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TITLE: **Hormonal Resistance and Metastasis: ER-coregulator-Src Signaling Targeted therapy**  
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| 14. ABSTRACT<br>The estrogen receptor (ER), is implicated in the progression of breast cancer. Endocrine therapy is shown to have a positive effect on the treatment of breast cancer; however, initial or acquired resistance to endocrine therapies frequently occurs. In this study, we have generated <i>model</i> cells that have defects in either ER coregulator PELP1 or Src signaling axis. Using these models, we demonstrated that PELP1-Src axis play a key role in ER- extranuclear actions. Our data suggest that PELP1 and Src kinase axis also play an essential role in enhancing cell migration and metastasis. Pharmacological inhibition of Src kinase using dasatinib or Src knock down by shRNA significantly inhibited E2-mediated extranuclear actions in therapy resistant cells. The results from our study showed that ER-Src axis play an important role in promoting hormonal resistance by proto-oncogenes such as HER2, PELP1 and blocking this axis prevents the development of hormonal independence. Since PELP1 and Src kinase are commonly deregulated in breast cancers, combination therapies using both endocrine agents and dasatinib may have better therapeutic effect by delaying the development of hormonal resistance. |                  |                       |                                      |                                                          |                                            |
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## Table of Contents

|                                   | <u>Page</u> |
|-----------------------------------|-------------|
| Introduction.....                 | 4           |
| Body.....                         | 4           |
| Key Research Accomplishments..... | 12          |
| Reportable Outcomes.....          | 12          |
| Conclusion.....                   | 13          |
| References.....                   | 14          |
| Appendices.....                   | 15          |

**Award Number:** W81XWH-08-1-0604

**Project Period:** September 1, 2008 – August 31, 2011

**Title:** Hormonal Resistance and Metastasis: ER-coregulator-Src Targeted therapy

**PI:** Ratna K Vadlamudi

**Report Period:** September 1, 2008 – August 31, 2010

## INTRODUCTION:

The estrogen receptor (ER), is implicated in the progression of breast cancer(1). Endocrine therapy using Tamoxifen, a selective estrogen receptor modulator (SERM), has been shown to improve relapse-free and overall survival(2). More recently, aromatase inhibitors, which deplete peripheral estrogen (E2) synthesis, are shown to substantially improve disease-free survival in postmenopausal women(3). Furthermore, endocrine therapy also shown to have a positive effect on the treatment of advanced metastatic disease. Despite these positive effects, initial or acquired resistance to endocrine therapies frequently occurs. *Accumulating evidence suggests that ER-coregulators play an essential role in hormonal responsiveness and cancer progression*(4-6). Proline, Glutamic-acid and Leucine-rich Protein 1 (PELP1) is a recently identified novel ER coregulator (7, 8). Emerging evidence suggests that ER signaling cross talk with growth factors play an important role in hormonal resistance and metastasis. Since multiple signaling pathways in addition to hormone are involved in activating ERs, **combination therapies** using both endocrine and nonendocrine agents that block different pathways may have **better therapeutic effect** and may delay development of hormonal resistance. Recent evidence implicates ER-coregulator PELP1 play an essential role in coupling ER with Src kinases leading hormonal resistance. *Since PELP1 is the only ER-coregulator that is shown to couple ER with Src kinase, and because expression of PELP1 and Src are commonly deregulated in breast cancer, we hypothesize that deregulation of PELP1 promotes Src activation and excessive signaling crosstalk with ER, leading to hormonal therapy resistance and metastasis.* This proposal is aimed to determine whether PELP1-Src signaling is a rate limiting factor in the development of hormonal independence and metastasis and to test whether blocking of the PELP1-Src pathway in combination with endocrine therapies prevent hormonal therapy resistance and metastasis.

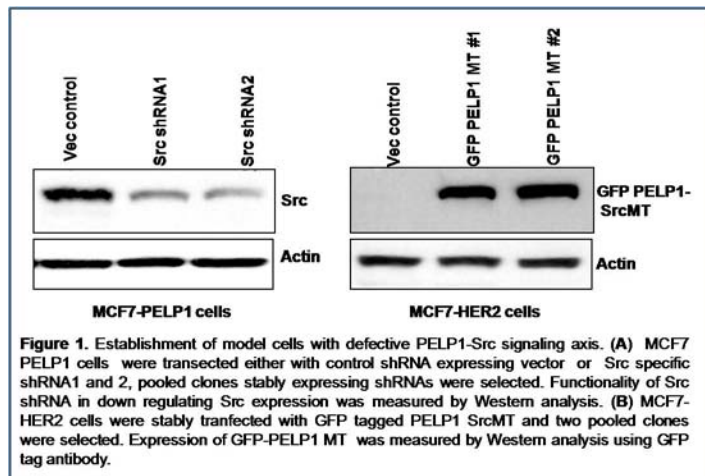
## BODY:

The scope of this proposal is to undertake the following three tasks outlined in the approved statement of work:

**Task 1. To establish the significance of ER-coregulator-Src axis in hormonal resistance and metastasis**

**Task2. To determine the efficacy of targeting of the ER-coregulator-Src axis on hormonal therapy and metastasis**

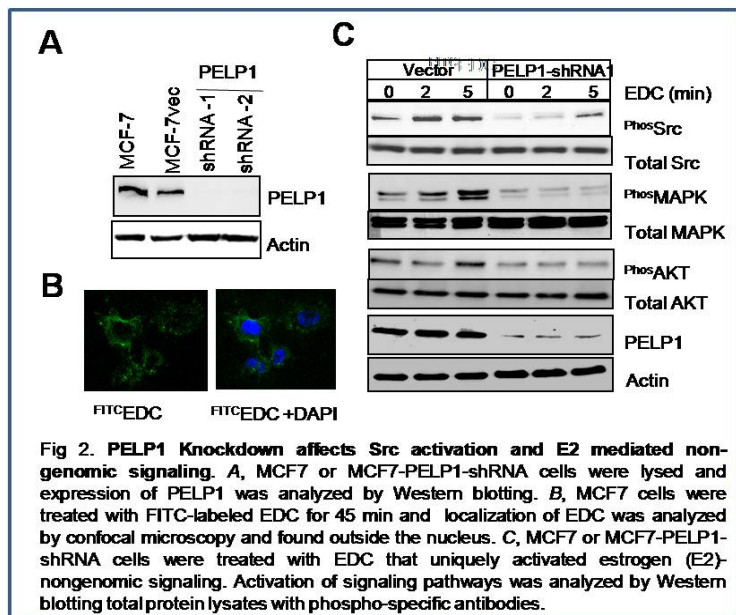
**Generation and characterization of model cells cell lines with or without functional ER-PELP1-Src axis.** To establish the significance of ER-PELP1-Src axis, during the first year, we established two additional breast cancer model cells (1) MCF7-GFPPELP1 clone that stably express Src-ShRNA (PELP1-Src-shRNA) and (2) MCF7-HER2 cell stably expressing PELP1-Src-mutant (HER2-PELP1-SrcMT).



PELP1-SrcMT contains mutation in the Src-SH3 binding on PELP1 (ProXXPro is mutated to AlaXXAla), thus functions as a dominant negative mutant. Clones were characterized for the functionality of Src down regulation by western analysis (Fig 1, left panel). Results showed that Src shRNA down regulates ~80% of endogenous Src expression. We have also characterized expression of PELP1 mutant using Western analysis (Fig. 1, right panel). These clones will be used

in the second year along with control MCF7-Her2 and MCF7-PELP1 clones to characterize the significance of PELP1-Src axis in therapy resistance and metastasis.

**Role of ER-PELP1-Src axis in ER non-genotropic signaling:** To study the *in vivo* significance of PELP1, we established MCF7 breast cancer model cells that stably expressed PELP1shRNA that specifically down regulate endogenous PELP1. MCF7 cells were transfected with shRNA vector were used as a control. Western blot analysis of total lysates revealed that the PELP1-shRNA clones down regulated PELP1 expression to ~80 % of the level seen in the parental and the vector-transfected clones (Fig. 2A). To further establish the role of PELP1 in E2-mediated non-genomic actions, we used EDC (nanoparticles coated with estrogen) that uniquely localize in the membrane/cytoplasm (Fig. 2B), and preferably activate ER-nongenomic signaling (9). MCF7 cells that expressed vector or PELP1 shRNA were treated with EDC for 2 or 5 min and signaling was analyzed by phosphor-specific antibodies.



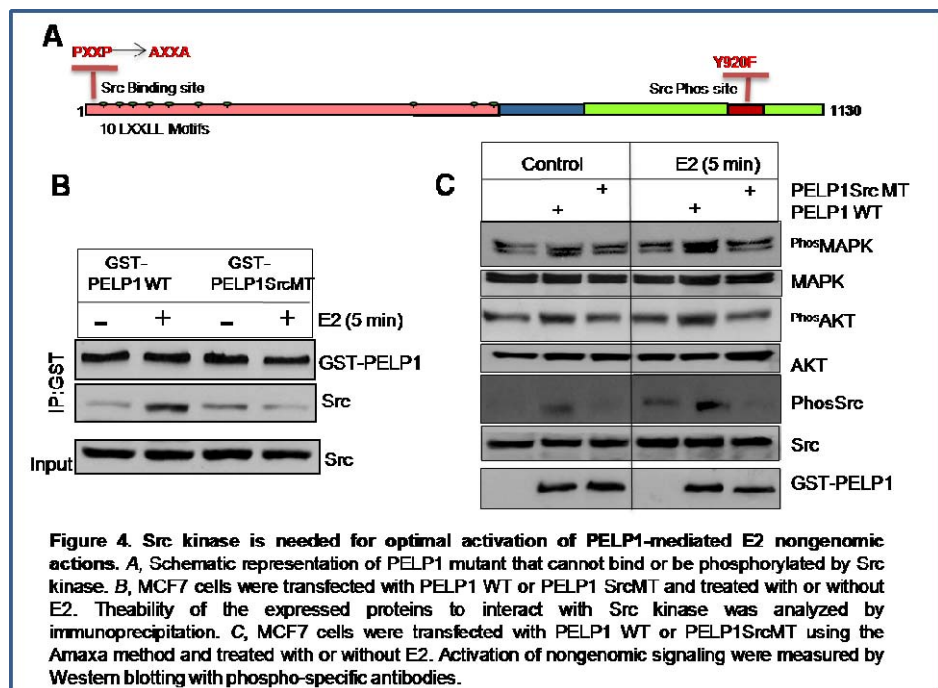
EDC addition uniquely promoted activation of Src and MAPK pathways. However, knock down of PELP1 by shRNA significantly affected the EDC-mediated increase in Src and MAPK activation (Fig. 2C). These results suggest that E2-mediated nongenomic actions play a key role

in the activation of Src and MAPK and that the functional PELP1 signaling axis is needed for E2-mediated non-genomic signaling.

**Significance of PELP1-Src axis on cytoskeleton signaling:** Because Src and PI3K play important role in cytoskeleton functions, cell attachment and migration, we asked whether E2-ER nongenomic actions contribute to cytoskeleton reorganization leading to cell migration. MCF7 cells that expressed vector or PELP1 shRNA were treated with either E2 or EDC for 10 min and cytoskeleton changes were analyzed by confocal microscopy. E2 or EDC addition uniquely promoted actin reorganization with filopodia and ruffle formations. However, knock down of PELP1 by shRNA significantly affected actin reorganization by E2 or EDC (Fig. 3). These studies demonstrate that ER

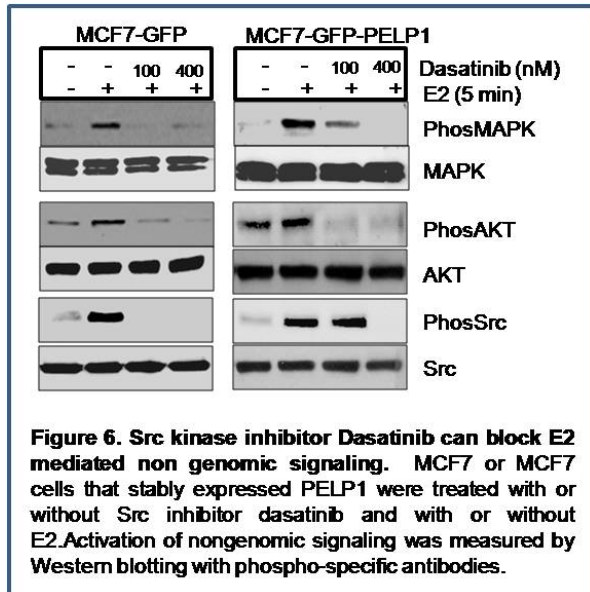
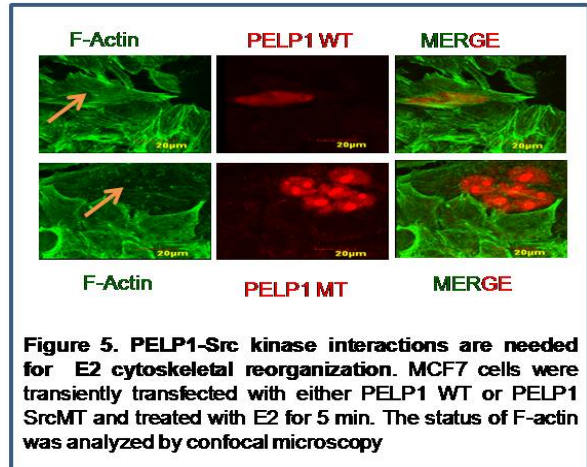
nongenomic actions have the potential to promote cytoskeleton changes.

**Src kinase plays a critical role in PELP1-mediated E2 nongenomic signaling:** To establish the significance of Src kinase in PELP1-mediated E2-ER nongenomic signaling, we generated a PELP1 mutant construct (PELP1SrcMT) that contains a mutation in the Src-SH3 binding site on PELP1 (ProXXPro is mutated to AlaXXAla) and a mutation in Src phosphorylation site (Tyr 920 is mutated to Phe, Fig. 4A). The PELP1SrcMT mutant is unable to interact with Src kinase and thus functions as a dominant negative mutant of PELP1. As expected, PELP1 WT but not the PELP1SrcMT interacted with Src kinase (Fig. 4B). Transient expression of PELP1SrcMT substantially interfered with E2-mediated activation of Src and MAPK (Fig. 4C). These results suggest that Src interactions with PELP1 is needed for optimal E2 mediated non genomic actions.



**Src kinase plays a critical role in PELP1-mediated signaling to cytoskeleton reorganization:**

To examine whether the significance of Src kinase in PELP1-mediated cytoskeleton reorganization, we transfected MCF7 cells with PELP1 WT and PELP1SrcMT. After 72 hours cells were stimulated with E2 and actin reorganization was measured using confocal microscopy. Expression of PELP1SrcMT substantially effected the E2-mediated cytoskeleton reorganization in a dominant negative fashion (Fig. 5). These results suggest that PELP1-Src axis play an important role in PELP1-mediated E2 nongenomic actions that contribute to cytoskeletal reorganization.

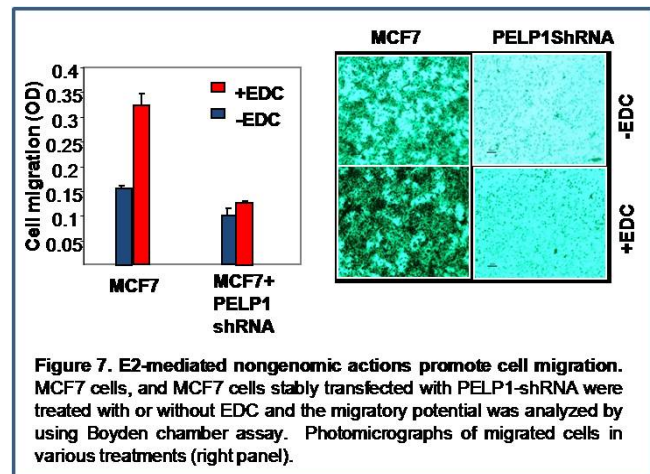


**Effect of Dasatinib on ER-nongenotrophic signaling:**

Since Src kinase appears to play a key role in E2 nongenomic signaling, we examined effect of inhibition of Src kinase using dasatinib, a well-established orally available inhibitor of Src family tyrosine kinases (10). For these studies, we used MCF7 control cells or MCF7-PELP1 model cells that overexpress PELP1 and exhibit increased E2-ER nongenomic signaling. Pharmacological inhibition of Src kinase using dasatinib abolished the E2-mediated activation of AKT and MAPK pathways both in MCF7 as well as in PELP1-overexpressing MCF7 cells (Fig. 6). These results suggest that pharmacological inhibitor dasatinib can be used to block E2-driven PELP1-mediated nongenomic signaling.

**PELP1 is needed for optimal cell migration promoted by E2 nongenomic actions:**

Because activation of PELP1-src axis by E2 nongenomic actions played a role in cytoskeleton reorganization, we examined whether E2-mediated nongenomic actions contribute to cell migration. In Boyden chamber assays, parental MCF7 cells showed low motility, and EDC further increased the migratory potential of those cells. The knockdown of PELP1 expression by siRNA substantially reduced EDC-mediated cell motility (Fig. 7). These data suggest that E2-PELP1 signaling potentially play a role in cell migration.



**Role of PELP1-Src axis in local E2 synthesis;** Our earlier studies showed that PELP1 promotes local E2 synthesis and Src plays a critical role in the activation of aromatase gene. In aromatase promoter based reporter gene assays, PELP1 enhances expression of aromatase and enhanced aromatase induction also occurs in Her2 overexpressing or therapy resistant cells such as LTLT that are resistant to letrozole (Fig. 8A). To understand the mechanism by which PELP1 enhances aromatase expression, in the initial set of experiments we examined whether PELP1-Src axis promotes epigenetic changes at the aromatase promoter leading to expression of aromatase. ChIP analysis revealed that MCF7 cells that donot express aromatase showed increased H3K9 methylation (a marker of repression), while MCF7-PELP1 model cells (that overexpress PELP1) which exhibit local E2 synthesis showed decreased histone H3K9 with a concomitant H3K4 methylation (a marker of activation) at the aromatase promoter (Fig. 8B). Interestingly, other model cells that exhibit increased local E2 synthesis (MCF7-Her2, SKBR3) also showed increased H3K4 methylation. ***These results suggest that PELP1 axis may play a role in modulating histone methylation by modulating epigenetic modifications at aromatase gene promoter.***

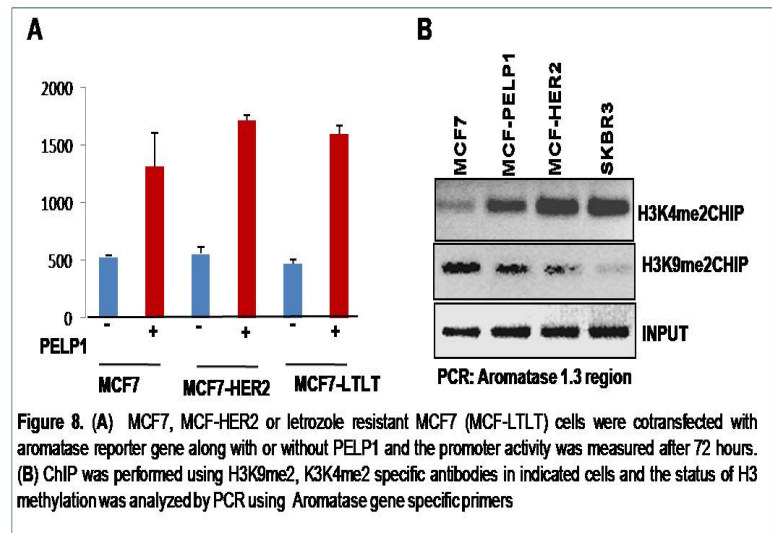


**Figure 8. (A)** MCF7, MCF-HER2 or letrozole resistant MCF7 (MCF-LTLT) cells were cotransfected with aromatase reporter gene along with or without PELP1 and the promoter activity was measured after 72 hours. **(B)** ChIP was performed using H3K9me2, K3K4me2 specific antibodies in indicated cells and the status of H3 methylation was analyzed by PCR using Aromatase gene specific primers

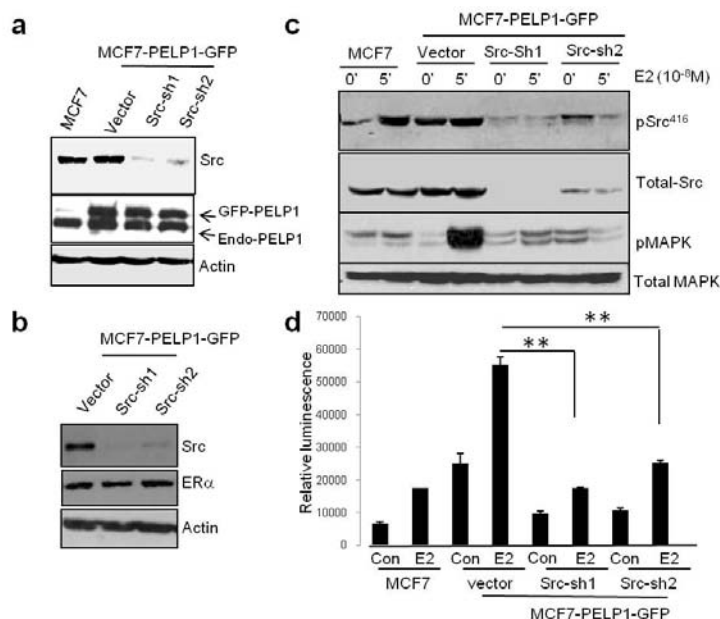


**Figure 9. Down regulation of Src kinase reduces PELP1 mediated ER extra-nuclear signaling.** a. MCF7-shRNA, MCF7-PELP1, and MCF7-PELP1-Src shRNA cells were lysed and expression of Src was analyzed by western blotting. b. Total lysates from MCF7-PELP1 and MCF7-PELP1-Src-shRNA model cells were analyzed for the expression of ER by Western blotting c. MCF7, MCF7-PELP1 and MCF7-PELP1-Src-shRNA cells were cultured in 5% DCC serum containing medium treated with or without estrogen. Activation of Src and MAPK signaling pathways was analyzed by Western blotting. d. Cell proliferation capacity of MCF7, MCF7-PELP1 and MCF7-PELP1-Src-shRNA stable cells were analyzed after treating the cells with or without E2.

