**PELP1 Overexpression in the Mouse Mammary Gland Results in the Development of Hyperplasia and Carcinoma**

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**Abstract**
Estrogen receptor (ER) coregulator overexpression promotes carcinogenesis and/or progression of endocrine related-cancers in which steroid hormones are powerful mitogenic agents. Recent studies in our laboratory, as well as others, demonstrated that the estrogen receptor coregulator PELP1 is a proto-oncogene. PELP1 interactions with histone demethylase KDM1 play a critical role in its oncogenic functions and PELP1 is a prognostic indicator of decreased survival in patients with breast cancer. However, the in vivo significance of PELP1 deregulation during initiation and progression of breast cancer remains unknown. We generated an inducible, mammary gland-specific PELP1-expressing transgenic (Tg) mouse (MMTVrtTA-TetOPELP1). We found more proliferation, extensive side branching, and precocious differentation in PELP1-overexpressing mammary glands than in control glands. Aged MMTVrtTA-TetOPELP1 Tg mice had hyperplasia and preneoplastic changes as early as 12 weeks, and ER-positive mammary tumors occurred at a latency of 14 to 16 months. Mechanistic studies revealed that PELP1 deregulation altered expression of a number of known ER target genes involved in cellular proliferation (cyclin D1, CDKs) and morphogenesis (EGFR, MMPs) and such changes facilitated altered mammary gland morphogenesis and tumor progression. Furthermore, PELP1 was hyper-phosphorylated at its CDK phosphorylation site, suggesting an autocrine loop involving the CDK–cyclin D1–PELP1 axis in promoting mammary tumorigenesis. Treatment of PELP1 Tg mice with a KDM1 inhibitor significantly reduced PELP1-driven hyperbranching, reversed alterations in cyclin D1 expression levels, and reduced CDK-driven PELP1 phosphorylation. These results further support the hypothesis that PELP1 deregulation has the potential to promote breast tumorigenesis in vivo and represent a novel model for future investigation into molecular mechanisms of PELP1-mediated tumorigenesis. Cancer Res; 74(24): 1–11. ©2014 AACR.

**Introduction**
Breast cancer is the most common neoplasm and the second most common cause of cancer-related deaths in women, posing a significant public health challenge. Given the hormone-dependent nature of breast cancer and the central function of estrogen receptor (ER)-α in estrogen signaling (1), the ER status of breast tumors is an important biomarker for both prognosis and prediction of treatment response (2). A majority of tumors exhibit estrogen dependency (ER positive), and therefore are suitable candidates for targeted endocrine therapies. Despite the well-documented benefits of endocrine treatment in patients with breast cancer, not all ER-positive tumors respond to endocrine manipulation and a substantial number of initially responding tumors later become refractory to treatment due to acquired resistance (3, 4). Such major clinical problems have highlighted a critical need for identifying novel therapeutic targets and additional diagnostic/prognostic biomarkers, and prompted a deeper investigation into the regulation and function of ER by coregulatory proteins. Transcriptional activity of ER is not only regulated by steroid hormones alone, but also by several coregulatory proteins (5, 6), which associate with the ER in response to hormone binding to activate transcription (5). ER coregulator levels are tightly regulated under normal conditions with deregulation primarily reported in the literature in association with a number of disease states. More than 100 of the nearly 300 distinct coregulators identified were revealed to be over- or underexpressed in human cancers; in breast cancer, 38% of the coregulators identified were found to be overexpressed (7). Coregulator overexpression may promote carcinogenesis and/or progression of endocrine related-cancers where steroid hormones are powerful mitogenic agents (8). ER signaling is intact in the...
therapy-resistant tumors, and ER interactions with critical coregulator proteins containing an LXXLL motif (including PELP1, SRC1, SRC3) appear to mediate ER signaling in these therapy-resistant and ER-positive metastatic tumors (9).

