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

Valerie Cortez1,2, Cathy Samayo1,2, Andrea Zamora1, Lizatte Martinez1, Rajeshwar R. Tekmal1,3, and Ratna K. Vadlamudi1,3

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 differentiation 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); 7395–405. ©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) 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 indicated that PELP1 is needed for optimal epigenetic modifications at 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 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 C57Bl6 females. Founder mice harboring the transgene construct were identified by PCR analysis of TetO-PELP1–specific primers and confirmed through Southern blot analysis of genomic DNA isolated from tail biopsies. TetO-PELP1 mice were bred with MMTV-rTA transgenic mice (18) to generate MMTVrTA-TetO-PELP1 transgenic mice. Offsprings were screened for both rTA and TetO-PELP1 transgenes by PCR and confirmed by using Southern blot analysis of tail DNA. Mice carrying MMTVrTA-TetO-PELP1 transgene were used to establish two independent bitransgenic founder lines. Trans-

gene induction via doxycycline treatment (MP Biomedicals) began in nulliparous female bitransgenic offspring at 8 weeks of age at 200 μg/mL in the drinking water.

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 manufacturer’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 in 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 genes 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 lysis 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). Mouse cyclin D1-591-F:ccagcgaggaggaatagatg; R:ggacttggctgtttctgctc. Mouse cyclin D1-2707F:tgaaatcgc-gaggtaac; R:ggacttgcgtttcttc.

Histology of whole mounts and side-branching quantification

The entire number 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 tissue recorded. The entire number four inguinal mammary gland was evaluated for presence of tumors and recorded. Tumor formation quantification was computed as follows: 100 × (number of tumors/number of mice). Statistical analysis was performed using Student’s t test.

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), phosMAPK (1:50), phos-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 o-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 (SA Bioscences), 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...
in vivo PELP1 expression

Epithelial hyperplasia and tumor development

PELP1 transgene overexpression promotes mammary phenotype

proliferation could in part be contributing to the hyperbranching phenotype. Our data suggest that the increase in mammary epithelial proliferation was consistent with the hyperbranching phenotype observed in PELP1-overexpressing transgenic mice. The hyperbranching phenotype in nulliparous mice (quiescent state of mammary epithelium) suggests an active role of PELP1 in mammary gland morphogenesis by inducing hyperbranching and epithelial remodeling. Because PELP1 functions as a coregulator of ER, 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 transgenic 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 ERβ 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 ERβ 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

Figure 2. Effect of PELP1 overexpression on mammary gland morphogenesis. The entire number four-inguinal mammary gland was removed, fixed in Carnoy’s solution, and stained with carmine alum overnight. A, representative whole-mount images (magnification, ×3) of mammary glands from 6-month-old nulliparous female mice of the indicated genetic controls (top) and doxycycline-treated bitransgenic animals (bottom). B, side-branching quantification (mean ± SEM). All experimental data points were generated from three biologic replicates per transgenic line. Statistical significance was determined by using a Student t test. ****, P < 0.0001. WT, wild type; M+, MMTV-rtTA transgenic mice; P+, TetO-PELP1 mice; and M+P+, MMTV-rtTA-TetO-PELP1 bitransgenic mice.

Figure 3. Effect of PELP1 overexpression on mammary epithelial proliferation. The entire number nine inguinal mammary gland from wild-type and PELP1 bitransgenic animals at 8 and 18 months of age were removed, formalin-fixed, paraffin-embedded, and sectioned every 4 μm. A, mammary epithelial proliferation as reflected by IHC analysis of Ki-67 expression. B, proliferation index was calculated relative to a percentage of total epithelial cell nuclei counts in five ×20 fields per slide. All experimental data points were generated from three biologic replicates per transgenic line. Statistical significance was determined by using a Student t test. ****, P < 0.0001. WT, wild type; and M+P+, MMTV-rtTA-TetO-PELP1 bitransgenic mice.
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-rTA-TetO-PELP1 transgenic 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
PELP1 expression was maintained in aged PELP1 Tg mice up to levels detected in human breast tumors. Furthermore, transgene over endogenous levels, which is very similar to the latency in multiparous animals compared with nulliparous way. We failed to see any significant difference in the tumor 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-rTA-TetO-PELP1 Tg mice had premalignant lesions. Control MMTV-rTA 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.
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 $G_1$–$S$ 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.