Our ongoing experiments will test whether Src inhibitor can be used to down regulate aromatase expression in PELP1 and Her2 overexpressing cells.

### **Functional Src kinase axis is needed for PELP1 dependent ER actions:**

Recent studies established PELP1 as an independent prognostic indicator of shorter breast cancer specific survival and disease-free intervals (11). In earlier studies, we have established breast cancer model cells with stable expression of PELP1 (MCF7-PELP1) to mimic the situation commonly seen in a subset of breast tumors. These model cells express 2-3 fold more expression PELP1 compared to endogenous levels of PELP1 and

exhibit increased E2 mediated proliferation (12), therapy resistance (13) and tumorigenesis in xenograft models (14). To study the *in vivo* significance of Src kinase in PELP1 mediated actions, we established MCF7-PELP1 model cells (pooled clones) stably expressing Src shRNA using a lentivirus system with puromycin selection. Western blot analysis of total lysates from PELP1-Src shRNA clones revealed that the Src shRNA down regulated Src expression to ~75 % of the level seen in the parental MCF7-PELP1 and the vector-transfected control clones (Fig. 9a). Src knockdown did not affect the expression of ER and PELP1 in these clones (Fig. 9b). Since PELP1 participates in ER $\alpha$ -extranuclear actions, we examined the significance of endogenous Src in the activation of ER $\alpha$ -extranuclear signaling pathways. We measured the activation of signaling pathways including Src, and MAPK after treating cells with estrogen (E2) for 5 min. Estrogen addition uniquely promoted activation of Src and MAPK pathways in MCF7 cells. As observed before, MCF7-PELP1 cells showed further increase in activation of MAPK compared to MCF7 cells. Src-shRNA-expressing MCF7-PELP1 cells had significantly less Src, and MAPK activation (Fig. 9c). We then examined whether Src down regulation affected PELP1-mediated increase in E2 driven proliferation using a Cell Titer-Glo assay. PELP1 expression increased estrogen mediated cellular proliferation compared to MCF7 cells, while Src downregulation in MCF7-PELP1 clones diminished its ability to increase cell proliferation (Fig. 9d).

**HER2-mediated ER extranuclear actions requires Src kinase:** Deregulation of HER2 expression/signaling has emerged as the most significant factor in the development of hormonal resistance (9,20) and cross-talk between the ER and HER2 pathways has been shown to promotes endocrine therapy resistance (20,21). ER $\alpha$  coregulator PELP1 interacts with HER2 and

is implicated in facilitating the ER crosstalk with HER2 signaling pathways (13). To examine whether Src axis plays a role in HER2 mediated ER $\alpha$  extranuclear actions, we have down regulated Src kinase using shRNA delivery. We have established two pooled clones of Src-shRNA in a MCF7-HER2 background, a well established model cell for HER2 deregulation (15). Western blot analysis of HER2-SrcShRNA clones revealed 85-90%

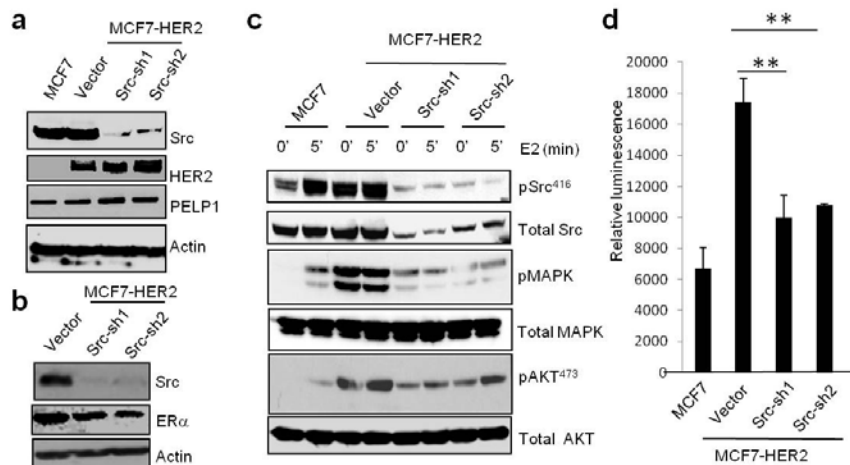


**Figure 10.** Down regulation of Src kinase reduces HER2 mediated ER extra-nuclear signaling. **a**, MCF7-shRNA, MCF7-HER2, and MCF7-HER2-Src-shRNA cells were lysed and expression of Src was analyzed by Western blotting. **b**, Total lysates from MCF7-shRNA, MCF7-HER2, and MCF7-HER2-Src-shRNA were analyzed for the expression of ER $\alpha$ , by Western blotting. **c**, MCF7-shRNA, MCF7-HER2, and MCF7-HER2-Src-shRNA cells were cultured in 5% DCC serum containing medium treated with or without E2. The activation of Src, AKT and MAPK signaling pathways was analyzed by western blotting of total protein lysates with phospho-specific antibodies. **d**, Cell proliferation capacity of MCF7-shRNA, MCF7-HER2, and MCF7-HER2-Src-shRNA stable cells were analyzed using Cell Titer-Glo assay. \*\*, P<0.001.

decrease in Src expression compared to the level seen in the parental MCF7-HER2 clones (Fig. 10a). Src knockdown did not significantly affect the expression of ER $\alpha$  in these clones (Fig.

10b). To examine the significance of endogenous Src in the HER2 mediated activation of ER $\alpha$ -extranuclear signaling, we measured the activation of Src, and MAPK after treating cells with estrogen for 5 min. Estrogen addition uniquely promoted activation of Src and MAPK pathways in MCF7 cells and MCF7-HER2 cells showed excessive activation of MAPK and AKT pathways. However, Src-shRNA-expressing MCF7-HER2 cells had significantly less AKT and MAPK activation (Fig. 10c). In cell proliferation assays, MCF7-HER2 cells showed significantly increased proliferation compared MCF7 cells, while SrcshRNA expressing MCF7-HER2 clones showed decreased proliferation compared to parental MCF7-Her2 cells (Fig. 10d). Collectively, these results suggest that functional Src axis is necessary for HER2 mediated ER extranuclear actions and proliferation.

**Dasatinib blocks estrogen mediated ER extranuclear actions in therapy resistant cells:** Earlier studies shown that MCF7-PELP1 and MCF7-HER2 model cells exhibit hormonal therapy resistance. Since our findings suggested that Src kinase plays a key role in estrogen mediated extranuclear signaling in these cells, we examined the effect of pharmacological inhibition of Src kinase using dasatinib, a well-established orally available inhibitor of Src family tyrosine

kinases (10). In addition, we have also used MCF7-tam model cells, a well studied model cells that exhibit acquired resistance to tamoxifen (15). Short time estrogen treatment of MCF7 cells resulted in increased activation Src and MAPK pathways (Fig. 11a). Interestingly, all three resistant model cells have constitutively higher levels of Src activation and estrogen treatment substantially increased activation of MAPK in these model cells compared to therapy sensitive MCF7 cells (Fig. 11a). Dasatinib pretreatment abolished estrogen mediated activation of Src and MAPK pathways in therapy sensitive MCF7 and also in all three therapy resistant models (Fig. 11a). In estrogen driven proliferation assays, dasatinib (200 nM) treatment substantially reduced PELP1 mediated increase in estrogen driven cell proliferation (Fig 11b). Similarly, dasatinib (200 nM) treatment also reduced the proliferation of therapy resistant MCF-Tam and MCF7-HER2 cells. Collectively, these results suggest that Src

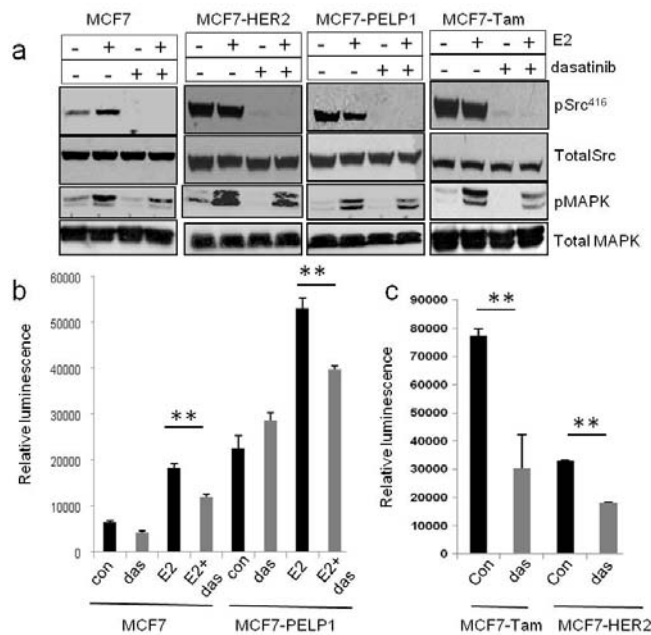


**Figure 11.** Dasatinib reduces E2 mediated ER extranuclear actions on therapy resistant cells. a, MCF7, MCF7-HER2, MCF7-PELP1 and MCF7-Tam cells were cultured in 5% DCC serum containing medium treated with or without E2 in the presence or absence of dasatinib. The activation of Src, and MAPK signaling pathways was analyzed by western blotting of total protein lysates with phospho-specific antibodies. b, Cell proliferation capacity of MCF7 and MCF7-PELP1 cells were analyzed after treating the cells with or without E2 in the presence or absence of dasatinib using Cell Titer-Glo assay. c, Cell proliferation capacity of MCF7-Tam, and MCF7-HER2 cells were analyzed after treating the cells with or without dasatinib using Cell Titer-Glo assay. \*, P<0.05; \*\*, P<0.001.

signaling plays a role in proliferation of therapy resistant cells and dasatinib can potentially be used to reduce estrogen-mediated extranuclear signaling in therapy resistant cells.

**PELP1 over expression enhances in vivo metastatic potential of ER-positive ZR75 cells:** We examined whether E2-mediated extranuclear actions contribute to cell migration and whether

EDC-mediated cell migratory potential can be blocked by pharmacological inhibition of Src kinase. Dasatinib effectively blocked the EDC-mediated cell migration in Boyden chamber assays (Fig. 12a). Similarly dasatinib also inhibited E2-mediated cell migration in wound healing assays (Fig. 12b). Since PELP1 expression is deregulated in metastatic tumors (14), we hypothesized that PELP1 overexpression may play a role in metastasis by promoting E2 extranuclear actions.

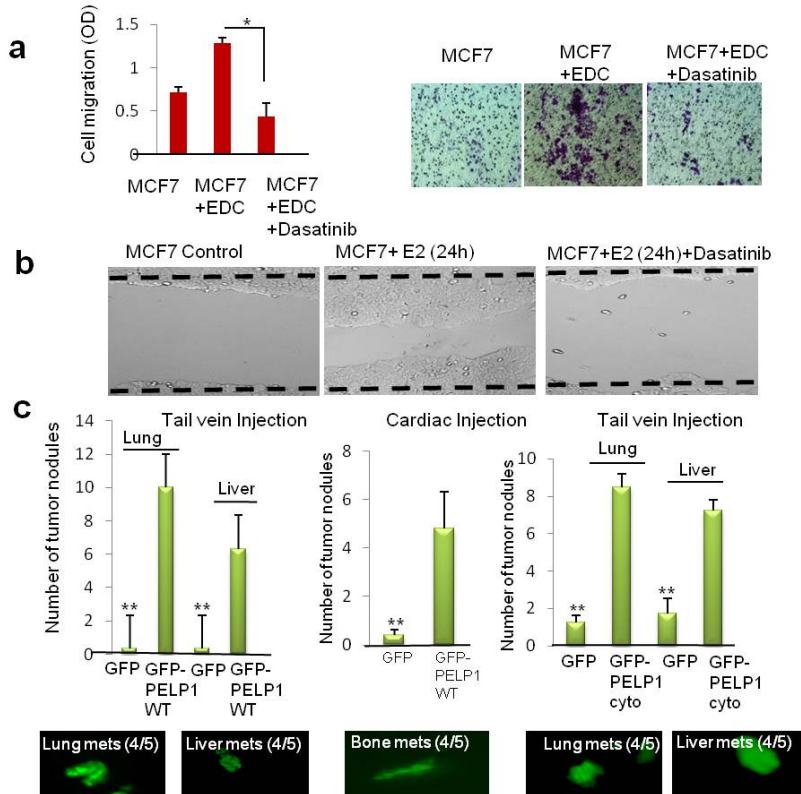


**Figure 12.** E2-mediated extranuclear actions promote cell migration and metastasis. **a**, MCF7 cells were treated with EDC in the presence or absence of Src inhibitor dasatinib (100 nM). The cell migratory potential was analyzed by using Boyden chamber assay. Data shown are the means of  $\pm$  SEM from three independent experiments performed in triplicate wells. \*,  $p < 0.05$ . **b**, Wound healing assay was performed in the presence or absence of E2 and in presence or absence of dasatinib. **c**, ZR75 cells expressing GFPvector or GFP-PELP1WT were injected into nude mice either via tail vein (**c**, Left panel) or cardiac route (**c**, middle panel) and metastases were recorded after 8 weeks. MCF7 cells expressing control GFP-vector or GFP+PELP1cyto were injected into nude mice ( $n=5$ ) via tail vein (**c**, right panel). Representative images of metastatic nodules as observed by fluorescence microscope are shown. The average number of tumor nodules is shown in the graph. Bars, SEM, \*\*,  $P < 0.0001$ .

(Fig. 12c, middle panel). To examine the significance of PELP1 extranuclear signaling in metastasis, we have repeated xenograft assay using PELP1cyto cells (12) that uniquely express PELP1 in the cytoplasm and are shown to excessively promote ER extranuclear signaling. Similar to PELP1WT cells, PELP1cyto cells also showed increased propensity to metastasize compared to MCF7 control cells (Fig. 12c, right panel). These results further suggest that ER-extranuclear actions have potential to promote metastasis.

We performed a proof-of-principle experiment using ER-positive ZR75 cells that exhibit poor metastasis in nude mice models. ZR75 cells were stably transfected with a GFP control or PELP1WT-GFP vector. PELP1WT-GFP cells had 3 fold higher expression of PELP1 than the control cells (data not shown). Mice injected with GFP control cells showed 0-1 metastatic nodules. However, PELP1-overexpressing cells had an increased propensity for metastases with 8-12 nodules identified in lungs (4 of 5 mice) and 6-8 nodules in liver (4 of 5 mice) (Fig. 12c). To validate these findings further, we also injected GFP-vector and GFP-PELP1WT cells via a cardiac route into nude mice. Earlier studies found that this route facilitates bone metastasis (16). GFP-PELP1WT-overexpressing cells, but not GFP vector-expressing cells, had metastases in the bone

## KEY RESEARCH ACCOMPLISHMENTS:

- Establishment of breast model cells with functional and defective PELP1 signaling axis
- Demonstration that endogenous PELP1 is needed for E2 mediated ER- extranuclear signaling
- Demonstration of the significance of ER extranuclear signaling on the migratory potential of breast cancer cells
- Demonstration that dasatinib have therapeutic utility in blocking ER-nongenomic actions
- Establishment of MCF7-PELP1 and MCF7-HER2 model cells with functional and defective Src signaling axis
- Demonstration that functional Src axis is necessary for HER2 mediated ER extranuclear actions and proliferation.
- Demonstration that dasatinib have therapeutic utility in sensitizing therapy resistant cells
- Demonstration that ER-extranuclear actions play an important role in metastases in vivo using xenograft models

**REPORTABLE OUTCOMES:** This study produced the following publications:

1. Chandrasekharan Nair B and Vadlamudi RK. Regulation of hormonal therapy resistance by cell cycle machinery *Gene Therapy and Molecular Biology* 2008 Dec;12:395-404.
2. Vadlamudi RK, Rajhans R, Chakravarty D, Chandrasekharan Nair B, Nair SS, Evans DB, Chen S, Tekmal RR.. Regulation of aromatase induction by nuclear receptor coregulator PELP1. *J Steroid Biochem Mol Biol*. 118:211-218, 2010
3. Chakravarty D, Nair SS, Santhanama B, Nair BC, Wang L, Bandyopadhyay A, Agyin JA, Brann D, Sun L, Yeh I, Lee FY, Tekmal R, Kumar R and Vadlamudi RK. Extranuclear functions of ER impact invasive migration and metastases of breast cancer cells. *Cancer Research*, 2010, 70(10):4092-101.
4. Chakravarty D, Tekmal R and Vadlamudi RK. PELP1: A novel therapeutic target for hormonal cancers. *IUBMB Life*. 2010 Mar;62(3):162-9.
5. Chakravarty D, Nair S, Nair BC, Wang L, Bandyopadhyay A, Agyin JK, Lee F, Sun L, Yeh I, Tekmal RR, Vadlamudi RK. Therapeutic potential of blocking ER non-genomic actions on ER positive metastasis. *Proceedings of the 100th Annual Meeting of the American Association for Cancer Research*; 2009 Apr 18-22; Denver, CO. Philadelphia (PA): AACR; 2009:2387.
6. Chakravarty D, Nair SS, Cortez V, Dandamudi R, Challa RB, Tekmal RR, Kumar R, Vadlamudi RK, Lee FY. Pathological Significance and Therapeutic Implications of ER Non-Genomic Actions. *Proceedings of the CTRC-AACR Breast Cancer Symposium*, San Antonio, TX. AACR?SABCS, 2009:3127.