Proline glutamic acid leucine rich protein (PELP1) is a proto-oncogene that interacts with ER through the LXXLL motif and provides breast cancer cells with a distinct growth and survival advantage by functioning as a critical coregulator (10, 11). PELP1 is overexpressed in many hormone-related cancers, promotes E2-mediated cell proliferation (12), and is prognostically linked to shorter breast cancer-specific survival (13). Therapy resistance (14), and metastasis (15, 16). Recent studies indicated that PELP1 is needed for optimal epigenetic modifications at ER target genes, that PELP1 interactions with KDM1 (LSD1, KDM1A) play a key role in PELP1-mediated oncogenic functions (17), and that PELP1 deregulation promotes tumor proliferation in xenograft models (16, 17). Collectively, these data provide evidence for PELP1-mediated ER coregulatory functions playing a role in hormonal tumorigenesis by providing cancer cells with a growth and survival advantage. However, the in vivo significance, functional role, and mechanism(s) by which PELP1 overexpression promotes initiation and progression of breast cancer have yet to be fully resolved.

In this present study, we generated a pathologically relevant murine breast cancer model by utilizing the tetracycline regulatory system to achieve inducible expression of PELP1 and the MMTV promoter to generate mammary epithelium-specific expression (18). PELP1-expressing mammary glands had more proliferation, extensive side branching, precocious differentiation, and mammary tumors. Mechanistic studies revealed that PELP1 deregulation modulates expression of a number of known ER target genes and cancer promoting genes. Our results provide the first direct evidence that defines the in vivo role of PELP1 in oncogenesis and demonstrates that PELP1 is an ER coregulator with tumorigenic potential.

**Materials and Methods**

**Generation of transgenic mice**

Full-length T7-His-tagged human PELP1 gene was PCR amplified and subcloned into the TMILA-SP plasmid (18) downstream to the human cytomegalovirus minimal promoter combined with a tetracycline operator (tetO) sequence and upstream to a luciferase reporter gene translated from an internal ribosome entry site via the In-Fusion cloning method. TetO-PELP1 transgenic mice were generated by UTHSC-Houston Transgenic core facilities by pronuclear microinjection into fertilized oocytes and implantation in pseudopregnant ton. Transgenic core facilities by pronuclear microinjection transgenic mice were generated by UTHSC-Houston TetrO-PELP1 internal ribosome entry site upstream to a luciferase reporter gene translated from an downstream to the human cytomegalovirus minimal promoter amplified through graded series of ethanol, cleared in xylene, and then stored in methyl salicylate. Images of mammary gland whole mounts from adult nulliparous control mice were prepared through formaldehyde, and stained by hematoxylin and eosin. The chromatin was isolated and subjected to immunoprecipitation using the indicated antibodies. Isotype-specific IgG was used as a control. DNA was eluted and resuspended in 50 μL of TE buffer and used for PCR amplification using published primer sequences (20). Mouse cyclin D1-391-F:ccagcgaggaggaatagatg; R:ggacttggctgtttctgctc. Mouse cyclin D1-591-F:ccagcgaggaggaatagatg; R:agcgtccctgtcttctttca; mouse cyclin D1-2707F:tgaaatccgct-

**Real-time PCR analysis**

Mammary gland tissues from nulliparous bitransgenic and wild-type controls were mechanically homogenized in TRIzol reagent (1 mL TRIZOL for 200 mg of frozen tissue) using the manufacture’s protocol (Invitrogen). Assessment of RNA integrity was done using a NanoDrop 2000c spectrophotometer and indicated that the RNA extracted from snap-frozen mammary glands was of good quality and suitable for real-time RT-PCR. Total RNA (2 μg) was used to generate first-strand complementary DNA with random hexamers and SuperScript III reverse transcriptase according to supplier’s instructions (Invitrogen, cat no. 18080-051). Mouse-specific primers were obtained from Sigma. Real-time PCR mixtures contained 25 ng template cDNA, SYBR Green master mix buffer, and 300 nmol/L forward and reverse primers. The cycling conditions comprised 10-minute polymerase activation at 95°C and 40 cycles at 95°C for 15 seconds and 60°C for one minute. Results and the difference in fold expression were calculated by using ΔΔCt method. Validated primers for each of the analyzed gene were purchased from realtimeprimers.com.

**Chromatin immunoprecipitation analysis**

The chromatin immunoprecipitation (ChIP) analysis was performed as described previously (19). In brief, frozen-stored Tg mice mammary tissues (n = 3) were broken into powder with mortar and pestle, suspended in CHiP buffer, cross-linked using formaldehyde, and quenched by glycine. The chromatin was isolated and subjected to immunoprecipitation using the indicated antibodies. Isotype-specific IgG was used as a control. DNA was eluted and resuspended in 50 μL of TE buffer and used for PCR amplification using published primer sequences (20).