Figure 6. Effect of pargyline treatment on PELP1-induced hyperplasia. A, side-branching quantification (mean ± SEM) in the mammary glands of 6-month-old MMTV-rtTA-TetO-PELP1 bitransgenic mice treated with PBS or pargyline. All experimental data points were generated from three biologic replicates per transgenic line. Statistical significance was determined by using a Student t test. $^*^*^*^*^* , $P < 0.0001$. B, mammary epithelial proliferation as reflected by IHC analysis of Ki-67 expression in the mammary glands of 6-month-old MMTV-rtTA-TetO-PELP1 bitransgenic mice treated with PBS or pargyline. Proliferation index was calculated relative to a percentage of total epithelial cell nuclei counts in five $>$20 fields per slide. All experimental data points were generated from three biologic replicates per transgenic line. Statistical significance was determined by using a Student t test. $^*^*^*^*^* , $P < 0.0001$. C, total lysates from the mammary glands of 6-month-old MMTV-rtTA-TetO-PELP1 bitransgenic mice were subjected to immunoprecipitation using the PELP1 antibody and PELP1–KDM1 interaction was verified by Western blot analysis. D, ChIP assay was done using the DNA isolated from the mammary glands of 6-month-old MMTV-rtTA-TetO-PELP1 bitransgenic mice (n = 3) treated with PBS or pargyline (Par) with antibodies specific for dimethyl H3K9. DNA recovered from ChIP or input controls was subjected to real-time quantitative PCR with primers specific to −591 to −332 and −2707 to −2509 that represent the proximal and distal promoter regions of mouse cyclin D1 promoter. Statistical significance was determined by using a Student t test. $^* , P < 0.05$.  

Cortez et al.

Cancer Res; 74(24) December 15, 2014

Cancer Research

Published OnlineFirst November 6, 2014; DOI: 10.1158/0008-5472.CAN-14-0993
PELP1 promotes tumorigenesis in vivo

PELP1 promotes tumorigenesis in vivo...
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 pathophysiologic 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

development and design: V. Cortez, C. Samayoa, R.R. Tekmal, R.K. Vadlamudi
Development of methodology: V. Cortez, R.K. Vadlamudi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V. Cortez, C. Samayoa, A. Zamora, R.R. Tekmal
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V. Cortez, C. Samayoa, A. Zamora, L. Martinez, R.R. Tekmal, R.K. Vadlamudi
Writing, review, and/or revision of the manuscript: V. Cortez, C. Samayoa, L. Martinez, R.K. Vadlamudi

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Martinez

Study supervision: L. Martinez, R.K. Vadlamudi

Acknowledgments

The authors thank Dr. Lewis A. Chodosh, University of Pennsylvania School of Medicine for providing MMTV-βTα transgenic mice, Challa Ram Babu for managing mice colony, Dimple Chakravarty for help with vector construction, and Dominque Newall for initial characterization of the transgene line, UTHTSCSA Pathology Core for preparation of blocks, Dr. Martha Hanes for pathologic analysis, and UTHSC Houston for the generation of transgenic mice. 

Grant Support

This work was supported by the NIH/NCI grant CA095681 (R.K. Vadlamudi), Komen grant KG090447 (R.K. Vadlamudi), Komen grant KG110812 (R.R. Tekmal), and NIH F31 award F31CA168184 (V. Cortez) and the Cancer Therapy and Research Center at the University of Texas Health Science Center at San Antonio through the NCI Cancer Center Support Grant P30CA054174-17. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 4, 2014; revised October 6, 2014; accepted October 22, 2014; published OnlineFirst November 6, 2014.

References

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

Valerie Cortez, Cathy Samayoa, Andrea Zamora, et al.


Updated version
Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-14-0993

Supplementary Material
Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2014/11/07/0008-5472.CAN-14-0993.DC1

Cited articles
This article cites 49 articles, 20 of which you can access for free at: http://cancerres.aacrjournals.org/content/74/24/7395.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at: /content/74/24/7395.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.