## CONCLUSIONS:

In the first year of this study, we have generated *model* cells that have defects in PELP1-Src signaling axis. Using these models, we demonstrated that ER-extranuclear actions play an important role in cell motility, establishing for the first time that endogenous PELP1 has as a critical role in activating signaling events that lead to cell motility/invasion via ER- Src-PELP1 pathway. Our results using estrogen dendrimers (EDC) demonstrates that ER nongenomic signaling has potential to promote cytoskeletal changes, leading to increased cell migration. Our data suggest that PELP1 and Src kinase play an essential role in the activation of ER nongenomic signaling leading to cytoskeleton reorganization and migration. Since breast tumors overexpress Src kinase, deregulation of PELP1 seen in breast tumors can contribute to activation of Src kinase, leading to the progression to metastasis. Pharmacological inhibition of Src kinase using dasatinib significantly inhibited E2-mediated nongenomic actions. In the second year of the study we found that; (a) Functional Src axis is needed for optimal activation of ER $\alpha$  extranuclear actions, (b) Src plays a key role in PELP1 and HER2 oncogene mediated ER $\alpha$  extranuclear actions and proliferation, (c) Excessive ER $\alpha$  extranuclear signaling in therapy resistant cells is inhibited by pharmacological inhibition of Src. Collectively, these results suggests that deregulation of PELP1 axis has the potential to contribute to breast cancer progression and therapy resistance by accelerating ER extranuclear actions. Our data using Xenograft models provide the first evidence demonstrating the significance of ER-extranuclear signaling to the metastatic potential of breast cancer cells and suggest that PELP1 deregulation commonly seen in metastatic tumors may play a role in metastasis by enhancing ER-extranuclear signaling. These results suggest that the ER $\alpha$ -Src-PELP1 axis is a novel target for preventing the emergence of metastatic cells and that dasatinib may have therapeutic utility in blocking ER $\alpha$ -positive metastases. Our ongoing studies during the third year will address the role of dasatinib in sensitizing therapy resistant cells in vivo using xenograft model cells.

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## Regulation of hormonal therapy resistance by cell cycle machinery

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### Summary

Estrogen Receptor (ER) plays a central role in the development and progression of breast cancer. Hormonal therapy substantially improves disease-free survival of ER+ve breast tumors, however acquired resistance to endocrine therapies frequently occur. Emerging data implicate growth factor signaling pathways and their cross talk with ER as major cause of resistance. Both these pathways have been recently shown to use cell cycle machinery as downstream effectors in mediating therapy resistance. Several studies have demonstrated deregulation of cell cycle regulators and their cross talk with ER in therapy resistant tumors. The objective of this article is to review the underlying mechanisms by which tumor cells use cell cycle machinery to override hormonal therapy and to explore cell cycle machinery components as novel therapy targets for overcoming hormonal therapy resistance.

### Keywords

Cell Cycle; CDKs; Estrogen; Estrogen Receptor; Co-regulators; Breast cancer; Therapy resistance; Antiestrogens

### I. Introduction

Steroidal hormone estradiol (E2) and Estrogen Receptor (ER) plays a central role in the development and progression of breast cancer and 70–80% of breast tumors are ER positive at the time of presentation (McGuire and Clark, 1992). ER positive tumors respond well with therapeutic agents targeting ER functions (Ariazi et al, 2006). Endocrine therapy using Tamoxifen, a selective estrogen receptor modulator (SERM), has been shown to improve relapse-free and overall survival (Lewis-Wambi and Jordan, 2005). More recently, aromatase inhibitors, which deplete peripheral estrogen (E2) synthesis, are shown to substantially improve disease-free survival in postmenopausal women (Leary and Dowsett, 2006). Despite the success of antiestrogens, de novo and/or acquired resistance to endocrine therapies frequently occur. Approximately 30% of these patients acquire resistance to endocrine therapy in later stages and is a significant problem in the treatment regime (Riggins et al, 2007). Although mechanisms for hormonal therapy resistance remain elusive, emerging data implicate growth factor signaling pathways and its cross talk with ER as a major cause of resistance (Shou et al, 2004). Both these pathways have been recently shown to use cell cycle machinery as downstream effectors in mediating therapy resistance (Shou et al, 2004; Perez-Tenorio et al, 2006; Ru et al, 2006). The prime focus of this review is to recapitulate the literature elucidating

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the role of cell cycle machinery as downstream effectors of various pathways leading to hormone therapy resistance.

## II. Estrogen receptors and coregulators

The human estrogen receptor (ER) is a key transcriptional regulator in breast cancer biology (Green and Carroll, 2007; Heldring et al, 2007). The biological effects of estrogen is mediated by its binding to the structurally and functionally distinct ERs (ER $\alpha$  and ER $\beta$ ) (Warner et al, 1999). ER  $\alpha$  is the major ER in the mammary epithelium and this has been further shown by ER $\alpha$  (Esr1) knockout mice, which display grossly impaired ductal epithelial cell proliferation and branching (Lubahn et al, 1993; Bocchinfuso and Korach, 1997). ERs comprises an N-terminal activation function 1 (AF1) domain, a DNA-binding domain, and a C-terminal ligand binding region that contains an activation function 2 (AF2) domain (Kumar et al, 1987). The AF-2 of the ER is located in the ligand binding domain, while the N-terminal AF-1 functions in a ligand-independent manner. AF-1 and AF-2 exhibit cell type and promoter context specificity (Berry et al, 1990). Upon binding of E2 to ER, the ligand-activated ER translocates to the nucleus, binds to the responsive element in the target gene promoters, and stimulates gene transcription (genomic/nuclear signaling) (McKenna et al, 1999; McDonnell and Norris, 2002). In addition to its well-studied nuclear functions, ER also participates in non-genomic signaling events in the cytoplasm and membrane. Such signaling has been linked to rapid responses to E2 which generally involves the stimulation of the Src kinase, MAPK, and AKT (Pedram et al, 2002).

Transcriptional activity of ERs is regulated not only by hormones but also by several coregulatory proteins called coactivators and corepressors (McKenna et al, 1999). The transcription functions of ER are shown to be influenced by several coactivators, including SRC1, GRIP1, AIB1, PELP1 and corepressors including nuclear receptor corepressor (NCoR), silencing mediator for retinoic and thyroid receptor (SMRT) and MTA1 (Tsai and O'Malley, 1994; Barnes et al, 2004). Coactivators preferentially associate with ligand bound ER while corepressors have been shown to preferentially associate with antagonist occupied ERs (Jepsen and Rosenfeld, 2002). Evidence suggests that multi-protein complexes containing coactivators, ERs, and transcriptional regulators assemble in response to hormone binding and activate transcription (McKenna et al, 1999). Accumulating evidence suggests that ER-coregulators play an essential role in hormonal responsiveness and cancer progression (Bocchinfuso and Korach, 1997; McKenna et al, 1999).

## III. Estrogen and cell cycle progression

It is well accepted that estrogen induces mitogenesis by recruiting non-cycling cells into the cell cycle and by increasing the rate of progression from G<sub>0</sub> to S phase. However, the molecular mechanism by which E2-ER signaling controls cell proliferation is not completely understood. Induction of the early-response genes (such as c-myc and c-fos) is proposed as one mechanism of this process (Prall et al, 1998a; Lamb et al, 2000), whereas regulation of Cyclin Dependent Kinase (CDK2 and CDK4) activities was proposed as another (Neuman et al, 1997; Prall et al, 1997; Foster et al, 2001). Each phases of cell cycle (G<sub>1</sub>, S, G<sub>2</sub> and M) is strictly under the control of different Cyclins and CDKs. CDK4 and CDK2 enhance G<sub>1</sub>-S transition in the cell cycle and for tumorigenesis, indicating that phosphorylation of downstream effector proteins by CDKs is vital for cell proliferation. Previous studies have shown that Cyclin D1-CDK4 and Cyclin ECDK2 are major regulators of G<sub>1</sub>/S transition, while Cyclin A-CDK2 controls S-phase and Cyclin B1-CDK1 controls transition through M-phase. The kinases are traditionally known to phosphorylate many key downstream substrates, most notably retinoblastoma and exhibit strict and elegant control of cell cycle progression. In addition, Cyclin D1 was identified as a target of E<sub>2</sub> action, and estrogen treatment was shown to up-regulate Cyclin D1 levels (Altucci

et al, 1996). Up-regulation of Cyclin D1 by ER signaling is accompanied by an increased proliferative response in breast cancer cells. E2 is shown to induce Cdc25A, a tyrosine phosphatase that controls G1-S transition in cell cycle by regulating the dephosphorylation of Cyclin-dependent kinase complexes (Ru et al, 2006). Collectively, these findings suggest that Estrogen induces proliferation of ER-positive breast epithelial cells by stimulating G<sub>1</sub>/S transition, which is associated with increased cyclin D1 expression and activation of CDKs (Foster et al, 2001). Since CDK4 and CDK2 are key players for G1-S transition in the cell cycle and for tumorigenesis, ER crosstalk with CDKs will have implications in therapy resistance.

#### IV. ER coregulators and cell cycle progression

Evolving evidence suggests that many of the ER coregulators play a vital role in cell cycle progression. Emerging evidence suggest that oncogenic ER-coregulatory proteins such as AIB1, PELP1 modulate Cyclin D1 expression and function, thus may enhance tumorigenesis and therapy resistance. We have summarized below some of the ER coregulators that are shown to play a role in E2-ER mediated cell cycle progression.

##### A. AIB1

ER coregulator SRC3/AIB1 is shown to regulate cell cycle machinery in numerous ways. AIB1 is shown to enhance E<sub>2</sub>-dependent induction of Cyclin D1, suggesting a role for ER coregulators in modulating Cyclin D1 expression (Planas-Silva et al, 2001). AIB1 is also shown to interact with E2F directly and modulate its transactivation function and is required for E2F1-mediated gene expression (Louie et al, 2004). Recent evidence also suggests that AIB1 has oncogenic potential and the transformation ability of AIB1 has been ascribed to its ability to control the expression of genes important for initiating DNA replication like cdc6, MCM7, Cyclin E, and CDK2 (Louie et al, 2006). E2F regulates AIB1 expression by cooperating with the transcription factor specificity protein 1 (Sp1) without direct interaction with E2F consensus sites, suggesting a positive feedback regulatory loop comprising of E2F and AIB1 (Mussi et al, 2006).

##### B. Ciz1

Ciz1, a p21(Cip1/Waf1)-interacting zinc finger protein is shown to function as an ER co-regulator and Ciz1 over-expression confers estrogen hypersensitivity and promotes the growth rate, anchorage independency, and tumorigenic properties of breast cancer cells. These effects on cell cycle progression is shown to be ER dependent through upregulation of Cyclin D1 expression (Den et al, 2006). However, a direct role of Ciz1 in DNA replication process in S phase has also been suggested. Ciz1 co-localizes with PCNA during S phase while depletion of Ciz1 restrains cell proliferation by inhibiting entry to S phase (Coverley et al, 2005).

##### C. CARM1/PRMT4

CARM1 is a methyltransferase that associate with ER coregulators and regulate transcription by histone H3 methylation and is essential for estrogen induced cell cycle progression (Chen et al, 1999). SiRNA mediated depletion of CARM1 in ER positive MCF7 and T47D cells reduced E2 mediated cell cycle progression (Fietze et al, 2008). Recent evidence also suggest that CARM1 regulate not only E2 mediated E2F expression but also expression of E2F target genes. The recruitment of CARM1 to E2F target genes and associated increase in H3R17 dimethylation during transcriptional activation has been shown to be dependent on another ER coactivator AIB1 (Fietze et al, 2008). In a recent study, expression of Cyclin E gene has been shown to correlate with recruitment of CARM1 on its promoter and associated increase in H3-R26 and H3-R17 methylation at its promoter (El et al, 2006). Consistent with the role of

CARM1 in regulating cell cycle genes, CARM1 knockout mice show small embryos and perinatal lethality (Yadav et al, 2003).

#### D. PELP1/MNAR

PELP1 is another ER coregulator that is shown to play a role in E2-mediated G1/S-phase progression (Balasenthil and Vadlamudi, 2003). PELP1 is a pRb-interacting protein and PELP1 deregulation promotes cyclin D1 expression. Breast cancer model cells, which overexpressed PELP1 showed persistent hyperphosphorylation of the pRb protein in an E2 dependent manner accompanied with increase in proliferation rate (Balasenthil and Vadlamudi, 2003). Recent studies suggested that PELP1 is a phospho-protein and its phosphorylation changes during cell cycle progression. PELP1 interacts with G1/S phase CDKs (both CDK4 and CDK2), and is a novel substrate to both of these enzymes (Chandrasekharan Nair et al, 2008a). Furthermore, increased PELP1 expression in a mammary gland during pregnancy, when the rate of cell proliferation is high, supports a physiological role for PELP1 in E2-mediated cell cycle progression in mammary glands (Vadlamudi et al, 2001). PELP1 is also known to interact with key proteins like Src, PI3K, four and a half LIM only protein2 to mediate E2 dependent non-genomic functions of ER. Mitogenic stimulus promotes PELP1 interaction with growth factor signaling component, epidermal growth factor (EGFR), HER2, STAT3, and hepatocyte growth factor regulated tyrosine kinase substrate (HRS) (Vadlamudi and Kumar, 2007). PELP1 has highest tissue expression in brain, testes, ovary and uterus (Khan et al, 2005; Vadlamudi and Kumar, 2007) and studies from rodent biology suggest that PELP1 is developmentally regulated and expressed at classical steroid target sites in brain like hippocampus, cortex, hypothalamus, amygdala and septum (Khan et al, 2005). Collectively, these emerging findings suggest that PELP1 plays a key role in relaying mitogenic signals, both in cytoplasm and nucleus and therefore is an attractive therapeutic target. The fact that siRNA mediated knockdown of PELP1 reduces cell proliferation in MCF-7 breast cancer cells strongly suggest that blocking PELP1 functions will undeniably benefit cancer therapeutic regime (Chandrasekharan Nair et al, 2008).

#### V. Modulation of cell cycle progression by anti-estrogens

Estrogens and anti-estrogens both are shown to exert their functions in G1 phase, where they regulate Cyclin D1 and Cyclin E expression and hence modulate the kinase function of CDK4 and CDK2, respectively. Inhibition of CDK kinase function leads to accumulation of hypophosphorylated retinoblastoma and resulting in cell cycle arrest. In short, the consensus is that estrogen accelerates the G1 phase passage while antiestrogens inhibit cell cycle progression by affecting these key cell cycle proteins. On the contrary, a recent transcriptional profiling presented a rather intriguing result regarding functioning of tamoxifen at the molecular level. Tamoxifen and estrogen both positively regulated a large set of cell cycle genes like cmyc, myb, fos, cdc25a, Cyclin E, Cyclin A2, and stk15 while the differential effect was on only few cell cycle genes, most notably on Cyclin D1 (Hodges et al, 2003). Interestingly, only Tamoxifen but not Raloxifene induced these key cell cycle regulators (Hodges et al, 2003).

Emerging evidence suggest that CDK inhibitors are also regulated by antiestrogens in mediating growth arrest. Tamoxifen treated breast cancer cell lines show a reduction in Cyclin D, increase p27 and simultaneous increase in Cyclin E-CDK2 bound p27 (Chu et al, 2005). In the same study, combination treatment of Tamoxifen along with a dual HER1/HER2 inhibitor, lapatinib (GW572016) showed more profound effect on these cell cycle regulators and rapid cell cycle arrest in all the three cell lines tested. Transduction of Tamoxifen treated cells with p-27 peptides (TAT-p27) helped in maintaining quiescence and made the cells resistant to mitogen stimulation (Carroll et al, 2003). These studies evoke the potential of using anti-p27 molecules in future to reverse Tamoxifen resistance. The recent findings that miRNAs also

regulate Tamoxifen response in cancer cells is an exciting advance in understanding therapy resistance. Upregulation of miR-221 and/or miR-222 has been directly shown to promote therapy resistance through downregulation of ER  $\alpha$  (Zhao et al, 2008).

Recent studies also implicated role of p53 in Tamoxifen mediated cell cycle arrest. Ichikawa et al. reported a concomitant increase in p53 expression and p21, a known CDK2 inhibitor in Tamoxifen treated MCF7 in time and dose dependent manner, suggesting possible role of p53 in mediating the G1 arrest caused by Tamoxifen (Ichikawa et al, 2008).

Future studies, however, are required to understand whether antiestrogens affect the expression of Cyclins at transcriptional level or whether unidentified intermediary players govern this pathway in a similar fashion as p53. Identifying key G1-S transition regulatory genes that are relieved of pRb mediated repression due to treatment with antiestrogens will be a priority to unravel more downstream players in antiestrogen mediated cell cycle arrest and such studies will further enhance understanding of antiestrogen resistance.

## VI. Cell cycle regulators and hormonal therapy resistance

There has been phenomenal advance in our understanding the role of cell cycle regulators in hormonal therapy resistance. Since tamoxifen mediate the cell cycle arrest by deregulating cell cycle regulators, it is perhaps not surprising that aberrant change in cell cycle machinery often contribute to induction of antiestrogen resistance. We have summarized below the evidence that showed potential role of the regulators of cell cycle machinery in promoting therapy resistance (Figure 1).

### A. Cyclin D1

Cyclin D1 was originally cloned as an oncogene (Motokura et al, 1991) and over-expression of Cyclin D1 has been noted in over 50% of human breast tumors of all histological types (Gillett et al, 1994; Kenny et al, 1999). There is surmounting evidence to suggest that altered Cyclin D1 expression promotes antiestrogen resistance (Wilcken et al, 1997; Pacilio et al, 1998; Hui et al, 2002). Cyclin D1 binds ER and increases its transcriptional activity (Neuman et al, 1997). This ability of Cyclin D1 to transactivate ER functions was independent of estrogen stimulation and interestingly, on its CDK4 association as well (Neuman et al, 1997). Over-expression of Cyclin D1 indeed was able to overcome the growth arrest mediated by antiestrogens but Cyclin D1 mutant that is unable to activate CDK4 but having intact ER transactivating potential was not able to promote cell proliferation in the presence of antiestrogens (Bindels et al, 2002). Cyclin D1 is shown to be over-expressed among different Tamoxifen resistant breast cancer cells (Kilker et al, 2004) and Cyclin D1 specific siRNAs restored the sensitivity of these cells to Tamoxifen suggesting therapies targeting Cyclin D1 may have therapeutic effect in hormonal therapy resistant cells (Kilker and Planas-Silva, 2006).

Furthermore, an alternative splice variant of Cyclin D1 named Cyclin D1b is reported to be over expressed in a variety of breast cancers (Betticher et al, 1995; Hosokawa et al, 1997; Wang et al, 2008) and appears to function as a nuclear oncogene (Lu et al, 2003). Cyclin D1b is also known to associate with CDK4 with a weaker kinase activity and over-expression of this alternative transcript Cyclin D1b is shown to overcome the antiestrogen mediated cell cycle arrest (Wang et al, 2008). Unlike Cyclin D1, this effect was independent of ER transactivation as Cyclin D1b lacks nuclear receptor interaction LXXLL motif but retains binding site for CDK4 (Wang et al, 2008).

In addition to activating CDK4, Cyclin D1 is also shown to promote hormonal therapy resistance through other pathways (Ishii et al, 2008). Cyclin D1 is known to mediate STAT3

repression but cells treated with Tamoxifen can potentially reverse this STAT3 repression by the redistribution of Cyclin D1 from STAT3 to ER-complex. This was confirmed by in vivo nude mice assays, where it was shown that growth of Cyclin D1–overexpressing tumors was stimulated by Tamoxifen treatment with concurrent elevation and activation of STAT3 (Ishii et al, 2008). PI3K/AKT or MAPK/ERK1 signaling is also reported to contribute to Cyclin D1 expression and promote to therapy resistance to Tamoxifen underscoring the importance of cross talk between various mitogenic pathways with cell cycle machinery in ultimately achieving antiestrogen resistance (Kilker et al, 2004).

Cyclin D1 negative tumor patients show better relapse free survival upon Tamoxifen-based therapy while Cyclin D1 expression correlated well with poor outcome upon antiestrogen treatment (Rudas et al, 2008). Clinical study with randomized post-menopausal breast cancer patients also show that Cyclin D1 over-expression correlates with poor outcome with Tamoxifen treatment (Stendahl et al, 2004). Similar results were obtained with premenopausal breast cancer patients with Cyclin D1 gene amplification (Jirstrom et al, 2005). Collectively these emerging finding suggest importance of Cyclin D1 as a useful predictive marker in the selection of Tamoxifen-based therapy regime.

## B. Cyclin E

Deregulation of Cyclin E in breast cancer model cells has been shown to resist cell cycle arrest mediated by Tamoxifen and this effect in part was attributed to the aberrant activation of E2F-Rb pathway (Dhillon and Mudryj, 2002). Subsequent studies showed that Cyclin E level showed good correlation with poor relapse-free-survival in patients treated with antiestrogens (Span et al, 2003). Interestingly, Cyclin E was not observed to be good prognostic marker for breast cancer as a whole, however, Cyclin E is a good predictor of antiestrogen resistance (Span et al, 2003; Desmedt et al, 2006). Another important feature of Cyclin E is its tumor specific proteolytic cleavage, yielding low molecular weight (LMW) forms of Cyclin E (Porter et al, 2001). Recent reports suggest that these LMW Cyclin E, lacking varying amount of amino terminal region of whole length Cyclin E, plays a vital role in promoting hormone therapy resistance (Akli et al, 2004). The LMW forms of Cyclin E could complex with CDK2 and accounts for increased CDK2 activity as compared to full length Cyclin E (Akli et al, 2004). LMW Cyclin E overexpressing MCF-7 cells showed greater resistance toward ICI-182,780 mediated growth arrest as compared to full length Cyclin E and this resistance was attributed to decreased inhibitory effects of p21 and p27 on these LMW-Cyclin E forms (Akli et al, 2004).