**Histology of whole mounts and side-branding quantification**

The entire four inguinal mammary gland was removed, fixed in Carnoy’s solution at room temperature for 48 hours, rehydrated, stained with carmine alum overnight (0.2% carmine 0.5% aluminum potassium sulfate), dehydrated through graded series of ethanol, cleared in xylene, and then stored in methyl salicylate. Images of mammary gland whole mounts from adult nulliparous control (n = 3) and bitransgenic animals (n = 3 per genotype) were taken for quantitative analysis. Secondary and tertiary branch points within five ×5 fields per gland were manually counted. For tumor formation analysis, aging mice were palpated every week and mammary glands containing tumors were isolated and formalin embedded as described above.

**Immunohistochemistry analysis**

Immunohistochemistry (IHC) and antigen retrieval were done according to a previously established protocol (17) with
primary antibodies overnight at 4°C PELP1 (1:1000), T7 (1:50), CK8 (1:50), phosphoMAPK (1:50), phospho-AKT (1:50), cyclin D1 (1:50), or Phospho PELP1 (1:50). Sections were then washed three times with 0.05% Tween in PBS, incubated with secondary antibody for 1 hour, washed three times with 0.05% Tween in PBS, visualized by 3,3′-diaminobenzidine substrate, and counterstained with hematoxylin QS (Vector Lab). The proliferative index was calculated as the percentage of Ki-67–positive cells in 10 randomly selected microscopic fields per slide at ×40. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) analysis was performed by using the In Situ Cell Death Detection Kit (Roche) as per the manufacturer’s protocol, and 10 randomly selected microscopic fields in each group were used to calculate the relative ratio of TUNEL-positive cells.

Luciferase assay
To monitor luciferase activity in vivo, mammary gland homogenates of age-matched experimental nulliparous adult female bitransgenic mice treated with or without doxycycline were assessed by using a Dual Luciferase Assay System (Promega) according to the manufacturer’s instructions. For whole animal in vivo bioluminescence imaging, the mice received 150 mg/kg of D-Luciferin (XenoLight Rediject; Caliper LifeSciences) intraperitoneally, and images were taken with an IVIS imaging system 10 minutes postinjection.

Immunoprecipitation and Western blot analysis
Mammary gland homogenates of age-matched experimental nulliparous adult female bitransgenic mice were prepared by using T-PER Tissue Protein Extraction Reagent (Thermo Scientific, cat. no. 78510) following the manufacturer’s protocol. Protein concentrations were determined using the Bradford assay. Equal amounts of total protein extracts of mammary glands were resolved using 8% or 10% SDS-PAGE gels. The proteins were transferred to a nitrocellulose membrane and immunoblotted with primary mouse antibodies against cyclin D1 (1:500), PTEN (1:500), or actin (1:1000) and developed using enhanced chemiluminescence methodology. Using T7-epitope antibody-coupled agarose beads, the PELP1 transgene was immunoprecipitated from homogenates of female integral mammary glands from double and single transgenic animals after 6 months with or without doxycycline treatment. Western blotting was done with an anti-T7 antibody (EMD BioSciences) to identify PELP1. For PELP1-KDM1 immunoprecipitation analysis, mammary gland lysates were prepared using a lysis buffer containing 50 mmol/L Tris-HCl-pH7.5, 0.2% Triton X-100, 0.3% NP-40, 150 mmol/L NaCl, 25 mmol/L NaF, 0.1 mmol/L sodium orthovanadate, along with a phosphatase and protease inhibitor cocktail. Immunoprecipitation was done using PELP1 antibody and Western blot analysis was done using KDM1 antibody (Bethyl Laboratories).

PCR array analysis
RNA was extracted from replicated sets of sections cut from formalin-fixed paraffin-embedded blocks of mouse mammary gland tissue using the ArrayGrade RNA Isolation Kit (SABiosciences), and the changes in RNA were profiled using the RT² Profiler Mouse Breast Cancer Pathway PCR Array (cat. no. 330231 Pamm-131ZA). Before the RT PCR step, 3 μg of total RNA was treated to digest possible gDNA contamination. Following the elimination step, cDNA was synthesized using RT² First Strand Kit (cat. no. 330401) in accordance with manufacturer’s protocol. Then, cDNA synthesized from 3 μg total RNA went through another round of amplification before being combined with RT² Real-Time SYBR Green PCR Master Mix (cat. no. 330500) and loaded onto the array. PCR reactions were executed by utilizing the ABI StepOnePlus machine.

