 25% C57BL/6 and 75% DBA or both. It is very likely that changing the strain background might result in tumor formation, as has been observed in other mammary gland models (24).

Next, we used this model to confirm the hypothesis that altered localization of PELP1 could participate in the development to resistance to tamoxifen. Transgenic and age-matched WT female virgin mice (n = 10 each group) were implanted with tamoxifen pellets (Innovative Research of America) at 10 weeks of age, before the development of detectable hyperplasia. The effect of tamoxifen was evaluated after 2.5 months of exposure (at 19 weeks of age; Supplementary Fig. 3). We found that, compared with intact transgenic mice, which exhibited 58% typical hyperplasia, tamoxifen treatment did not prevent or reduce the extent of hyperplasia in the treatment group (Fig. 3A). Consistent with this result, cell proliferation, as analyzed by proliferating cell nuclear antigen staining, was not significantly decreased in tamoxifen-treated mice compared with untreated transgenic mice (33 ± 9% versus 31 ± 9%; Fig. 3B). Furthermore, immunohistochemical analysis of mammary gland sections for phospho-MAPK revealed that tamoxifen exposure did not attenuate activation of MAPK induced by transgene (21 ± 6% versus 25 ± 7%; Fig. 3A and B). Taken together, these results

![Fig. 2. Cytoplasmic PELP1 in mammary epithelium causes hyperplasia and promotes nongenomic signaling.](image-url)
suggest that mammary epithelium harboring the PELP1-cyto transgene exhibits an increased nongenomic signaling and resists the antitumor activity of tamoxifen, which in turn could be due to the cumulative effects of the activation of the MAPK and Akt pathways, including posttranslational modification of estrogen receptor and its associated proteins.

Because PELP1-cyto transgenic mice did not form tumors, although they developed mammary hyperplasia, the role of cytoplasmic PELP1 in hormone resistance in mammary gland tumors remains unknown. To address this issue, we made use of the well-characterized MCF-7/cytoplasmic PELP1-expressing clones (PELP1-cyto; ref. 15). As expected, we found that PELP1-cyto was localized to the cytoplasm, whereas WT PELP1 was localized to the nucleus (Supplementary Fig. 1B). The PELP1-cyto clones are tamoxifen insensitive (Supplementary Fig. 1C) and form tumors in nude mice (15). Consistent with a previous report (15), PELP1-cyto clones readily formed tumors (14 of 14 mice) when injected into the mammary fat pads of nude mice. However, tamoxifen treatment did not prevent or reduce the extent of mammary tumorigenesis in these mice (Fig. 4A). Western blot analysis of lysates from these tumors consistently revealed that there was no change in the levels of activated MAPK after tamoxifen treatment (Fig. 4B). Immunohistochemical analysis revealed that there is also no reduction in the levels of phospho-Akt and proliferating cell nuclear antigen after tamoxifen treatment (Supplementary Fig. 4). Collectively, these findings suggest that cytoplasmic PELP1 expression is one of the important biological variables of hormone sensitivity in breast tumors.

Encouraged by the above results and to validate the clinical implication to the above data, we analyzed the cytoplasmic localization of PELP1 in a cohort of 331 breast tumor (234 estrogen receptor positive) samples (25) by immunohistochemistry. The cutoff limits between low and high tumors were as follows: tumors with no staining (i.e., negative), weak staining, or <50% tumor cells with a strong staining were classified as low PELP1,
and tumors with >50% tumor cells with a strong staining were classified as high PELP1. Results showed that 43% of the tumors had high levels of cytoplasmic PELP1 and that high PELP1 correlated with tumor grade (Nottingham histologic grade) and proliferation (Fig. 5A-D; Table 1). Nottingham histologic grade is assessed based upon three parameters: tubular structure, nuclear atypia, and mitotic count. The higher the score from these parameters, the more malignant the tumor is. This suggests that high PELP1 expression in the cytoplasm is coupled to a more aggressive tumor.

**Fig. 4.** Cytoplasmic PELP1–induced mammary gland tumors are insensitive to tamoxifen. A, mammary tumor xenografts induced with MCF-7/PELP1-cyto clones in nude mice are insensitive to tamoxifen. B, Western blot analysis showing levels of activated MAPK in lysates from the above tumors.

**Fig. 5.** Immunohistochemical analysis of cytoplasmic PELP1 in breast cancer specimens. A-D, breast tumors arranged in a tissue microarray were stained with PELP1 antibody (dilution, 1:2,500). A, a tumor negative for PELP1. B, low staining. C and D, strong staining.
Table 1. Distribution of cytoplasmic PELP1 staining in breast tumors according to tumor grade and proliferation

<table>
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<th>Variable</th>
<th>PELP1 cytoplasmic staining</th>
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<tr>
<td></td>
<td>Negative/low, n = 188 (57%)</td>
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<td>High, n = 143 (43%)</td>
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<td>III</td>
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<td>0.001</td>
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<tr>
<td>Ki-67</td>
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<td>0.001</td>
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<tr>
<td>0-10%</td>
<td>83</td>
<td>0.044</td>
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<tr>
<td>11-25%</td>
<td>51</td>
<td>0.401</td>
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<td>&gt;25%</td>
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Abbreviation: NHG, Nottingham histologic grade.
*Correlations were calculated using Spearman’s p.

References


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