## C. Cyclin A

Emerging evidences suggest that Cyclin A also play important role in hormone therapy resistance. Detection of Cyclin A over expression by immuno-histochemical methods correlated well with early breast cancer relapse and can be considered a good marker of Tamoxifen resistance (Michalides et al, 2002). Cyclin A is also known to associate with CDK2 and phosphorylates ER and thereby increase its transactivation potential (Trowbridge et al, 1997). Cyclin A/CDK2 complex phosphorylates Ser-104 and Ser-106 located in the AF-1 domain of ER and increase its transcriptional activity (Rogatsky et al, 1999). The ER transactivation through CDK2-Cyclin A phosphorylation is evident in presence and the absence of estrogen stimulation and also with Tamoxifen treatment (Rogatsky et al, 1999). Large scale randomized trials are however required to understand the potential of CDK2-Cyclin A mediated phosphorylation of ER as a prognostic marker for assessing the efficacy of antiestrogen therapy regime.

## D. Cyclin dependent kinases

Most downstream events in antiestrogen resistance signaling pathways, like upregulation of various Cyclins ultimately converge upon modulation of Cyclin Dependent Kinases; the most conspicuous of which is the activation of Cyclin Dependent Kinase 2 (CDK2) (Dhillon and Mudryj, 2002; Akli et al, 2004). Apart from CDK2, CDK10 has been recently implicated in hormone resistance. CDK10 is a newly reported player in mediating antiestrogen therapy resistance, identified by functional genomics approach (siRNA screen) (Iorns et al, 2008; Swanton and Downward, 2008). An unbiased loss of function siRNA screen performed by Iorns et al, identified modulators of Tamoxifen sensitivity and found that RNAi mediated downregulation of CDK10 increases ETS2-driven transcription of c-RAF, resulting in MAPK pathway activation and independence from ER pathway. Loss of CDK10 in ER positive breast cancer was shown to be associated with relapse of cancer after anti-hormone therapy. CDK10 is cdc2 related kinase found to play important role in G2-M progression. While no Cyclins have been identified to associate with CDK10, it is known that ETS2 is interacting partner of CDK10 (Kasten and Giordano, 2001). This low amount of CDK10 in antiestrogen resistant cells were attributed to the methylation of CDK10 promoter in vivo, underscoring the importance of epigenetic changes accompanying the hormone resistance phenotype (Iorns et al, 2008).

## E. CDK inhibitors

Down regulation of p21 has been implicated with Tamoxifen resistant phenotype. Somatic deletion of p21 gene in human breast cancer cells demonstrated that these cells were resistant to Tamoxifen mediated growth arrest (Abukhdeir et al, 2008). The mechanism behind this effect was attributed to increased ER phosphorylation at serine 118 by CDK complex upon p21 decrease. Role of ER phosphorylation as an effector of Tamoxifen resistance was elucidated by transfecting p21 null-MCF10A cells with ER cDNA constructs with Serine118 mutated to alanine. These transfected cells became responsive to Tamoxifen, proving that ER activation is the downstream element in p21 mediated Tamoxifen growth resistant phenotype (Abukhdeir et al, 2008). Antiestrogen resistance could be abolished by treating cells with antisense p21 or p27 oligonucleotides, leading to activation of Cyclin Dependent Kinase 2 (Cariou et al, 2000).

Among various molecular pathways implicated in down regulating CDK inhibitors, MAPK/MEK activation is notable (Donovan et al, 2001). MEK inhibitor, U0126 was used to inhibit MEK pathway and re-sensitized to growth arrest by antiestrogen in LY-2 model cells of antiestrogen resistance. Different phospho-isoforms of p27 were detected in these antiestrogen resistant model cells that may contribute toward generating resistance phenotype (Donovan et al, 2001). Detailed studies are however warranted to delineate and correlate specific sites of phosphorylation on p27 with clinical outcome with antiestrogen therapy.

Localization of CDK inhibitors has also been implicated in the development of antiestrogen resistance. Studies have shown that heregulin  $\beta$ 1 over-expression that activates PI3K and MAPK pathway, also promotes p21 localization into cytoplasm (Perez-Tenorio et al, 2006). Tumors with increased cytoplasmic localization of p21 respond poorly with Tamoxifen treatment (Perez-Tenorio et al, 2006). In premenopausal women with early breast cancer, an increase in p27/KIP1 expression was able to predict better relapse free survival upon Tamoxifen combination treatment (Pohl et al, 2003). This trial included 512 randomized patients wherein multivariate analysis revealed decreased p27 expression to be correlated with poor outcome upon combination endocrine therapy. A recent study indicated that p27kip1 is another important target of miR-221 that promotes mediate resistance to hormonal therapy (Miller et al, 2008).

## F. Retinoblastoma and E2Fs

Rb-E2F pathway plays a fundamental role in cell proliferation and deregulation is frequently observed in breast cancer. siRNA mediated Rb ablation is able to overcome the growth arrest by antiestrogen treatment and using in vivo xenograft model, Rb deficient tumors were shown to retain the ability to grow in spite of Tamoxifen treatment (Bosco et al, 2007). Furthermore, the same study included analysis of 60 human breast cancer patients treated with Tamoxifen to generate a Rb gene expression signature (Bosco et al, 2007). Another study found that expression of viral T-antigens in breast cancer cells (MCF7) that promote inactivation of endogenous Rb, elicited antiestrogen resistance (Varma and Conrad, 2000). P53 binding ability of T-antigen was however shown not required for this phenotype. In continuation of this work, Conrad and colleagues elucidate the molecular mechanism behind Rb's role in promoting antiestrogen resistance (Varma et al, 2007). Inducible pLT cell lines were utilized to demonstrate that functional inactivation of pRb can lead to CDK2/Cyclin A activation and reversal of antiestrogen mediated cell cycle arrest. The new hypothesis put forward was that ER<sup>+</sup> Rb<sup>-</sup> tumors showing increased CDK2 activity and resulting hormone therapy resistance can be targeted by agents blocking CDK2. Currently many such CDK2 targeting drugs (although not very specific ones) are available in clinical trials and need to be evaluated in the context.

## G. c-Myc

c-Myc is a well known cell cycle regulator and oncogene frequently up regulated in breast cancer. It is also one of the earliest estrogen responsive gene, showing a noticeable increase in protein level within 15 min of estrogen treatment (Dubik et al, 1987). C-myc expression when induced in MCF-7 using Tet-on expression system could potentially abrogate antiestrogen mediated growth arrest (Venditti et al, 2002). Similar results were obtained in a different study, wherein over expression of c-myc down regulated p21 expression and mediated antiestrogen resistance (Mukherjee and Conrad, 2005). C-myc expression can rescue the G1 arrest mediated by Tamoxifen by activating CDK2/Cyclin E complex and further phosphorylation of p130 (Prall et al, 1998a). Involvement of c-myc in regulating p21 expression levels and contributing to emergence of antiestrogen resistance is also reported (Mukherjee and Conrad, 2005). p21 levels in antiestrogen resistant cells increased when treated with cmyc siRNAs, suggesting important role of c-myc in downregulating p21 levels and promoting hormonal therapy resistance (Mukherjee and Conrad, 2005).

From the above mentioned studies, we present an interesting case that cell cycle regulators play a vital role in the emergence of hormone therapy resistance. However, the studies performed so far do not provide clear distinction of using cell cycle regulators as prognostic markers of therapy resistance or therapeutic targets against resistant cells. The key challenge in this area is to unequivocally show that targeting cell cycle regulators can potentially reverse the hormone therapy resistance but the side effects may limit their use as evidenced by recent studies. Targeting kinase functions of CDK2 is a feasible option and currently there are some ongoing clinical studies employing pan CDK inhibitors against non-small cell lung cancer like r-roscovitine (Seliciclib or CYC202). Our lab has recently tested the efficacy of combinatorial usage of r-roscovitine with Tamoxifen against various hormone resistant cell lines like MCF-tam (resistant to Tamoxifen), MCF-7-Her2 (overexpressing Her2), and MCF7-PELP1 (overexpressing PELP1) and found encouraging results in sensitizing these cells to Tamoxifen treatment (Chandrasekharan Nair et al, 2008b). Another possibility to overcome toxic side effects would be to explore nanotechnology methods that allow cancer cell specific delivery of the cell cycle inhibitors reducing toxic side effects. Such combinatorial use of cell cycle inhibitors along with classical hormone therapy represents a novel therapeutic modality to circumvent the problem of toxicity and to enhance therapeutic success.

## VII. Conclusions and Future Direction

The estrogen receptor (ER) plays a central role in the progression of breast cancer and endocrine therapy is widely used to target ER+ve breast cancer. Despite the positive effects, de novo and/or acquired resistance to endocrine therapies frequently occur. Most downstream events in the resistance signaling pathways appear to converge upon modulation of cell cycle regulatory proteins. Evolving evidence suggests that cell cycle machinery cross talk with estrogen receptors, ER-coregulators and growth factor receptors and such interaction play a role in the development of therapy resistance. It is therefore of great interest to understand how cell cycle machinery promotes therapy resistance. Since cell cycle dependent kinases cross talk with nuclear receptors and coregulators to regulate various downstream genes, we believe that associated nucleosomal histone modification via methylation and acetylation could play a vital role in therapy resistance. There is scarcity of studies toward understanding cell cycle dependent histone/DNA modifications and epigenetic changes that contribute toward acquiring hormone therapy resistance. Similarly, identifying newer substrates of CDKs and investigating their potential role in therapy resistance will provide novel insights into the mechanistic basis of Tamoxifen resistance. Combinatorial therapy using CDK inhibitors along with conventional hormone therapy is a feasible option to resensitize the cells against hormone therapy resistance. Future microRNA profiling studies is expected to identify new miRNAs that regulate cell cycle machinery, thus increase the repertoire of novel targets for interfering hormone therapy resistance. Future studies are also warranted in safe delivery of cell cycle inhibitors utilizing new technologies (such as targeted nano particles) to enable to use these new drugs with less side effects. We strongly believe that further understanding of the molecular mechanisms by which tumor cells use cell cycle machinery to acquire therapy resistance will provide novel therapeutic targets, which in conjunction with conventional hormone therapy will be useful in targeting therapy resistant tumors.

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## Biography

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## Abbreviations

|      |                                                    |
|------|----------------------------------------------------|
| CDK  | cyclin-dependent kinase                            |
| EGF  | epidermal growth factor                            |
| ER   | estrogen receptor                                  |
| MAPK | mitogen-activated protein kinase                   |
| MNAR | modulator of nongenomic actions of the ER          |
| NR   | nuclear receptor                                   |
| PI3K | phosphatidylinositol-3 kinase                      |
| PELP | proline-, glutamic acid-, and leucine-rich protein |
| PKA  | protein kinase A                                   |
| pRb  | retinoblastoma protein                             |

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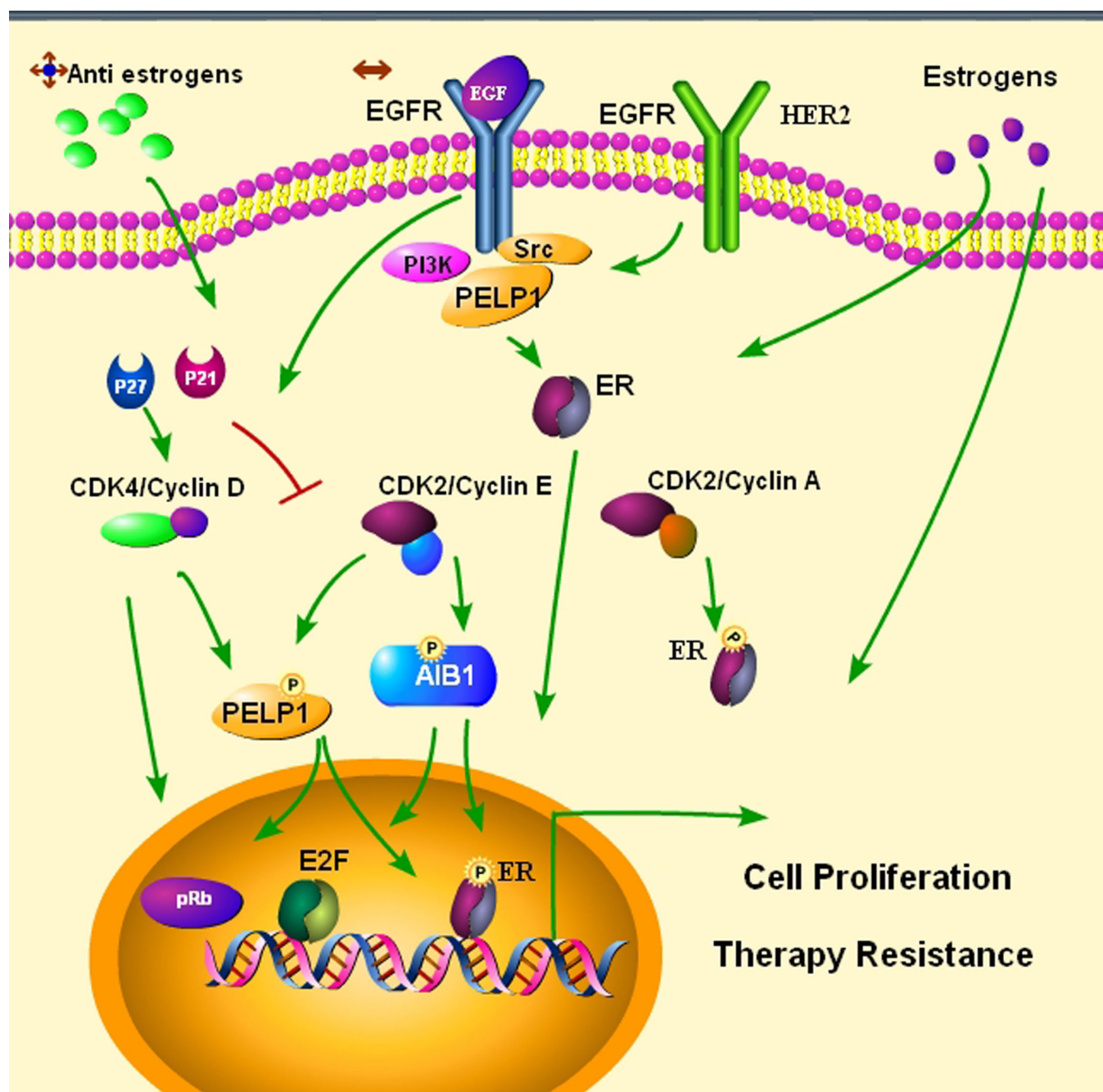


**Figure 1.** Schematic representation of the current understanding of regulation of hormonal therapy resistance by cell cycle machinery. Convergence of growth factors and estrogen receptor signaling pathways in therapy resistant cells suggest that deregulation cell cycle regulators are likely to contribute to the development of therapy resistance in breast cancer cells.

Regulation of aromatase induction by nuclear receptor coregulator PELP1<sup>☆</sup>

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## ABSTRACT

Estradiol (E2), estrogen receptor (ER), ER-coregulators have been implicated in the development and progression of breast cancer. In situ E2 synthesis is implicated in tumor cell proliferation through autocrine or paracrine mechanisms, especially in post-menopausal women. Several recent studies demonstrated activity of aromatase P450 (*Cyp19*), a key enzyme that plays critical role in E2 synthesis in breast tumors. The mechanism by which tumors enhance aromatase expression is not completely understood. Recent studies from our laboratory suggested that PELP1 (Proline, Glutamic acid, Leucine rich Protein 1), a novel ER-coregulator, functions as a potential proto-oncogene and promotes tumor growth in nude mice models without exogenous E2 supplementation. In this study, we found that PELP1 deregulation contributes to increased expression of aromatase, local E2 synthesis and PELP1 cooperates with growth factor signaling components in the activation of *aromatase*. PELP1 deregulation uniquely up-regulated aromatase expression via activation of *aromatase* promoter I.3/II. Analysis of PELP1 driven mammary tumors in xenograft as well as in transgenic mouse models revealed increased aromatase expression. PELP1-mediated induction of aromatase requires functional Src and PI3K pathways. Chromatin immuno precipitation (ChIP) assays revealed that PELP1 is recruited to the Aro 1.3/II aromatase promoter. HER2 signaling enhances PELP1 recruitment to the *aromatase* promoter and PELP1 plays a critical role in HER2-mediated induction of *aromatase* expression. Mechanistic studies revealed that PELP1 interactions with orphan receptor ERR $\alpha$ , and histone demethylases play a role in the activation of aromatase promoter. Accordingly, ChIP analysis showed alterations in histone modifications at the aromatase promoter in the model cells that exhibit local E2 synthesis. Immunohistochemical analysis of breast tumor progression tissue arrays suggested that deregulation of aromatase expression occurs in advanced-stage and node-positive tumors, and that cooverexpression of PELP1 and aromatase occur in a sub set of tumors. Collectively, our results suggest that PELP1 regulation of aromatase represent a novel mechanism for in situ estrogen synthesis leading to tumor proliferation by autocrine loop and open a new avenue for ablating local aromatase activity in breast tumors.

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## 1. Introduction

Mammary tumorigenesis is accelerated by the action of ovarian hormones, and approximately 70% of breast tumors are ER-positive at the time of presentation. Endocrine therapy is the most important component of adjuvant therapy for patients with early stage ER-positive breast cancer [1]. The biological functions of estrogens are mediated by the nuclear receptor ER, a ligand-dependent transcription factor that modulates gene transcription via direct

recruitment to the target gene chromatin. In addition, ER also participates in cytoplasmic and membrane-mediated signaling events (non-genomic signaling) and generally involves cytosolic kinases including Src, MAPK, PI3K [2,3]. Accumulating evidence strongly suggest that ER signaling requires coregulatory proteins and their composition in a given cell determine the magnitude and specificity of the ER signaling [4,5]. Coregulators function as multitasking molecules and appear to participate in a wide variety of actions including remodeling and modification of chromatin [6]. Coregulators appear to have the potential to sense the physiological signals [7] and activate appropriate set of genes, thus have potential to function as master regulators, and their deregulation is likely to provide cancer cells an advantage in survival, growth and metastasis [8,9]. A commonly emerging theme is that marked alteration in the levels and functions of coregulators occurs during the

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progression of tumorigenesis [10]. Although much is known about the molecular basis of interaction between ER and coregulators, very little is known about the physiological role of coregulators in the initiation and progression of cancer.

## 2. Regulation of aromatase in breast

Aromatase (Cyp19), a key enzyme involved in E2 synthesis [11], is expressed in breast tumors and locally produced E2 might act in a paracrine or autocrine fashion [12]. Breast tumors from post-menopausal women are shown to contain higher amounts of E2 than would be predicted from levels circulating in plasma [13]. Expression of the aromatase gene is under the control of several distinct and tissue-specific promoters; however, the coding region of aromatase transcripts and the resulting protein is identical [14]. In disease free breast, aromatase expression is directed via distal 1.4 promoter, while aromatase expression is shown to be activated via PII and 1.3 in adipose tissue and epithelial cells in breast bearing tumor [15–17]. Recently, aromatase inhibitors that inhibit peripheral E2 synthesis are shown to be more effective in enhancing the survival of post-menopausal women with ER+ve breast cancer [18]. Even though these new treatments appear successful, emerging data suggest that tumors evade this treatment by developing “adaptive hypersensitivity” manifested as hormone-independent tumorigenesis through increased non-genomic signaling and growth factor signaling cross-talk [19–21]. Recent studies also demonstrated that HER2 status plays an important role in tumor-induced aromatase activity via the COX-2 pathway [22]. Further, HER2 overexpression can also promote ligand-independent recruitment of co-activator complexes to E2-responsive promoters, and thus may play a role in the development of letrozole resistance [21]. Accumulating evidence also suggest that a variety of different factors may regulate expression and activity of aromatase under pathological conditions and local production of estrogen may enhance tumor growth and may also interfere with hormone therapy [23]. The molecular mechanism by which breast tumors enhance local aromatase expression and whether epigenetic changes play a role in activation of aromatase in tumors remain unknown and is an active area of research investigation.