Statistical analyses
GraphPad Prism software (GraphPad Software) was used to analyze all data. Results (mean ± SEM) were analyzed using a two-tailed t test. Survival data are presented as Kaplan–Meier plots and were analyzed by using a log-rank (Mantel–Haenszel) method.

Results
Expression of PELP1 in mammary glands of bitransgenic mice
To determine the role of PELP1 in mammary tumorigenesis in vivo, we generated an inducible transgenic mouse model by crossing transgenic mice that express human PELP1 under the control of a tet-responsive promoter (TetO-PELP1) with transgenic mice expressing tetracycline-responsive trans-activator protein under the control of the murine mammary tumor virus (MMTV-rtTA; Supplementary Fig. S1A; ref. 18). To facilitate transgene detection, TetO-PELP1 construct encodes a bicistronic mRNA comprising a luciferase reporter gene translated from an internal ribosome entry site downstream of the PELP1-coding sequences as well as T7 epitope tag before ATG. Founder mice harboring the transgene construct were identified using PCR with TetO-PELP1–specific primers and were confirmed by Southern blotting genomic DNA isolated from tail biopsies. Two independent transgenic lines were established (Supplementary Fig. S1B and data not shown). The Tg line 1 has four copies and the Tg line 2 has six copies of the PELP1 transgene (data not shown). Tg line 2 exhibited poor breeding with small litter size; therefore, we used Tg line 1 for this study and key phenotypes were validated using Tg line 2. PELP1 transgene induction was initiated via doxycycline treatment (200 μg/mL) in the drinking water in nulliparous female bitransgenic offspring at 8 weeks of age and in vivo bioluminescence imaging was performed. Luciferase activity was only observed in bitransgenic animals treated with doxycycline (Fig. 1A and Supplementary Fig. S1C). The human PELP1 transcripts in the transgenic mammary glands were 3- to 4-fold higher than the transcripts in mammary glands with endogenous PELP1 (Supplementary Fig. S1D). Immunoprecipitation followed by Western blot analysis revealed that expression of T7-PELP1 transgene only occurred in mammary tissues extracted from the doxycycline-treated bitransgenic mice (Fig. 1B). IHC using the T7-epitope tag also revealed greater PELP1 transgene protein expression in these mice (Fig. 1C, left panels), confirming our Western data. PELP1-overexpressing cells are of epithelial
origin as these cells expressed CK8 (Fig. 1C, right panels), thus confirming spatial regulation of PELP1 expression in our Tg mice model.

**PELP1 overexpression disrupts mammary gland morphogenesis by inducing hyperbranching and epithelial proliferation**

To determine the effect of PELP1 on mammary gland morphology, we examined Carmine-stained whole mount preparations from female nulliparous transgenic animals (Fig. 2A). Differences in the amount of side branching after PELP1 induction were observed as early 2 months; these differences became significant at 4 months (59.9 ± 3.2 branch points in transgenic mice vs. 15.88 ± 0.93 control mice; Fig. 2B). The hyperbranching phenotype in nulliparous mice (quiescent state of mammary epithelium) suggests an active role of PELP1 in proliferation and epithelial remodeling. Because published studies using *in vitro* models showed that PELP1 plays a role in cell proliferation, we hypothesized that the extensive hyperbranching phenotype mediated by PELP1 overexpression could be due to alterations in proliferation. To test this hypothesis, we analyzed mammary epithelial proliferation and apoptosis. Although TUNEL analysis revealed no change in the number of cells undergoing apoptosis (data not shown) at both 8- and 18-month time points, the Ki-67 labeling index increased significantly (52% and 68%, respectively; Fig. 3A and B). Our data suggest that the increase in mammary epithelial proliferation could in part be contributing to the hyperbranching phenotype *in vivo*.