## 3. PELP1, a novel ER-coregulator

Proline, glutamic acid, leucine rich protein 1 (PELP1), also called as a modulator of non-genomic actions of estrogen receptor (MNAR) is a novel ER-coregulator [24,25]. PELP1 contains several motifs and domains that are commonly present in many transcriptional co-activators, including 10 nuclear receptor (NR)-interacting boxes (LXXLL motifs), a zinc finger motif, a glutamic acid-rich domain, and 2 proline-rich domains (Fig. 1) [24,25]. A unique feature of PELP1 is the presence of an unusual stretch of 70 acidic amino acids in the C-terminus that functions as a histone-binding region [26,27]. PELP1 is localized both in the nuclear and cytoplasmic compartments. In the nuclear compartment PELP1 interacts with histones and histone modifying enzymes, suggesting that PELP1 has some function in these complexes [28,29] and thus plays a role in chromatin remodeling for ligand-bound ERs [27]. Emerging evidence also indicates that PELP1 plays a key role in extra nuclear actions of nuclear receptors and thus represents a unique ER-coregulator that participates in both genomic and non-genomic actions of ER. PELP1 modulates the interaction of estrogen receptor with Src, stimulates Src enzymatic activity and thus enhances MAPK pathway activation [25]. PELP1 is also shown to directly interact with the p85 subunit of PI3K and enhances PI3K activity, leading to activation of the PKB/AKT pathway [30]. Mechanistic studies showed that PELP1 interacts with the SH3 domain of c-

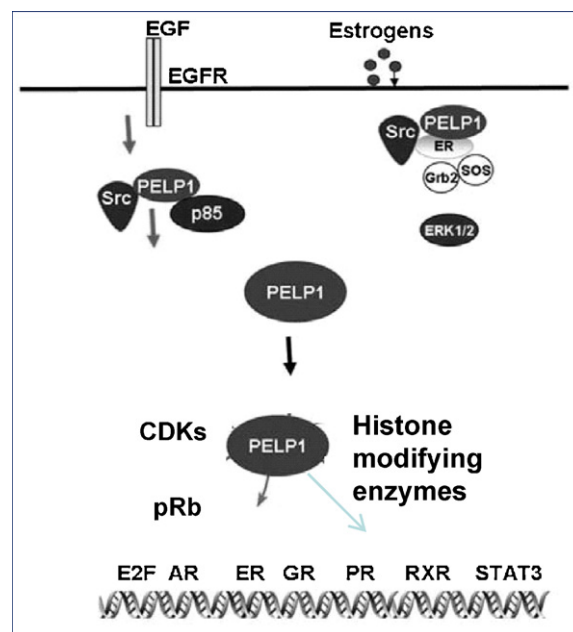


**Fig. 1.** Schematic representation of the current understanding of PELP1 signaling pathway. PELP1 participates in NR non-genomic signaling by coupling NRs with cytosolic kinases (Src and PI3K) and growth factor signaling components. PELP1 contains 10 LXXLL (NR interacting motifs) and interacts with several nuclear receptors (ER, ERR, GR, PR, AR, RXR). PELP1 interacts with histones and histone modifying enzymes (including CBP, P300, HDAC2, LSD1) and promotes chromatin remodeling. PELP1 interacts with pRb, is phosphorylated by CDKs and plays a role in NR mediated cell cycle progression.

Src via its N-terminal PXXP motif, and ER interacts with the SH2 domain of Src at phosphotyrosine 537; the PELP1-ER interaction further stabilizes this trimeric complex, leading to activation of Src kinase. Activated Src kinase then phosphorylates PELP1, which in turn acts as a docking site for PI3K leading to activation of PKB/AKT pathway [31]. PELP1 interacts with and modulates functions of several nuclear receptors and transcriptional activators including AR, ERR, GR, PR, RXR, FHL2 and STAT3 [28]. PELP1 promotes E2-mediated cell proliferation by sensitizing cells to G1 > S progression via its interactions with the pRb pathway [32]. PELP1 is shown to be phosphorylated by several kinases including PKA, HER2, Src, CDKs and its phosphorylation is modulated by estrogen and growth factors [28]. Collectively, these findings suggest that PELP1 serves as a scaffolding protein that couples various signaling complexes with estrogen receptor and participates in genomic and non-genomic functions (Fig. 1).

## 4. PELP1 expression in hormonal cancers

Emerging studies suggest that PELP1 is a proto-oncogene and its expression is deregulated in hormone-dependent cancers including cancers of breast [24,33], endometrium [34] and ovary [35]. Although PELP1 is predominantly localized in the nuclei of hormonally responsive tissue cells, in a subset of tumors it localizes in the cytoplasm alone [30]. Altered localization of PELP1 appears to contribute to tamoxifen resistance via excessive activation of Src/AKT pathways leading to follow-up modifications of ER [30]. Such modifications of the ER pathway may lead to the activation of ER target genes in a ligand-independent manner. Thus, deregulation of PELP1 expression has the potential to contribute to hormonal therapy resistance seen in patients with hormone-dependent neoplasm by excessively activating extra nuclear signaling pathways.

## 5. PELP1 deregulation promotes local induction of aromatase

Recent studies from our laboratory showed that PELP1 functions as a potential proto-oncogene [36]. In this study, we found that breast cancer cells stably overexpressing PELP1 showed a rapid tumor growth in xenograft studies compared to control vector transfectants and tumor growth in PELP1 clones occurred in the absence of external estrogen supplementation. These findings raised a hypothesis that PELP1 deregulation modulates local aromatase to produce local estrogen thus promoting tumor growth without exogenous E2 supplementation. Immunohistochemistry (IHC) analysis of the PELP1-induced xenograft tumors using aromatase specific antibody showed that PELP1 driven tumors have increased aromatase expression compared to control E2 induced MCF-7 tumors (Fig. 2A). Results from studies using exon specific primers showed that MCF7 clones that overexpress PELP1 showed increased levels of exon I.3/II transcripts compared to MCF7 parental clones. In reporter gene assays utilizing Aro 1.3/II-luc, MCF7-PELP1 cells showed a 5-fold increase in the reporter gene activity (Fig. 2B). Western blot analysis showed that MCF7-PELP1 clones have increased levels of aromatase compared to the aromatase levels in the control MCF7 cells (Fig. 2B). PELP1 expressing MCF7 cells also showed increased aromatase activity suggesting the functionality of induced aromatase (Fig. 2C). Collectively, these results suggest that PELP1 deregulation has potential to regulate the aromatase gene expression via the I.3/II promoter and such deregulation could contribute to local E2 synthesis.

## 6. Role of PELP1 in growth factor regulation of aromatase

Deregulation of HER2 oncogene expression/signaling has emerged as the most significant factor in the development of hormone resistance. ER expression occurs in ~50% HER2-positive breast cancers and cross-talk between the ER and HER2 pathways promotes endocrine therapy resistance [37]. ER-coregulators are targeted by excessive ER-HER2 cross-talk leading to hormone resistance in a subset of breast tumors [38]. Recent studies also demonstrated that HER2 status plays an important role in tumor-induced aromatase activity via the COX-2 pathway [22]. Earlier studies showed that PELP1 interacts with HER2 and EGFR signaling components, and HER signaling promotes tyrosine phosphorylation of PELP1 [34,39]. In our studies, we found that growth factor signaling enhances PELP1 regulation of the *aromatase* promoter and resulted in increased aromatase activity [40]. We also found that PELP1 overexpression, or growth factor signaling enhances PELP1 recruitment to the silencer regions of the promoter I.3/II, suggesting that PELP1 could be one of those factors that promote *aromatase* expression via activation of the I.3/II promoters under conditions of growth factor deregulation (Fig. 3).

## 7. Expression of PELP1 and aromatase in breast tumors

Since PELP1 deregulation promotes aromatase expression in breast epithelial cells, we investigated whether aromatase expression is deregulated in breast tumors and whether its expression correlates with PELP1 expression using a breast cancer tissue microarrays (TMAs) obtained from the Cooperative Breast Cancer Tissue Resource (CBCTR) of the National Cancer Institute (NCI). IHC analysis of the breast tumor arrays showed increased expression of aromatase in DCIS and node-positive tumors compared to no or weak expression in normal breast tissue. PELP1 expression positively correlated with cancer grade and node status. The number of samples with a high level (score 3) of PELP1 staining increased as tumors progressed from grade 1 to grade 2 or 3. Interestingly,

tumors that showed increased expression of PELP1 also showed increased aromatase expression compared to PELP1 low expressing tumors (Fig. 2D). Collectively, these results suggested that deregulation of aromatase expression occurs in advanced-stage and node-positive tumors, and that cooverexpression of PELP1 and aromatase may occur in a sub set of tumors [40].

## 8. PELP1 regulation of aromatase expression in vivo

To test whether PELP1 deregulation in vivo has potential to regulate aromatase expression, our laboratory recently developed a transgenic mice (Tg) model. As a means of targeting the expression of the PELP1 transgene to the mammary gland, we placed the PELP1-cDNA under the control of the MMTV promoter. In this MMTV-PELP1 Tg model, mammary tumors were observed as early as 24 weeks and at this stage >40% of mice ( $n = 16$ ) developed mammary tumors by 8 months. No spontaneous mammary tumors were found in the wild type cohort. Pathological analysis revealed that these tumor masses represent full blown mammary adenocarcinomas. PELP1 driven tumors are ER+ve, and express aromatase, while wild type age matched control did not show any aromatase expression (Fig. 2E). These results thus provide evidence for in vivo potential of PELP1 deregulation in enhancing local E2 synthesis.

## 9. PELP1 and aromatase expression in endometriosis

Several lines of evidence demonstrate local estradiol (E2) production in endometriosis lesions [41,42]. Aberrant expression of steroidogenic acute regulatory protein (StAR) and aromatase in endometriotic tissue is shown to result in up-regulation of estrogen production [43]. Evolving evidence indicate that in cancers of breast, endometrium and ovary, *aromatase* expression is primarily regulated by increased activity of the proximally located promoter Aro1.3/II region [44]. To examine whether PELP1 has potential to regulate *aromatase* expression in endometrial cells, we performed reporter gene activation assays. Cotransfection of GFP-PELP1 but not GFP vector in human endometrial stromal cells (HESC) showed increased *aromatase* reporter activity and expression (Fig. 2F). Since PELP1 expression is deregulated in some ER driven pathological situations, we examined the expression status of PELP1 and aromatase in a small number ( $n = 5$ ) of eutopic and ectopic endometrium. Results showed increased staining intensity of PELP1 in ectopic endometrium compared to eutopic endometrium (Fig. 2G). In addition, ectopic endometrium also showed increase in aromatase staining. Collectively, these results suggest a possibility that PELP1 has potential to modulate aromatase expression in endometrial cells and its expression may be deregulated in endometriosis.

## 10. PELP1 regulation of aromatase in ovarian cancer cells

Our recently completed study using ovarian cancer tissue arrays suggested that PELP1 deregulation occurs in different types of ovarian cancer [35]. Results suggested that deregulation of PELP1 occurs in all subtypes of ovarian cancer (including serous, endometrioid, clear cell carcinoma, and mucinous tumors) and 60% of the tumors have 2–3-fold increase in PELP1 staining intensity (Fig. 2H). Since emerging evidence implicates that local estrogen synthesis also play a role in ovarian tumorigenesis, we have examined whether PELP1 regulates aromatase activation in ovarian cancer cells using Aro1.3/II promoter that is shown to be active in ovarian cells. In reporter gene assays, PELP1 enhanced the activation of Aro 1.3/II promoter activity in BG1 cells in a dose dependent manner (Fig. 2I). Western analysis of PELP1 overexpressing BG1 clones showed that PELP1 overexpression increases aromatase expression in ovarian cancer cells (Fig. 2J). These results suggest that PELP1 deregulation

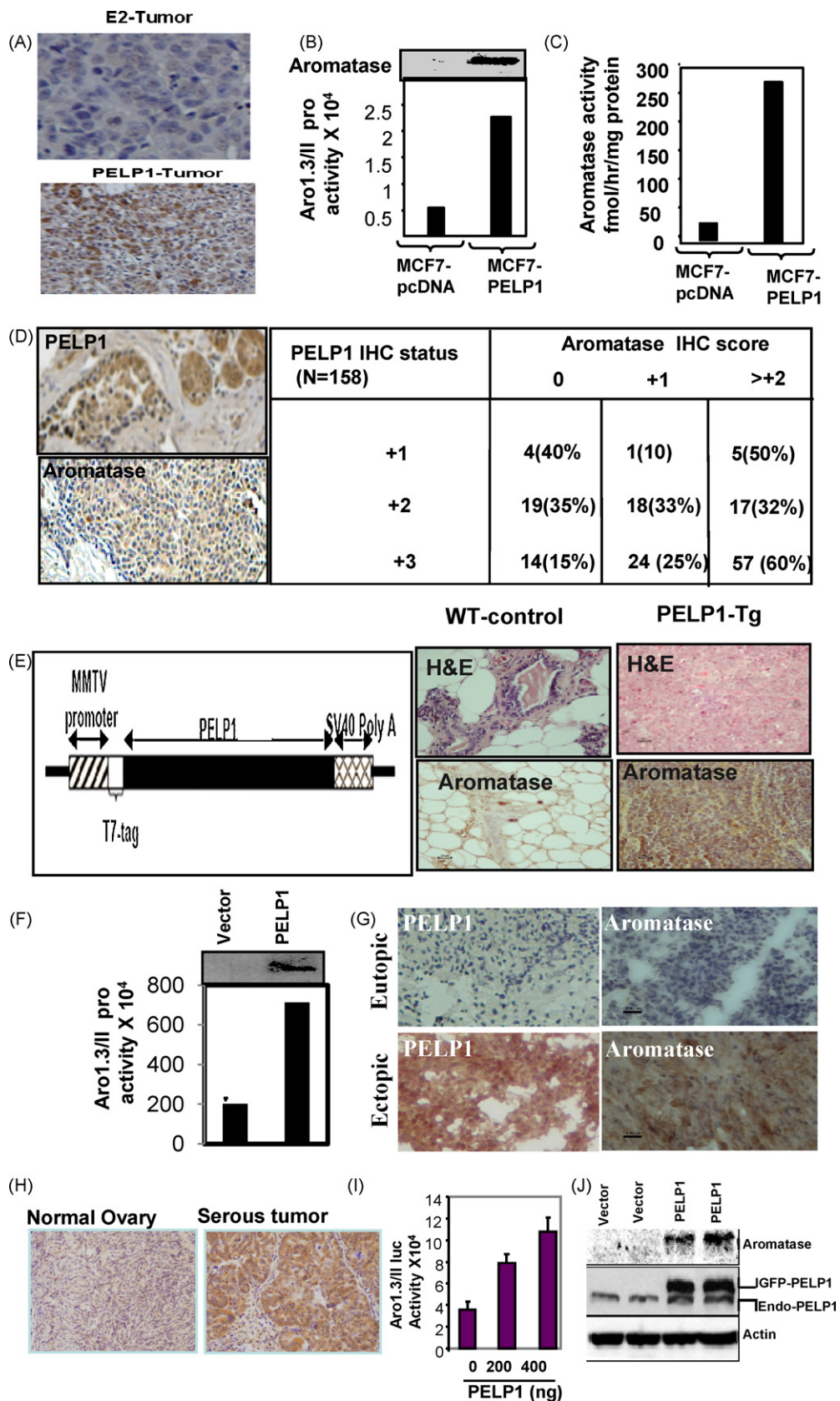


**Fig. 2.** PELP1 deregulation promotes local E2 synthesis. (A) IHC staining of aromatase in E2 induced and PELP1-induced xenograft tumors. (B) MCF7 cells or MCF7-PELP1 were transiently transfected with Aro1.3/II promoter and after 48 h, reporter activity was measured. Total cell lysates from MCF7 cells stably expressing vector or PELP1 were analyzed for aromatase expression by Western. (C) Aromatase activity in control MCF7 cells and MCF7-PELP1 clones was measured by tritiated-water release assay. (D) Co-expression of PELP1 and aromatase in breast tumors. PELP1 and aromatase expression was determined by IHC using breast cancer TMA arrays. Sections were scored according to IHC intensity in a range from 0 to 3, in which 0 indicated no expression; 1, low expression; and 2, moderate expression; 3 high expression. Summary of the staining is shown as a table. A representative sample of one tumor with high expression of PELP1 and aromatase is shown. (E) Schematic representation of construct used to

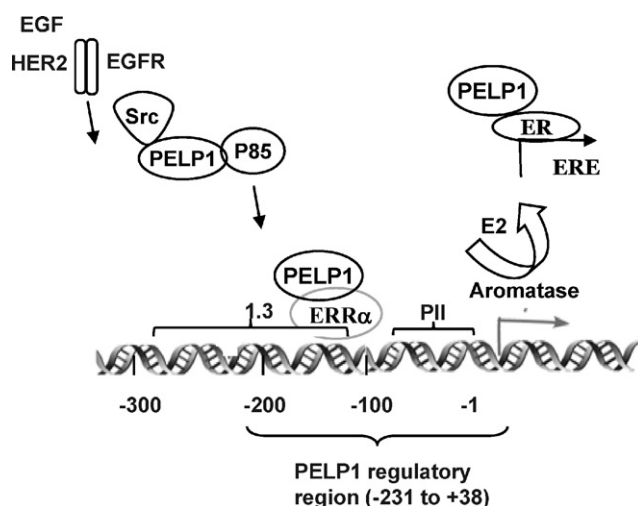


**Fig. 3.** Schematic representation of PELP1 regulation of *aromatase* promoter. Growth factor signals or PELP1 deregulation via overexpression promotes PELP1 recruitment to aromatase promoter (–213 to +38 region). At the aromatase promoter, PELP1 interactions with orphan receptor ERRα in conjunction with histone modifying enzymes promotes aromatase expression leading to E2-ER autocrine signaling loop.

also has potential to promote local E2 synthesis in ovarian cancer cells.

### 11. Role of PELP1-mediated non-genomic actions in aromatase induction

PELP1 is a unique regulator of nuclear receptor that participates in genomic as well as in non-genomic actions [28]. To examine whether PELP1-mediated non-genomic signaling pathways are involved in PELP1-mediated induction of *aromatase* expression, we pretreated MCF7-PELP1 cells with various signaling inhibitors that block specific pathways: PD98059, mitogen-activated protein/extracellular signal-regulated kinase inhibitor; PP2, the Src family tyrosine kinase inhibitor; LY-294002, the PI3K inhibitor; SB203580, and the p38MAPK inhibitor. Results from these assays showed that PELP1-induced aromatase promoter activity can be abolished by pretreatment with c-Src or PI3K pathway inhibitors while pretreatment of MAPK pathway inhibitors had no effect on PELP1-induced aromatase expression. These results suggest that functional c-Src and PI3K pathways are required for PELP1-mediated induction of *aromatase* (Fig. 3). Similarly, HER2 regulation of PELP1-mediated activation of *aromatase* was also abolished by pretreatment of MCF7-HER2 cells with the Src inhibitor PP2. Collectively, the findings from this published study suggest that c-Src signaling plays a vital role in PELP1-mediated induction of *aromatase* [40].

### 12. Role of PELP1 genomic functions in aromatase induction

PELP1 is predominantly nuclear in localization and earlier studies showed that PELP1 is recruited to several nuclear receptor target genes and play a role in chromatin modifications. Using various deletion constructs of aromatase promoter reporter gene and

by ChIP analysis of *aromatase* promoter, we found that 269 base region located in the –231/+38 Aro P1.3/II promoter is required for PELP1 regulation of aromatase (Fig. 3). In addition, ChIP analysis showed that PELP1 is specifically recruited to the –231/+38 region. Further analysis revealed that HER2 signaling also required Aro 1.3/II –231/+38 region for PELP1-mediated activation of *aromatase*. Earlier studies showed that this region possess binding regions for ERRα, BRCA1 and a transcriptional silencer element (S1) [45]. Immunoprecipitation analysis revealed that PELP1 interacts with ERRα but not with BRCA1. Using reporter gene assays, ERRα specific siRNA and ERRα antagonist, we found that PELP1 promotes activation of Aro1.3/II promoter via interactions with the ERRα [40]. Earlier studies found that ERRα up-regulates aromatase expression via the I.3/II promoters [46]. Since PELP1 does not have a DNA binding domain, it is possible that ERRα serves as a docking site for PELP1 recruitment and PELP1 ability to interact with histones and histone modifying enzymes, may play a role in chromatin remodeling at *aromatase* promoter (Fig. 3).