**PELP1 transgene overexpression promotes mammary epithelial hyperplasia and tumor development**

To directly test the tumorigenic potential of deregulated PELP1 expression *in vivo*, we assessed the premalignant changes within the epithelium and tumor ontogeny. Histologic examination of mammary glands in PELP1 bitransgenic mice treated with doxycycline revealed distended primary ducts with areas of hyperplastic growth and structures with multiple cell layers (Fig. 4A). Between 6 to 10 months of age, precancerous mammary lesions (atypical hyperplasia, mammary intraepithelial neoplasia, and carcinoma *in situ*) were observed. Mammary gland adenocarcinomas were observed in 14- to 18-month-old mice. (Fig. 4A). The presence of ERα in PELP1-driven hyperplasia and mammary tumors was analyzed by using IHC. Approximately, 100% of mammary glands and 90% of hyperplasia and mammary tumors from PELP1 transgenic mice had ERα expression (Fig. 4B). Further analysis revealed that mammary glands, hyperplasia, and mammary tumors from PELP1 transgenic mice had low/undetectable levels of ERβ expression (data not shown). Palpable mammary tumor development was first detected between 6 months and 8 months of age. The Kaplan–Meier survival curve demonstrates that PELP1 overexpression significantly correlates with shorter mammary tumor-free survival (Fig. 4C). Collectively, these results suggest that PELP1 deregulation has potential to promote mammary tumorigenesis *in vivo*.

**PELP1 transgene overexpression in mammary gland alters expression of genes involved in cell-cycle progression and morphogenesis**

Because PELP1 functions as a coregulator of ER, we hypothesized that PELP1-mediated mammary tumorigenesis could be mediated by alterations of specific genes in the mammary gland. To test this, we investigated the differential expression of breast cancer-focused genes between control and PELP1 transgenic mammary glands using the real-time RT² Profiler PCR array. Out of the 84 mouse breast cancer pathway-focused genes, 67 genes demonstrated at least a 3-fold difference (up or
downregulation) in expression (Fig. 5A). Of the several genes identified with significantly higher expression, we focused on validating the top candidate genes associated with the cell-cycle progression and mammary gland ductal elongation. Specifically, we focused on genes that are shown in previous studies using in vitro model cells to be regulated by PELP1 and ERα. The changes in the expression pattern that were detected by PCR array were confirmed by using qRT-PCR (Fig. 5A). Western blot analysis confirmed upregulation of cyclin D1 and downregulation of PTEN in the PELP1 transgene expressing mammary glands (Fig. 5B). Furthermore, IHC analysis revealed greater expression of cyclin D1 in PELP1 transgenic mammary glands, validating our qRT-PCR and Western data (Supplementary Fig. S2A). Earlier studies from our laboratory suggested that PELP1 recruits to the promoters of a number of ER target genes including cyclin D1 and promotes epigenetic changes. To examine whether PELP1 is recruited to the cyclinD1 gene promoter in mouse mammary glands, we performed ChIP analysis. Results from this experiment showed that both PELP1 and ERα are recruited to the proximal region (−591 to −332) of mouse cyclinD1 proximal promoter (Fig. 5C; ref. 20) in PELP1 Tg mouse mammary glands, with no recruitment seen at distal region (−2707 to −2509; data not shown).

Because PELP1 is a substrate of both CDK4 and CDK2, and because we observed more expression of cyclin D1 and CDK2 in the PELP1 transgene-expressing mammary glands, we...
examined whether increased expression of cell-cycle regulators potentiated an autocrine loop by phosphorylating PELP1. IHC analysis revealed more phosphorylation of PELP1 at ser991, a site phosphorylated by CDKs (Supplementary Fig. S2B). Because PELP1 transgene-expressing mammary glands exhibited more EGFR and EGF gene expression, we also analyzed activation of downstream effectors of EGFR pathways such as Phos-AKT and PhosMAPK. Higher levels of phosphorylated MAPK as well as phosphorylated Akt were detected in the PELP1 transgene-expressing mammary glands than in the control mammary glands (Supplementary Fig. S3A and S3B).