### 13. Significance of PELP1 in epigenetic modifications of aromatase promoter

Emerging evidence suggest that histone methylation, an epigenetic phenomena, could play a vital role in many neoplastic processes by silencing or activation of genes [47]. However, unlike genetic alterations, epigenetic changes are reversible. Recent studies showed that demethylase LSD1 can demethylate H3-K4 and H3-K9, recruits to a significant fraction of ER target genes and is shown to be required to demethylate proximal histones to enable ER-mediated transcription [48]. Evolving studies in our laboratory suggest that PELP1 interacts with LSD1 and also recognizes methyl modified histones [49] [28]. Because PELP1 is recruited to aromatase promoter and interacts with histone demethylase, it is possible that PELP1 modulate H3 methyl modifications at the aromatase promoter. ChIP analysis revealed that MCF7 cells that do not express aromatase showed increased H3K9 methylation (a marker of repression), while MCF7-PELP1 model cells (that overexpress PELP1) that exhibit local E2 synthesis showed decreased histone H3K9 with a concomitant increase in H3K4 methylation (a marker of activation) at the aromatase promoter. Interestingly, other model cells that exhibit increased local E2 synthesis (MCF7-HER2, SKBR3) also showed increased H3K4 methylation at aromatase promoter (Fig. 4A). These results suggest that epigenetic modification may play a role in the local aromatase expression and PELP1 deregulation could play a role in modulating histone methylation at the aromatase promoter region.

### 14. Targeting local estrogen synthesis by blocking PELP1-LSD1 axis

Pargyline is a selective monoamine oxidase inhibitor that blocks LSD1 activity [50] and is approved by FDA for treatment of moderate to severe hypertension. Pargyline is commercially available from many sources and the safety and efficacy of this drug is well established. Since PELP1 expression is deregulated in hormonal dependent tumors, and because PELP1 interacts with LSD1 and promotes local E2 synthesis, inhibition of PELP1-LSD1 axis by inhibitor Pargyline will probably affect growth advantage seen in the PELP1 overexpressing cells by reducing local E2 synthesis. To test this,

generate Tg mouse. B, PELP1 Tg mice (8 months old) with mammary tumor and age matched WT mice control mammary glands were analyzed for morphological changes using H&E and aromatase expression by IHC. (F) PELP1 modulates aromatase expression endometrial model cells. Immortalized human endometrial stromal cells (HESC) were transiently transfected with GFP vector or GFP-PELP1 expression vectors along with aromatase 1.3/II luciferase reporter. Reporter activity and expression of aromatase was measured after 72 h. (G) Expression status of PELP1 and aromatase in eutopic and ectopic endometrium determined by IHC. H, A representative sample of PELP1 staining in normal and serous ovarian tumor is shown. (I) BG 1 ovarian cancer cells were transiently transfected with Aro1.3/II promoter along with increasing amount of PELP1. After 72 h, reporter activity was measured. J, Total cell lysates from BG1 cells stably expressing vector or PELP1 were analyzed for aromatase expression by Western.

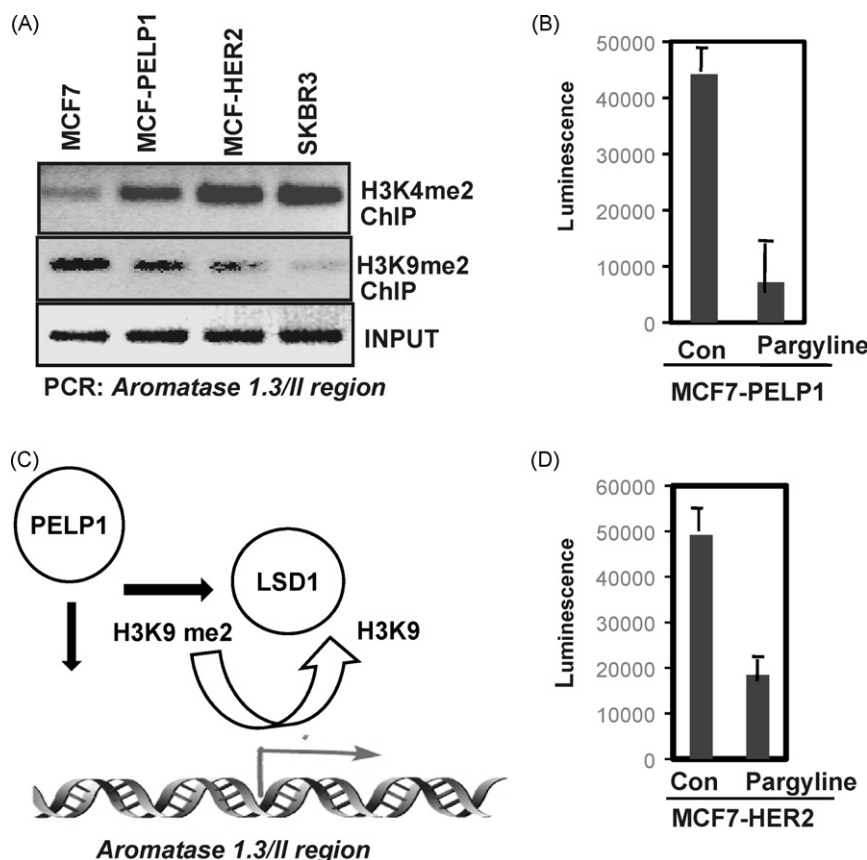


**Fig. 4.** PELP1 promotes epigenetic modifications at the aromatase promoter. (A) Chromatin immune precipitation (ChIP) was performed using H3K9me2, K3K4me2 specific antibodies in indicated cells and the status of H3 methylation was analyzed by PCR using aromatase 1.3/II promoter specific primers. (B) MCF7-PELP1, MCF7-HER2 cells were cultured in a 96 well plate and treated with 3 mM of Pargyline for 72 h. Cell viability was measured by ATP assay (Promega, Cell Titer Glo ATP assay).

MCF7-PELP1 cells that over express PELP1, MCF7-HER2 cells that overexpress oncogene HER2, were treated with or without Pargyline (3 mM) for 72 h and the cell viability was determined by Cell titer-glo ATP assay (Promega). Pargyline substantially inhibited viability in both model cells (Fig. 4B, C). These results suggest that PELP1-mediated epigenetic modifications may play a role in local E2 synthesis and blocking PELP1-LSD1 axis will have therapeutic utility (Fig. 4D).

## 15. Concluding Comments

Understanding the molecular mechanism by which tumors enhance aromatase expression is clinically important. Accumulating evidence suggest that a variety of different factors may regulate expression and activity of aromatase under pathological conditions and that aromatase promoter I.3 and II as the main promoters that regulate aromatase expression in breast tumors. Earlier studies using elegant methodology identified several nuclear factors (BRCA, ERR $\alpha$ ), signals (Cytokines, PGE2), oncogenes (HER2) and epigenetic modifications at the aromatase promoters to play a role in induction of aromatase. Although, it is not completely understood, the molecules that connect physiological/oncogenic signals to the nuclear receptors may play a role in the activation of normally suppressed aromatase promoter in the tumor cells. Recent evidence suggests that nuclear receptor coregulators have potential to function as major regulators of hormone receptor physiology because of their ability to sense physiological signals and due to their ability to convey those signals to the nuclear receptors at the target gene promoters. PELP1 is novel nuclear receptor coregulator whose expression is deregulated in hormonally driven cancers. Our results suggest that PELP1 overexpression or deregulated growth factor signaling enhances PELP1 recruitment to the silencer regions of the promoter I.3/II, suggesting PELP1 could be one of those factors that promote aromatase expression in breast tumor cells leading local E2 synthesis in breast epithelial cells (Fig. 5). PELP1 ability to interact with growth factors, nuclear receptors and epigenetic modifiers, suggest that deregulation of PELP1 could enhance tumor growth by promoting autocrine ER signaling loop. Future studies using larger number of tumor samples are warranted to examine whether PELP1

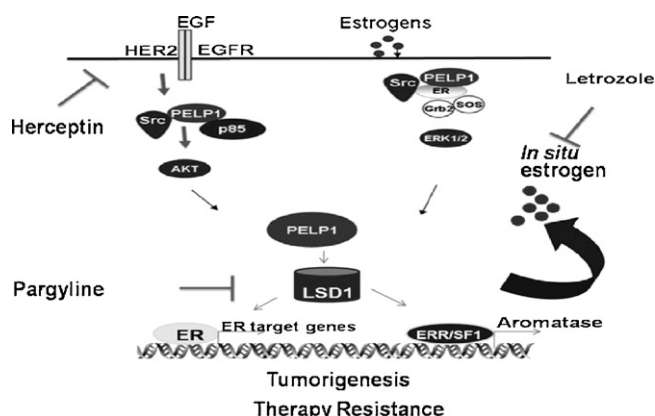


**Fig. 5.** Schematic representation of potential PELP1 autocrine signaling loop in cancer cells. Growth factor signaling and/or activation of non-genomic signaling pathways promotes ER co-activator PELP1 to form a complex with ERR $\alpha$  and PNRC2, leading to activation of aromatase expression promoting formation of a positive feedback loop locally synthesizing E2, results in the activation of E2-ER-PELP1 signaling in breast cancer cells.

could serve as prognostic marker/diagnostic marker for predicting local E2 synthesis. Discovering novel pathways that contribute to local E2 synthesis in breast tumors will enable to develop new therapeutic agents that block these pathways with fewer side effects.

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# Extranuclear Functions of ER Impact Invasive Migration and Metastasis by Breast Cancer Cells

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## Abstract

The molecular basis of breast cancer progression to metastasis and the role of estrogen receptor (ER) signaling in this process remain poorly understood. Emerging evidence suggests that ER participates in extranuclear signaling in addition to genomic functions. Recent studies identified proline-, glutamic acid-, and leucine-rich protein-1 (PELP1) as one of the components of ER signalosome in the cytoplasm. PELP1 expression is deregulated in metastatic breast tumors. We examined the mechanism and significance of ER-PELP1-mediated extranuclear signals in the cytoskeletal remodeling and metastasis. Using estrogen dendrimer conjugate (EDC) that uniquely activate ER extranuclear signaling and by using model cells that stably express PELP1 short hairpin RNA (shRNA), we show that PELP1 is required for optimal activation of ER extranuclear actions. Using a yeast two-hybrid screen, we identified integrin-linked kinase 1 (ILK1) as a novel PELP1-binding protein. Activation of extranuclear signaling by EDC uniquely enhanced E2-mediated ruffles and filopodia-like structures. Using dominant-negative and dominant-active reagents, we found that estrogen-mediated extranuclear signaling promotes cytoskeleton reorganization through the ER-Src-PELP1-phosphoinositide 3-kinase-ILK1 pathway. Using *in vitro* Boyden chamber assays and *in vivo* xenograft assays, we found that ER extranuclear actions contribute to cell migration. Collectively, our results suggest that ER extranuclear actions play a role in cell motility/metastasis, establishing for the first time that endogenous PELP1 serves as a critical component of ER extranuclear actions leading to cell motility/invasion and that the ER-Src-PELP1-ILK1 pathway represents a novel therapeutic target for preventing the emergence of ER-positive metastasis.

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## Introduction

The estrogen receptor (ER) is implicated in breast cancer progression. The majority of human breast cancers start out as ER positive (1) and a large portion of metastases retain their ER (2). Although initial endocrine therapy has a positive effect on the treatment of advanced metastatic disease (3), acquired resistance to endocrine therapies frequently occurs, with tumors recurring as metastases, which is the leading cause of death from breast cancer. Tumor metastasis consists of a series of discrete biological processes that move tumor cells from the primary neoplasm to a distant location

and involves a multistep cascade (4). The process of migration is orchestrated through the activation of biochemical pathways that involves multiple cytoskeleton proteins (5). Although substantial information is available on the process of metastasis, the role of E2-ER signaling in breast metastasis remains controversial.

ER extranuclear signaling has been linked to rapid responses to E2 through stimulation of the Src kinase, mitogen-activated protein kinase (MAPK), and phosphatidylinositol-3-kinase (PI3K) pathways in the cytosol. Emerging evidence suggests that ER participates in extranuclear signaling through the formation of a multiprotein complex collectively called a “signalosome” (6). The use of novel ligands that uniquely activate extranuclear signals showed that extranuclear pathways have distinct biological outcomes (7). The molecular mechanism(s) of ER extranuclear signaling and the pathobiology of ER extranuclear actions remain unknown.

Proline-, glutamic acid-, leucine-rich protein-1 (PELP1; ref. 8) is also known as the modulator of the nongenomic actions of the ER (9). PELP1 plays important roles both in the genomic (10) and the nongenomic actions of the ER (11, 12). Recent evidence also suggests that PELP1 couples ER to several signaling pathways such as Src-MAPK, PI3K-AKT, and epidermal growth factor receptor (EGFR)-signal transducers and activators of transcription 3 (12, 13), and that PELP1

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expression is deregulated in metastatic breast tumors (14). Although these studies suggested that PELP1 has tumorigenic potential, whether PELP1-mediated extranuclear signaling plays a role in cell invasion and/or metastasis has not yet been defined.

In this study, we examined whether PELP1-mediated ER extranuclear signaling play a role in cytoskeletal remodeling, invasion, and metastasis. Our results suggest that ER extranuclear signaling has the potential to contribute to the tumor cell motility and that targeting the ER-PELP1 axis represents a novel therapeutic target to combat breast cancer progression to metastasis in ER-positive breast tumors.

## Materials and Methods

**Cell cultures and reagents.** MCF7 cells were purchased from the American Type Culture Collection. ZR75 cells were maintained as previously described (15). Antibodies against vinculin, actin, and the steroid hormone 17 $\beta$  estradiol were purchased from Sigma. Green fluorescent protein (GFP)-epitope antibody was purchased from Clontech and anti-T7-epitope antibody was purchased from EMD BioSciences. PELP1 antibody was purchased from Bethyl Laboratories. Antibodies against phospho-AKT, phospho-MAPK, phospho-GSK3, phospho-Src, and PI3K inhibitor LY294002 were purchased from Cell Signaling. Dasatinib was obtained from Bristol-Myers Squibb Pharmaceutical Research Institute. MCF7 cells stably expressing PELP1 short hairpin RNA (shRNA) were generated using FuGENE-6 transfection (Roche) and by G418 selection (500  $\mu$ g/mL). PELP-specific shRNA and control shRNA vectors were purchased from SuperArray. MCF7-PELP1cyto cells were earlier described (12).

**Plasmids, generation of mutants, and transfection.** PELP1-Src mutant (PELP1SrcMT) that contains a mutation in the SH2 binding site and the Src phosphorylation site Y920F was generated by site-directed mutagenesis using the Quick Change Lightning Mutagenesis kit (Stratagene). Plasmids for the myr-p110 subunit of PI3K (active PI3K) and SrcY527F (active Src kinase) were described earlier (16, 17). RFP-myr-p110 (Active PI3K) was constructed by PCR-based cloning of open reading frame of myr-p110 into pDsRed-monomer-N1 vector. Expression vector for integrin linked kinase 1 (ILK1) dominant active (DA; ILKS423D) and dominant negative (DN; ILKE359K) were provided by Dr. Dedhar (Department of Cancer Genetics, Vancouver, British Columbia, Canada; ref. 18). The plasmid for Active CDC42 [pcDNA3-EGFP-cdc42 (Q61L)] was received from Addgene (19).

**Yeast two-hybrid library screening and bait construction.** PELP1 bait was constructed by amplifying DNA corresponding to amino acids 600 to 866 through PCR and subcloning into Gal4-binding domain vectors (pGBD vector; Clontech). pGBD-PELP1 (amino acids 600–866) was used as a bait to screen a mammary gland cDNA library fused to a Gal4 activation domain (Clontech) as described (20).

**Preparation of estrogen dendrimers.** The estrogen dendrimer conjugates (EDC) were prepared following a published procedure by Katzenellenbogen and colleagues (11).

A poly amido amine dendrimer (G-6) was used for the preparation of EDCs. The EDCs were characterized by nuclear magnetic resonance analysis and the data were found to be consistent with that of the published report (7). A small aliquot was FITC labeled by using the Sure Link Fluorescein (FITC) labeling kit (KPL).

**Cell migration and metastasis assays.** Wound healing and Boyden chamber assays were performed as described (21). For determining *in vivo* metastatic potential, xenograft studies were performed as described (22). Briefly,  $1 \times 10^5$  model cells in 100  $\mu$ L PBS were injected into the tail vein or left cardiac ventricle of 5- to 6-week-old ovariectomized nude mice ( $n = 5$ ) that were each implanted with one E2 pellet (60-d release, 0.72 mg, Innovative Research of America). After 8 weeks, the mice were euthanized and metastatic nodules on the surface of lung and liver were identified by color and counted under a fluorescent microscope.

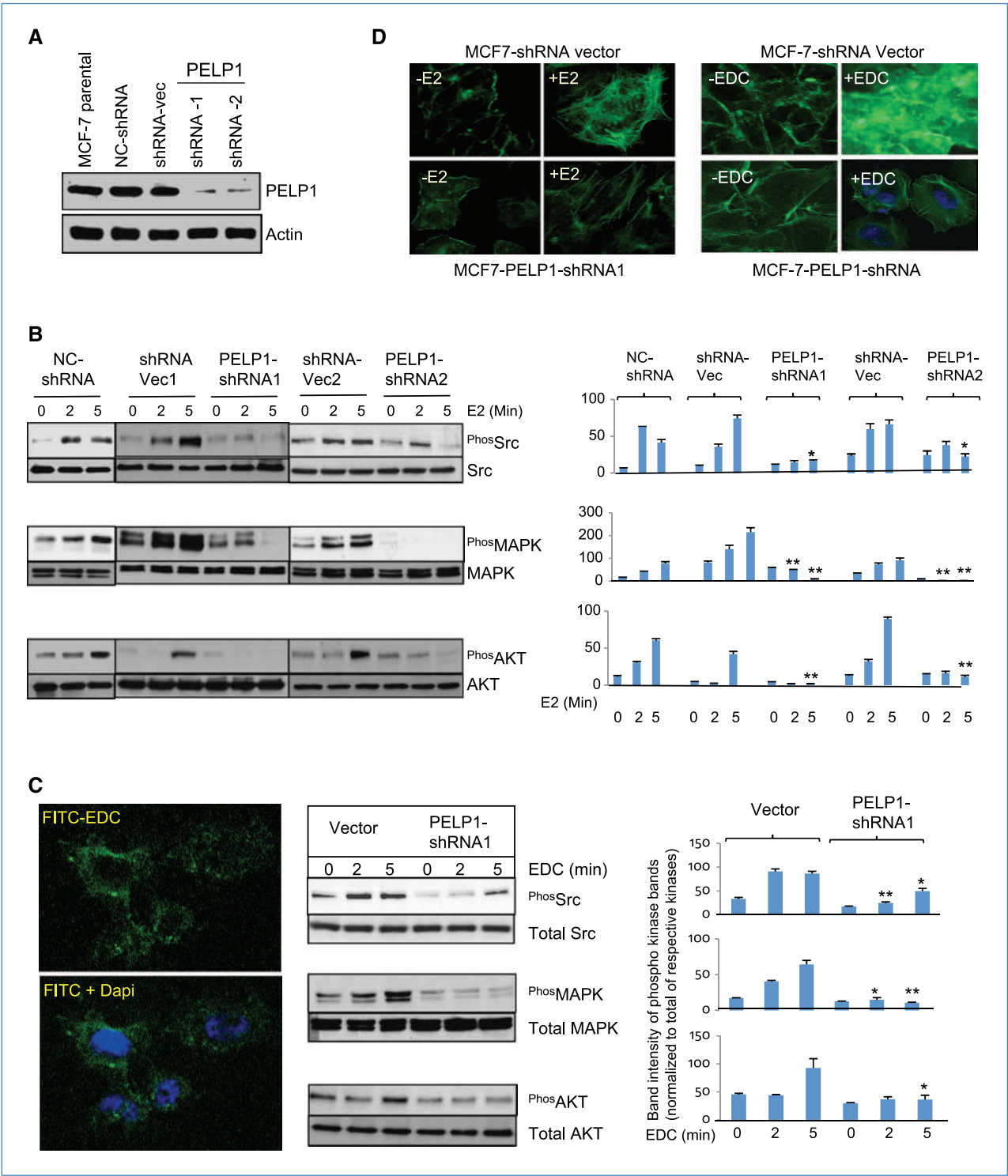
**Western blotting and immunoprecipitation.** Cell lysis, immunoprecipitation, and Western blot analysis with phospho antibodies were performed as described (23).