Earlier studies using in vitro models suggested that PELP1 deregulation promotes changes in the epigenetic mark such as H3K9me2 that contributes to the activation of genes via its association with KDM1 (17), and that PELP1-mediated alterations in H3K9me2 can be reversed by pargyline, an inhibitor of KDM1. Accordingly, treatment of MMTV-rtTA-TetO-PELP1 bitransgenic mice with pargyline (80 mg/kg/every other day/i.p.) for 4 months reversed the PELP1 transgene-mediated effects, including decreased hyperplasia and hyperbranching were observed and were similar to the control mice (Fig. 6A). Furthermore, pargyline treatment resulted in decreased mammary epithelial proliferation (Fig. 6B). Immunoprecipitation results showed that PELP1 can form complex with KDM1 in transgenic mammary gland (Fig. 6C). ChIP studies on cyclin D1 promoter showed increased dimethyl modification at H3K9, a mark that is removed by histone demethylase KDM1 (Fig. 6D). These results further support the hypothesis that PELP1-mediated oncogenic functions may involve KDM1 functions. Accordingly, IHC examination revealed more expression of the inhibitory histone methyl mark H3K9me2, lower cyclin D1 levels, and less phosphorylation of PELP1 in pargyline-treated PELP1 transgene-expressing mammary glands compared with control Tg mammary glands (Supplementary Fig. S4A and S4B).

Collectively, the results from these studies revealed that PELP1 overexpression leads to activation of several ERα target genes involved in cellular proliferation (cyclin D1 and CDKs) and morphogenesis (EGFR and MMPs), and that PELP1-mediated epigenetic changes may play a role in PELP1-mediated mammary tumor initiation.

Discussion

Changes in ERα-associated coregulator expression have been demonstrated to substantially contribute to ERα activity and often correlate with a poor prognosis for patients with
breast cancer (21–23). PELP1 is a proto-oncogene that functions as an ER coregulator and exhibits overexpression in breast tumors. Despite significant progress in understanding the role of PELP1 in ER signaling using in vitro models, no in vivo models were available to elucidate its significance in the initiation and progression of breast cancer. Thus, we used an engineered mouse model (MMTV-rtTA-TetO-PELP1) to assess the functional role of PELP1 in breast tumor initiation and progression. Our results demonstrated that PELP1 is a critical regulator of ductal morphogenesis and that deregulation of PELP1 expression is sufficient to initiate mammary tumorigenesis.

In our Tg mouse model, PELP1 expression is under control of a doxycycline-regulated promoter. To avoid developmental artifacts, we initiated induction of the PELP1 transgene at 8 weeks of age. We observed a 3- to 4-fold induction of PELP1 transgene over endogenous levels, which is very similar to the levels detected in human breast tumors. Furthermore, transgene expression was maintained in aged PELP1 Tg mice up to 22 months. Mammary glands of MMTV-rtTA-TetO-PELP1 Tg mice had premalignant lesions. Control MMTV-rtTA and TetO-PELP1 mice did not have any signs of premalignant lesions. PELP1 Tg mammary glands and mammary lesions showed increased Ki-67 expression, which is indicative of high proliferation. Many of the premalignant lesions progressed to mammary tumors as the mice aged. PELP1 premalignant lesions and mammary tumors had high levels of cyclin D1 expression. Earlier in vitro studies using breast cancer models showed that cyclin D1 is a PELP1 target gene (24). Our in vivo data also support this observation. Because cyclin D1 is frequently overexpressed in breast cancers and implicated in cancer progression (25), high cyclin D1 level in the PELP1-induced mammary lesions indicated that PELP1-mediated tumorigenesis may involve modulation of the cyclin D1 pathway. We failed to see any significant differences in the tumor latency in multiparous animals compared with nulliparous animals. Since our earlier studies showed that PELP1 deregulation induces local E2 synthesis via aromatase regulation...
and such findings may explain to some extent the lack of exacerbate phenotype in PELP1 Tg multiparous animals, however, future studies are needed. Our ongoing studies will address the role of local estrogen in PELP1-mediated tumorigenesis and also test the effect of PELP1 overexpression on various stages of development of mammary gland.

Estrogens induce proliferation of ER-positive breast epithelial cells by stimulating G1–S transition (27). Our recent results identified the ER coregulator PELP1 as a novel substrate of CDKs, and PELP1 is sequentially phosphorylated by the CDK4/cyclin D1, CDK2/cyclin E, and CDK2/cyclin A complexes (12). PELP1 can couple E2 signaling to the E2F axis, and CDK phosphorylation plays a key role in the PELP1 oncogenic functions (12). EGFs activate the CDK4/cyclin D1 complexes (28) and PELP1 is shown to be phosphorylated at CDK sites when treated with EGF (29). Gene expression analysis revealed more expression in a number of cell-cycle regulator genes including CDK2, cyclin A1, and CDK2 with concomitantly less expression of the CDK inhibitor CDKN2a and PTEN in PELP1 Tg mammary glands than in the control glands. Furthermore, PELP1 Tg mammary glands exhibited increased phosphorylation of PELP1 at the CDK site, suggesting functional activation of upregulated CDK pathways. These results also suggest that PELP1 deregulation may enhance tumor growth by promoting a CDK-PELP1 functional autocrine signaling loop.

The steroid hormone estradiol (E2) plays an important role in the development of the mammary gland. ERα is the major ER subtype in the mammary epithelium and its importance in mammary gland biology and development has been confirmed by studying ERα knockout (KO) mice. These mice display...
grosely impaired ductal epithelial cell proliferation and branching (30, 31). EGFR and EGF play critical roles in mammary gland branching, and extensive cross-talk in signaling occurs between ERα and EGFR signaling (32). Mammary gland branching morphogenesis is also dependent, in part, on the extracellular matrix-degrading enzymes, including matrix metalloproteinasises (MMPs; ref. 33). Earlier studies suggested that PELP1 has the potential to regulate expression of several genes involved in the epithelial–mesenchymal transition including MMPs (34, 35). In our transgenic mice model, induction of PELP1 expression by doxycycline significantly enhanced mammary ductal branching and morphogenesis. Gene expression studies revealed higher expression levels in a number of genes involved in morphogenesis including EGFR, MMP9, MMP2, and Notch in PELP1-overexpressing mammary glands than in control mammary glands. These findings, in part, explain the mammary morphogenesis phenotype observed in PELP1 Tg mice. However, future studies are needed to examine the mechanisms by which PELP1 modulates the expression of genes involved in morphogenesis. Since we analyzed gene expression changes using a focused microarray containing 84 genes, additional studies using whole-genome approaches such as RNAseq and ChIPseq are needed to further understand the mechanisms by which PELP1 deregulation promotes branching and morphogenesis phenotypes.

Deregulation of the ER coactivator SRC3 (AIB1) was reported in breast tumors (21, 22). ER coregulatory proteins have been suggested to play roles in the generally observed tissue-specific effects of tamoxifen (36). SRC3 (AIB1) KO mice studies demonstrated that normal expression of the coactivator SRC3 (AIB1) is required for initiation of tumorigenesis by carcinogens and oncogenes (37, 38). Using inducible expression of PELP1 directed into the mammary gland of Tg mice, we provided evidence that PELP1 is another ER coactivator whose deregulation has potential to promote mammary tumor initiation. PELP1 interacts with and functions as a coactivator of a number of transcription factors that play a critical role in mammary gland biology including ER, E2F, and STAT3 (10, 11). Furthermore, PELP1 interacts with and recruits a number of epigenetic modifiers (such as KDM1) in the target chromatin and thus facilitates activation of a number of genes involved in proliferation and cancer progression (19). Our recent RNAseq studies identified 318 genes as PELP1-regulated genes and many of them are involved in breast cancer progression (39) and that PELP1 is also involved in splicing of genes. Collectively, multiple roles played by PELP1 in cellular signaling may contribute to important changes in seen the mammary gland of PELP1 transgenic mice.

Approximately 70% of breast tumors are ER positive at the time of presentation (6, 40, 41). PELP1 expression provides ER-positive breast cancer cells with a distinct growth and survival advantage (12). PELP1 overexpression has been noted in many hormone-related cancers (10, 42), and PELP1 promotes E2-mediated cell proliferation. In the ER-positive/luminal-like group of tumors, PELP1 is prognostically linked to shorter breast cancer-specific survival (13, 43) and to therapy resistance (14, 44). In our studies, PELP1 deregulation promoted hyperplasia and mammary tumors that were predominantly ER positive. Even though hyperplasia was seen as early as 3 months, progression to mammary adenocarcinomas had a long latency of between 14 and 18 months. This long latency in the development of mammary tumors in PELP1 Tg mice could be due to the nature of slow growth characteristics of ER-positive mammary tumors. Similar long latencies were also reported in other Tg mouse models such as MMTV AIB1 (21–23). Another possibility is that delay in tumorigenesis in PELP1 Tg mice may indicate need for acquiring additional mutations. This is supported by the fact that some of PELP1 Tg mice developed tumors as early as 8 months. Since coregulators are shared by multiple transcription factors and some of these coactivation functions may promote tumor suppression, tumor promotion may be delayed until such pathways are inactivated. In support of this, our recent study suggested that PELP1 also functions as coactivator of tumor suppressor p53 and such activation may counteract PELP1 oncogenic functions and could contribute to long latency (45).