**ILK kinase assays.** Exogenously expressed GFP-ILK1 or endogenous ILK1 was immunoprecipitated and was used as a source of ILK enzyme. *In vitro* kinase assays using MBP protein were performed in HEPES buffer (50 mmol/L HEPES, 10 mmol/L MgCl<sub>2</sub>, 10 mmol/L MnCl<sub>2</sub>, 1 mmol/L NaF, and 0.2 mmol/L Na<sub>3</sub>VO<sub>4</sub>) containing immunoprecipitated ILK1 enzyme, 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P] ATP, and 25  $\mu$ mol/L ATP in 30  $\mu$ L reaction.

**Immunofluorescence studies.** The immunofluorescence studies were performed as previously described (13). Secondary antibodies conjugated with Alexa 488 (green) or Alexa 546 (red), or Alexa 633 (Blue) dye was used to recognize different primary antibodies (Molecular Probes). The filamentous actin (F-actin) status was analyzed by phalloidin staining.

## Results

**PELP1 knockdown affects E2-ER-mediated extranuclear signaling and cytoskeletal reorganization.** To study the *in vivo* significance of PELP1 in the extranuclear actions of ER, we established MCF7 breast cancer model cells that stably expressed PELP1shRNA that specifically downregulate endogenous PELP1. MCF7 cells were transfected with shRNA vector and negative control shRNA vector (that express shRNA targeting scrambled artificial sequence with no sequence identity to the human genome) were used as a control. Western blot analysis of total lysates revealed that the PELP1-shRNA clones downregulated PELP1 expression to ~80% of the level seen in the parental and the vector-transfected clones (Fig. 1A). To examine the significance of endogenous PELP1 in the activation of ER extranuclear signaling pathways, we measured the activation of signaling pathways including Src, AKT, and MAPK after treating cells with E2 for short time periods. Compared with shRNA vector-transfected cells, PELP1-shRNA-expressing cells had significantly less Src, AKT, and MAPK activation (Fig. 1B). To further establish the role of PELP1 in E2-mediated



nongenomic actions, we used EDC (nanoparticles coated with estrogen) that uniquely localize in the membrane/cytoplasm (Fig. 1C, left) and preferably activate ER extranuclear signaling (7). MCF-7 cells that express vector or PELP1-shRNA were treated with EDC for 2 and 5 minutes, and signaling was analyzed by phospho-specific antibodies. EDC addition uniquely promoted the activation of Src and MAPK pathways. However, knockdown of PELP1 by shRNA significantly affected the EDC-mediated increase in Src and MAPK activation (Fig. 1C, middle and right). These results suggest that E2-mediated extranuclear actions play a key role in the activation of Src and MAPK and that the functional PELP1 signaling axis is needed for E2-mediated extranuclear signaling. Because Src and PI3K play important role in cytoskeletal functions, cell attachment, and migration, we asked whether E2-ER extranuclear actions contribute to cytoskeletal reorganization leading to cell migration. MCF7 cells that expressed vector or PELP1-shRNA were treated with either E2 or EDC for 10 minutes and cytoskeletal changes were analyzed by confocal microscopy. E2 or EDC addition uniquely promoted actin reorganization with filopodia and ruffle formations. However, knockdown of PELP1 by shRNA substantially affected actin reorganization by E2 or EDC with little ruffles/filopodia formations and predominantly showed cortical actin and stress fibers (Fig. 1D). These studies show that ER extranuclear actions have the potential to promote cytoskeletal changes leading to ruffle and filopodia formation.

**Src kinase plays a critical role in PELP1-mediated E2 extranuclear signaling leading to cytoskeletal reorganization.** PELP1 acts as a scaffolding protein coupling ER with Src kinase, leading to the activation of ER-Src-MAPK and ER-Src-AKT pathways (11). Earlier studies also revealed that PELP1 interacts with c-Src SH3 domain through its NH<sub>2</sub>-terminal PXXP motif and Src phosphorylates PELP1 at its COOH terminal (tyrosine 920) domain (9). To establish the significance of Src kinase in PELP1-mediated E2-ER extranuclear signaling, we generated a PELP1 mutant construct (PELP1SrcMT) that contains a mutation in the Src-SH3 binding site on PELP1 (ProXXPro is mutated to AlaXXAla) and a mutation in Src phosphorylation site (Tyr 920 is mutated to Phe; Fig. 2A). The PELP1SrcMT is unable to interact with Src kinase and thus functions as a DN mutant of PELP1. As expected, PELP1 wild-type (WT) but not the PELP1SrcMT interacted with Src kinase (Fig. 2B). Transient expression of PELP1SrcMT substantially effected the E2-mediated cytoskeletal reorganization in a DN fashion (Fig. 2B, right) and also interfered with the E2-mediated activation of Src and MAPK (Fig. 2C). Because Src kinase seems to play a key role in E2 extranuclear signaling, we examined the effect of inhibition of Src kinase using dasatinib, a well-established orally available inhibitor of Src family tyrosine kinases (24). For these studies, we used MCF7 control cells or MCF7-PELP1WT model cells that overexpress PELP1 and exhibit increased E2-ER extranuclear signaling. Pharmacologic inhibition of Src kinase using dasatinib abolished the E2-mediated activation of AKT and MAPK pathways both in MCF7 as well as in PELP1-overexpressing MCF7 cells (Fig. 2D). Collectively, these results

suggest that Src kinase play an important role in PELP1-mediated E2 extranuclear actions.

**Integrin-linked kinase 1 is a novel PELP1-interacting protein.** To identify the novel components of the PELP1 signalsome that contribute to ER extranuclear signaling leading to cytoskeletal reorganization, we performed a yeast two-hybrid screen using a mammary gland cDNA expression library. One of the positive clone sequences matched with that of ILK1. The specificity of ILK1 and PELP1 interaction was confirmed further using cotransformation followed by a survival assay in selection medium using yeast cells that stably expressed histidine, tryptophan, leucine nutrient reporter genes under the control of GAL response elements. The GBD-PELP1- and GAD-ILK1-transformed colonies grew in the medium lacking adenosine, histidine, tryptophan, and leucine, whereas the cells cotransformed with the control GBD vector and GAD-ILK1 did not grow (Fig. 3A, left). Deletion experiments revealed that the possible interaction of ILK1 and PELP1 involved amino acids 601 to 886 (Fig. 3A, right). To further verify the interaction between ILK1 and PELP1, we transfected T7-tagged PELP1 and GFP-tagged ILK1 into MCF7 cells. Total lysates were subjected to immunoprecipitation with PELP1-tagged antibody followed by Western blotting with ILK1. Results showed that PELP1 can interact with ILK1 *in vivo*. (Fig. 3B, left). Similarly, the PELP1 and ILK1 interaction was also observed in PELP1cyto model cells that express PELP1 exclusively in the cytoplasm, suggesting the physiologic significance of such an interaction in the cytoplasm (Fig. 3B, right). Confocal analysis of EDC-treated MCF7 cells showed colocalization of PELP1 with ILK1 upon EDC treatment (Fig. 3C). Coimmunoprecipitation assay results showed that PELP1 interaction with ILK1 is dependent on ligand (Fig. 3D, left). Immunoprecipitation of PELP1 also showed the presence of ILK1 in the precipitates along with Src, ER, and the P85 subunit of PI3K, which are the known PELP1 signalsome components (Fig. 3D, right). These data suggest that ILK1 is a novel component of the PELP1 signalsome.

**ILK1 couples E2-mediated PELP1 signaling to cytoskeleton.** Because ILK1 is a novel component of PELP1 signalsome, we examined the significance of ILK1 in PELP1 signaling using DN and active ILK1 constructs (18), and monitored the formation of motility-related structures including the formation of stress fibers, lamellipodia (membrane ruffles), and filopodia (microspikes). Expression of DN ILK1 into MCF7 cells significantly reduced the formation of actin structures by E2 treatment (Fig. 4A, top). Accordingly, overexpression of DA ILK1 rescued the formation of F-actin structures including ruffles and filopodia in MCF7-PELP1shRNA cells (Fig. 4A, bottom). Overexpression of Src kinase failed to rescue the cytoskeleton defects in PELP1-shRNA clones (data not shown), suggesting that PELP1 is downstream of Src kinase. Because the Src kinase phosphorylation of PELP1 promotes downstream signaling by coupling with the PI3K axis, we introduced a membrane-targeted, RFP-tagged PI3K, a myristoylated subunit of p110 that functions as an active PI3K, into PELP1-shRNA cells. The expression of membrane-targeted PI3K rescued the actin structures

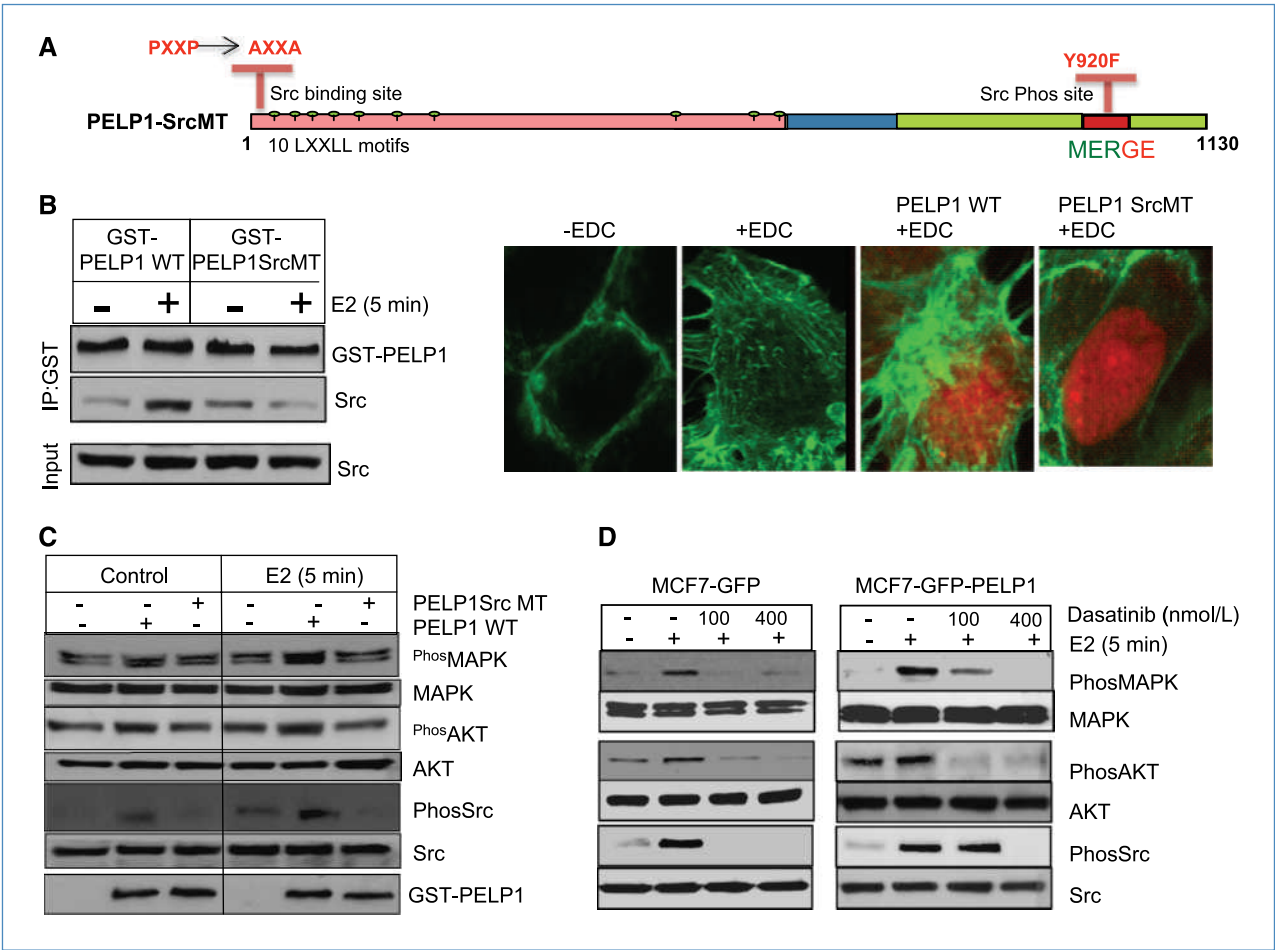


**Figure 2.** Src kinase is needed for the optimal activation of PELP1-mediated E2 extranuclear actions. A, schematic representation of PELP1 mutant that cannot bind or be phosphorylated by Src kinase. B, MCF7 cells were transfected with PELP1WT or PELP1SrcMT and treated with or without E2. The ability of the expressed proteins to interact with Src kinase was analyzed by immunoprecipitation (IP; left). MCF7 control and MCF7 cells were transiently transfected with either PELP1WT or PELP1SrcMT and were treated with EDC for 5 min. The status of F-actin was analyzed by confocal microscopy (right). C, MCF7 cells were transfected with PELP1WT or PELP1SrcMT using the Amaxa nucleofection method and treated with or without E2. Activation of extranuclear signaling was measured by Western blotting. D, MCF7 or MCF7 cells that stably expressed PELP1 were treated with or without dasatinib and with or without E2. Activation of extranuclear signaling was measured by Western blotting.

in PELP1-shRNA cells (Fig. 4B, top). However, cotransfection of membrane-targeted PI3K along with DN ILK1 inhibited the active PI3K-mediated rescue of actin structures (Fig. 4B, bottom), indicating that ILK1 functions downstream of PI3K in the PELP1 signaling axis. We also measured whether the expression of active CDC42, a known downstream target of ILK1, rescues the phenotype. Overexpression of active CDC42 indeed restored the actin structures in PELP1-shRNA cells (Fig. 4C, top) and DN ILK1 failed to interfere with active CDC42-mediated restoration (Fig. 4C, bottom). Such results suggest that E2>PELP1>PI3K>ILK1>CDc42 as a signaling pathway that contribute to E2-mediated cytoskeleton changes.

Because earlier studies showed that PELP1 interactions with Src enhance Src kinase activity, we examined whether PELP1 interactions with ILK1 also modulate its kinase activity. We performed *in vitro* kinase assay using immunopreci-

pitated ILK1 as the enzyme source and baculovirus-purified glutathione *S*-transferase (GST)-PELP1 as an activator. Incubation of ILK1 with GST-PELP1, but not GST alone, increased the ILK1 activity in *in vitro* assay (Fig. 4D, left). To examine the PELP1 regulation of ILK1 *in vivo*, we transfected ILK1 with or without PELP1 in 293T cells and activation of the ILK substrate GSK3 $\beta$  was detected. Compared with vector transfection, cotransfection of PELP1 increased GSK3 $\beta$  phosphorylation (Fig. 4D, left). Treatment of MCF7 model cells with EDC that stimulate E2 extranuclear signaling substantially increased ILK kinase activity; however, PELP1 knock-down substantially affect the EDC-mediated activation of ILK1 (Fig. 4D, middle). To examine, whether the PELP1-mediated activation of ILK1 is dependent on functional Src or PI3K pathways, we have used inhibitors of these pathways. Results showed that treatment of cells with PI3K inhibitor LY294002 abolishes the PELP1-mediated activation of ILK1,

whereas Src inhibitor dasatinib has no effect on the PELP1-mediated activation of ILK1 (Fig. 4D, right). Earlier studies have shown that ligand-induced phosphorylation of PELP1 by Src is critical for PELP1 coupling to the PI3K pathway by p85-SH2 domain (9). Similarly, overexpression of PELP1 (a situation that occurs in tumors) is also shown to constitutively activate PI3K pathway by p85-SH3 domain-mediated interactions (12), suggesting that PELP1 can potentially interact with and activate PI3K through two distinct mechanisms. Because in this experiment (Fig. 4D) we have used PELP1 overexpression, inhibition of ILK1 activity by PELP1 in the presence of PI3K inhibitor but not in the presence of Src kinase inhibitor suggests that the direct interactions of PELP1 with PI3K may lead to ILK1 activation. However, in the physiologic context, Src kinase does play a role in the ligand-mediated activation of ILK1 by promoting PELP1-PI3K-ILK1 complex formation.

**PELP1 is needed for optimal cell migration promoted by E2 extranuclear actions.** We examined whether E2-mediated extranuclear actions contribute to cell migration. In Boyden chamber assays, parental MCF7 cells showed low motility and EDC further increased the migratory potential of those cells. The knockdown of PELP1 expression by small interfering RNA substantially reduced EDC-mediated cell motility (Fig. 5A). Interestingly, model cells expressing DN ILK1 also

failed to migrate upon EDC stimulation (Fig. 5A). We also examined whether EDC-mediated cell migratory potential can be blocked by pharmacologic inhibition of Src kinase. Dasatinib effectively blocked the EDC-mediated cell migration in Boyden chamber assays (Fig. 5B). Similarly, dasatinib also inhibited E2-mediated cell migration in wound-healing assays (Fig. 5C).

**PELP1 overexpression enhances the in vivo metastatic potential of ER-positive ZR75 cells.** Because PELP1 expression is deregulated in metastatic tumors (14), we hypothesized that PELP1 overexpression may play a role in metastasis by promoting E2 extranuclear actions. We performed a proof-of-principle experiment using ER-positive ZR75 cells that exhibit poor metastasis in nude mice models. ZR75 cells were stably transfected with a GFP control or PELP1WT-GFP vector. PELP1WT-GFP cells had 3-fold higher expression of PELP1 than the control cells (data not shown). Mice injected with GFP control cells showed none to one metastatic nodule. However, PELP1-overexpressing cells had an increased propensity for metastases with 8 to 12 nodules identified in lungs (4 of 5 mice) and 6 to 8 nodules in liver (4 of 5 mice; Fig. 5D). To validate these findings further, we also injected GFP-vector and GFP-PELP1WT cells through a cardiac route into nude mice. Earlier studies found that this route facilitates bone metastasis (25). GFP-PELP1WT-overexpressing

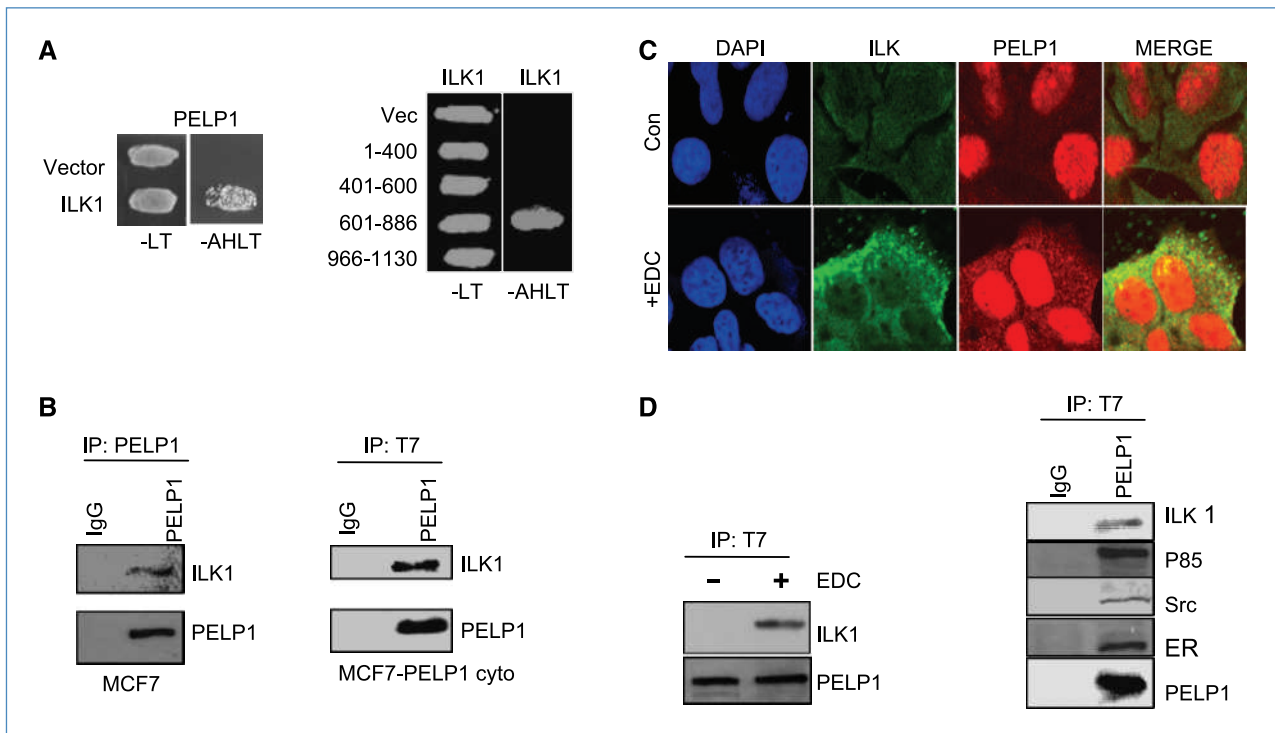


**Figure 3.** ILK1 is a novel PELP1-binding protein. A, confirmation of PELP1 interaction with ILK1 is shown in a yeast-based growth assay (left). Identification of the domain of interaction between PELP1 and ILK1 using a yeast-based growth assay (right). B, MCF7 cells that express T7-tagged PELP1WT (left) or T7-PELP1cyto mutant (right) were treated with EDC and the PELP1 and ILK1 interaction was confirmed by immunoprecipitation assay. C, MCF7 cells were treated with or without EDC for 5 min and the colocalization of PELP1 and ILK1 was analyzed by confocal microscopy. D, MCF7- T7-PELP1cyto cells were treated with or without EDC and total protein lysates were immunoprecipitated with T7-tagged antibody. The presence of PELP1, Src, ILK1, ER, and p85 in the immunoprecipitates was analyzed by Western blotting.