Recent evidence suggests that ER coactivators, in addition to ER, are targets of growth factor signaling and growth factor-mediated phosphorylation of ER, and ER-coregulatory proteins have been shown to have a role in tamoxifen resistance (46). ER coactivators are targeted by excessive ER-HER2 cross-talk leading to hormone resistance in a subset of breast tumors (46). PELP1 deregulation and/or its mislocalization in the cytoplasm can contribute to the activation of the MAPK and AKT pathways by coupling the ER-Src pathways (47). In our transgenic mouse model, wild-type PELP1 was used and it predominantly localized in the nuclear compartment, as we expected. Interestingly, gene expression studies revealed upregulation of EGF and downregulation of PTEN in the Tg mice mammary glands than in the control mammary glands. Furthermore, analysis revealed increased activation of the MAPK and AKT pathways in PELP1 Tg-expressing mammary glands. We failed to see any activation of Src kinase in the mammary glands (data not shown). Collectively, these results suggest that PELP1 deregulation has the potential to activate MAPK and AKT indirectly by enhancing expression of EGF/EGFR or by downregulating PTEN. Increased activation of MAPK and AKT could have contributed to the increased proliferation and survival of mammary epithelial cells in PELP1 transgene-expressing mammary gland.

Estrogen stimulation induces various histone modifications at ER target gene promoters and is mediated by recruitment of coregulators to the target gene promoters by ER. Histone methylation-dependent mechanisms impose ligand dependency for ER-mediated gene activation and may play a vital role in many neoplastic processes, representing a valuable therapeutic target (48). Previous studies showed KDM1 recruitment to a significant fraction of ER target genes, demethylation of H3-K4 and H3-K9 and demethylation of proximal histones enables ER-mediated transcription (49). Emerging evidence also suggests that PELP1 is involved in oncogenesis through its interaction with histones, acetyltransferases, deacetylases, and the demethylase KDM1 (9, 12). We observed decreased expression of H3K9me2 mark in PELP1-
Driven hyperplasia and mammary tumors. In our previous studies using preclinical models, we observed pargyline inhibited PELP1-driven tumorigenesis by blocking KDM1 functions (17). Similarly, pargyline treatment delayed the development of hyperplasia in PELP1 Tg mice and reduced the hyperbranching phenotype induced by PELP1 overexpression. Because pargyline also has ability to inhibit MAO in addition to inhibition of KDM1, we cannot rule out the possibility that part of the results could be due to inhibition of MAO. In our previous in vitro studies (19), under conditions of KDM1 knockdown, pargyline showed little or no effect on PELP1-mediated functions, suggesting pargyline effects on PELP1 signaling require functional KDM1. Because PELP1 interacts with KDM1 in Tg mouse mammary gland and because pargyline increased dimethyl H3K9 mark at cyclinD1 promoter, we reason that effects observed are due to pargyline inhibition of KDM1. However, future studies are clearly needed using a specific inhibitor of KDM1. These findings underscore the importance of epigenetic modifications in PELP1 oncogenic functions in vivo.

In summary, our data using novel inducible transgenic mouse model provided the first in vivo evidence that overexpression of PELP1 in mammary epithelial cells initiates a pathologic state, leading to breast cancer initiation and progression. The proposed PELP1 Tg model will provide an opportunity to study the biologic events that underlie the PELP1 actions in pathophysiological conditions and sets a stage for future investigations to test pharmacologic interventions in conditions of PELP1 deregulation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: V. Cortez, C. Samayoa, R.R. Tekmal, R.K. Vadlamudi
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