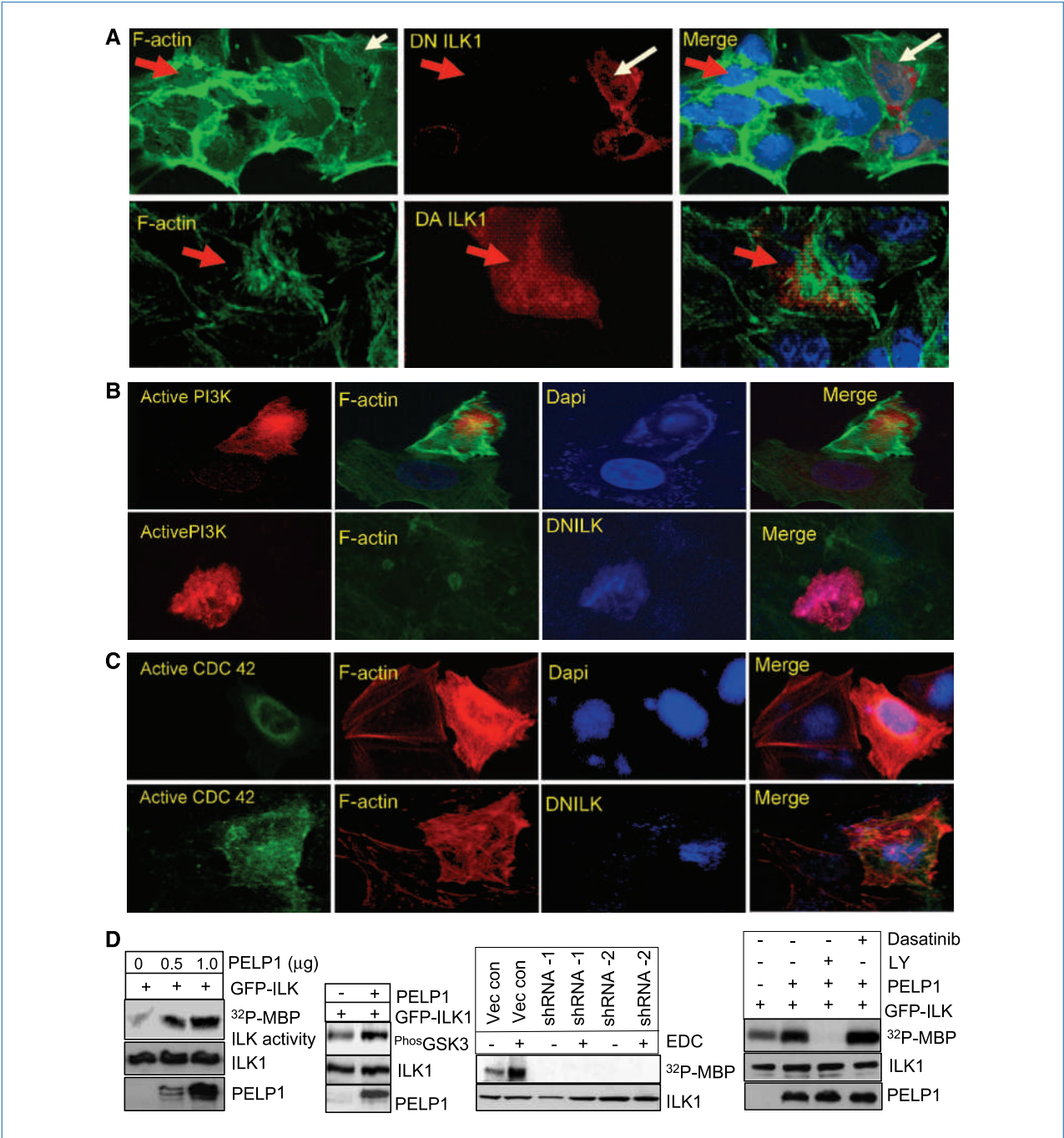


**Figure 4.** PELP1-ILK1 axis plays a productive role in E2-mediated cytoskeleton reorganization. Only one representative image for each experimental condition is shown and the results are representative of three independent replicates. **A**, MCF7 breast cancer cells were transfected with DN-ILK1 (red). After 72 h, the cells were treated with EDC for 10 min and F-actin (green) status was analyzed by confocal microscopy (top). MCF7-PELP1-shRNA cells were transiently transfected with DA-ILK1. F-actin status was verified by confocal microscopy (bottom). **B**, MCF7-PELP1-shRNA cells were transfected with constitutively active RFP-P110α subunit of PI3K (red) without (top) or with DN-ILK1 (bottom, blue) and F-actin (green) changes were analyzed by confocal microscopy. **C**, MCF7-PELP1-shRNA cells were transiently transfected with DA-CDC42 (green) without (top) or with DN-ILK1 (bottom, blue) and F-actin status was verified by confocal microscopy. **D**, the ability of PELP1 to enhance ILK1 activity was measured by an *in vitro* kinase assay by incubating immunoprecipitated GFP-ILK1 with increasing amounts of GST-PELP1 (left). MCF7 cells were transfected with ILK1 expression vector with or without PELP1 expression vector and the ability of PELP1 to enhance ILK1 downstream signaling was analyzed by Western blotting (left). MCF7 and MCF7-PELP1-shRNA cells were treated with or without EDC. ILK1 was immunoprecipitated and kinase activity was measured using an *in vitro* kinase assay (middle). Cells were transfected with ILK1 expression vector with or without PELP1 expression vector. After 72 h, cells were treated with the PI3K inhibitor (LY294002, 50 μmol/L) or Src kinase inhibitor (dasatinib, 100 nmol/L). ILK1 was immunoprecipitated and the ILK1 activity was measured by an *in vitro* kinase assay (right).

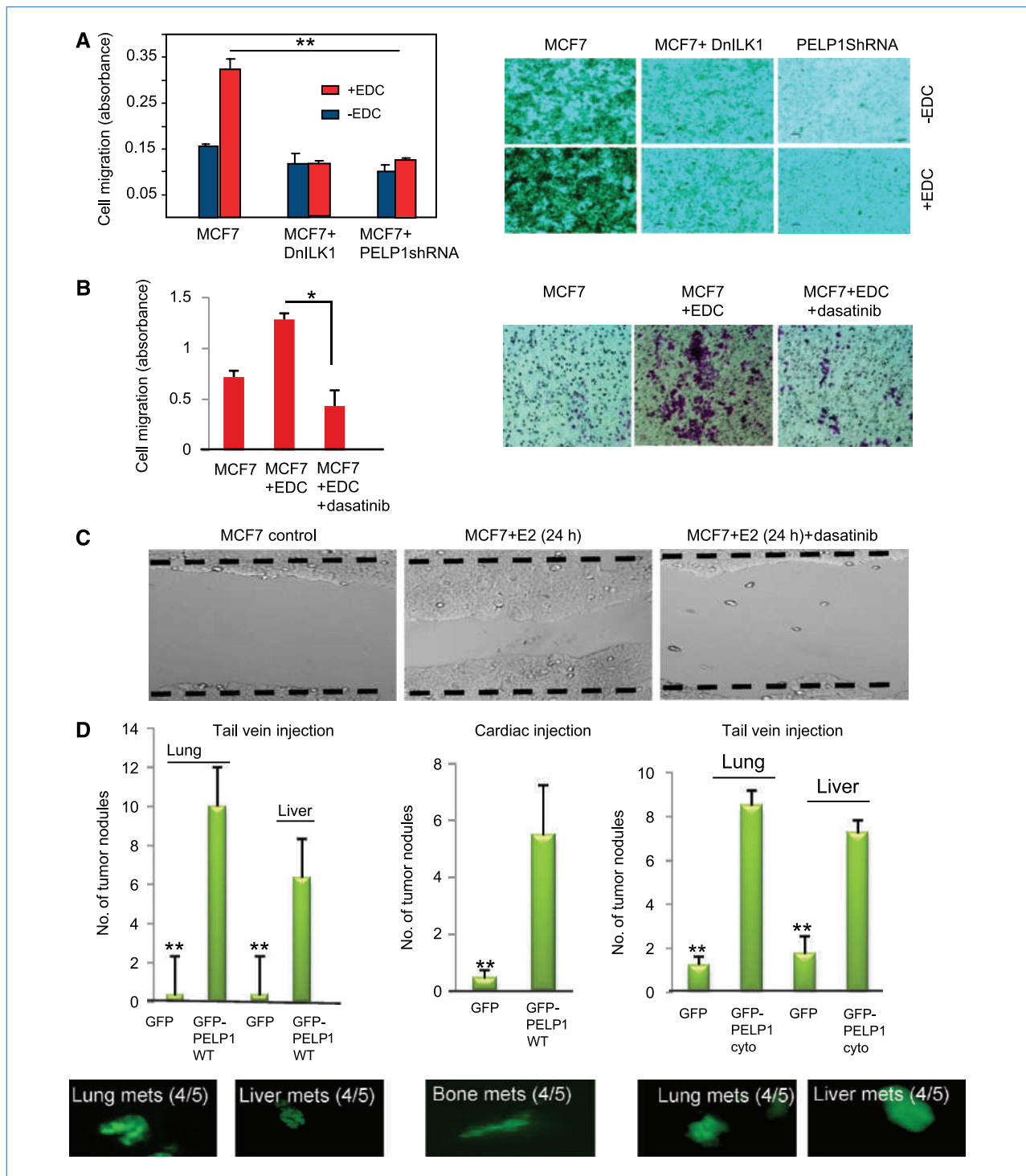


**Figure 5.** E2-mediated extracellular actions promote cell migration and metastasis. **A**, MCF7 cells, MCF7 cells transfected with DN-ILK1, and MCF7 cells stably transfected with PELP1-shRNA were treated with or without EDC and the migratory potential was analyzed by using Boyden chamber assay. Photomicrographs of migrated cells in various treatments (right). Columns, mean from three independent experiments performed in triplicate wells; bars, SEM. \*\*,  $P < 0.001$ . **B**, MCF7 cells were treated with EDC in the presence or absence of Src inhibitor dasatinib (100 nmol/L). The cell migratory potential was analyzed by using the Boyden chamber assay. Columns, mean from three independent experiments performed in triplicate wells; bars, SEM. \*,  $P < 0.05$ . **C**, wound-healing assay was performed in the presence or absence of E2 and in presence or absence of dasatinib. **D**, ZR75 cells expressing GFPvector or GFP-PELP1WT were injected into nude mice either through the tail vein (left) or cardiac route (middle) and metastases were recorded after 8 wk. MCF7 cells expressing control GFP-vector or GFP+PELP1cyto were injected into nude mice ( $n = 5$ ) through the tail vein (right). Representative images of metastatic nodules as observed by fluorescence microscope are shown. Columns, mean number of tumor nodules; bars, SEM; \*\*,  $P < 0.0001$ .

cells, but not GFP vector-expressing cells, had metastases in the bone (Fig. 5D, middle). To examine the significance of PELP1 extranuclear signaling in metastasis, we have repeated xenograft assay using PELP1cyto cells (12) that uniquely express PELP1 in the cytoplasm and are shown to excessively promote ER extranuclear signaling. Similar to PELP1WT cells, PELP1cyto cells also showed increased propensity to metastasize compared with MCF7 control cells (Fig. 5D, right). These results further suggest that ER extranuclear actions have potential to promote metastasis.

## Discussion

The pathologic significance of ER extranuclear signaling and its role in the progression to metastasis of breast cancer remain unknown. In this study, using estrogen dendrimers, DN reagents, and pharmacologic inhibitors of ER extranuclear signaling, we found that ER extranuclear actions play an important role in cell motility and metastases. In addition, we established for the first time that endogenous PELP1 play a critical role in coupling ER extranuclear signaling to cell motility through the ER-Src-PELP1-ILK-Rac/CDC42 pathway.

The proto-oncogene c-Src is a multifunctional intracellular tyrosine kinase implicated in the regulation of a variety of processes including proliferation, differentiation, survival, and motility (26). Src interacts with multiple cellular factors including human EGFR 2, EGFR, and ER, and breast tumors overexpress Src kinase (27). PELP1 acts as a scaffolding protein coupling the ER with Src kinase leading to the activation of the ER-Src-MAPK pathway (11). Our data suggest that PELP1 and Src kinase play an essential role in the activation of ER extranuclear signaling leading to cytoskeleton reorganization and migration. Because breast tumors overexpress Src kinase, deregulation of PELP1 seen in breast tumors can contribute to the activation of Src, leading to the progression to metastasis. Pharmacologic inhibition of Src using dasatinib significantly inhibited E2-mediated extranuclear actions and reduced E2-mediated migratory potential. These results suggest that the ER-Src-PELP1 axis is a novel target for preventing the emergence of metastatic cells and that dasatinib may have therapeutic utility in blocking ER-positive metastases.

ER $\alpha$  has been implicated in breast cancer progression and a majority of the human breast cancers starts out as hormone-dependent. Some evidence suggests that the extranuclear effects of estrogen can regulate different cellular processes, such as proliferation, survival, and apoptosis (28). Our results using EDC shows that ER extranuclear signaling has potential to promote cytoskeletal changes, leading to increased cell migration. Findings from these studies also showed that E2 extranuclear signaling promotes the formation of signaling complexes that contain PELP1, ER Src, and ILK1 and that extranuclear signaling from this axis play important roles in cytoskeletal rearrangements, motility, and metastasis.

We identified ILK1 as a novel interacting protein of PELP1 and showed that ILK1 functions as a downstream effector of ER extranuclear signaling, leading to cytoskeletal reorganiza-

tion. ILK1 is known to play an important role in cytoskeleton reorganization and in the activation of Rho GTPases (Rac and CDC42). These effects are reversible upon inhibition of ILK protein expression (29). The ability of PELP1 to modulate the ILK1 pathway and its potential deregulation in metastatic breast cancer suggest that the modulation of ILK1 pathway may represent one potential mechanism by which PELP1 promotes metastasis in breast cancer cells.

PELP1 is a key component of the ER signalsome in the cytoplasm and is shown to play a role in ER extranuclear actions (8, 9). PELP1 expression seems to be predominantly in the cytoplasm in a subset of breast tumors. Previous studies showed that PELP1 cytoplasmic localization excessively promotes ER extranuclear signaling and that such deregulation contributed to tamoxifen therapy resistance (12). A recent study showed that patients whose tumors had high levels of cytoplasmic PELP1 had a tendency to respond poorly to tamoxifen compared with patients whose tumors had low levels of cytoplasmic PELP1 (30). In this study, using ligands that uniquely activate ER extranuclear signaling (EDC), and PELP1shRNA or dominant mutants that block PELP1 signaling, we found that E2-driven PELP1-mediated ER extranuclear actions can promote the cell migratory potential.

Endocrine therapy has also been shown to have a positive effect on the treatment of advanced metastatic disease (3). A few earlier studies suggested a negative effect of ER signaling on motility and invasion of cells (31, 32), whereas several recent studies showed a positive effect of ER signaling on motility (32, 33). Many metastatic tumors retain ER (34), and if primary tumors are ER positive, >80% of lymph node metastases and 65% to 70% of distant metastases retain ER (2, 35). A clinical correlation has also been reported between ER-positive tumors and the development of bone metastasis (34, 36). Similarly, ER signaling has been shown to enhance lung metastasis by promoting host-compartment response (37). PELP1 expression is deregulated in metastatic tumors (14) and PELP1 protein expression is an independent prognostic predictor of shorter breast cancer-specific survival and its elevated expression is positively associated with markers of poor outcome (38). Our data suggest that ER extranuclear signaling plays a role in metastasis and PELP1 deregulation commonly seen in metastatic tumors may play a role in metastasis by enhancing ER extranuclear signaling.

In summary, our data provide the first evidence showing the significance of ER extranuclear signaling to the metastatic potential of breast cancer cells. Our findings also identified ILK1 as a novel component of ER-PELP1 signalsome that connects ER signaling to cytoskeleton. We hypothesize that the ER-Src-PELP1-PI3K-ILK1 pathway represents a novel target to prevent the emergence of ER-positive metastatic cells through blockage of ER extranuclear signals in combination with endocrine therapy.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Critical Review

# PELP1: A Novel Therapeutic Target for Hormonal Cancers

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### Summary

Recent studies implicate that the estrogen receptor (ER) coregulator proline-, glutamic acid-, and leucine-rich protein (PELP) 1 as playing critical roles in ER-genomic, ER-nongenomic, and ER-signaling cross talk with growth factor signaling pathways. PELP1 expression is deregulated in hormonal cancers and recent studies further elucidated the molecular mechanisms by which PELP1 regulates hormone therapy response. Although PELP1 is important for normal functions of the ER, the possibility to target ER-PELP1 axis appears to be an effective strategy for preventing hormonal carcinogenesis and therapy resistance. Thus, PELP1 may be useful as prognostic marker for hormonal cancers and PELP1 signaling may be useful to generate targeted therapeutics to overcome hormonal therapy resistance. © 2009 IUBMB

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**Keywords** estrogen receptor; coregulators; PELP1; hormonal signaling; therapy resistance.

### INTRODUCTION

The human estrogen receptor (ER) is a key transcriptional regulator in breast cancer biology. The majority of the human breast cancers start out as hormone-dependent. (1, 2). Endocrine therapy using tamoxifen, a selective estrogen receptor modulator (3) and aromatase inhibitors, which ablate estrogen (E2) biosynthesis, has been found to substantially improve disease-free survival (4). Despite these positive effects, initial or acquired resistance to endocrine therapies frequently occurs. Emerging evidence suggests that ER action is complex, involves genomic as well as nongenomic signaling events (5–7), and requires functional interactions with coregulators (8). As a modulator of ER functions, ER-coregulators are likely to play a role in breast

cancer progression and emerging data suggest that the level and activity of coregulators can specify hormonal sensitivity of tumors. Advances in research during the past decade have identified several novel proteins as being ER coregulators (9). Even though coregulators modulate ER functions, each coregulator appears to play an important and a nonoverlapping function *in vivo* (10–12). One of the ER coregulators, proline-, glutamic acid-, leucine-rich protein-1 (PELP1) (13), (also previously known as modulator of the nongenomic actions of the estrogen receptor, MNAR) (14), is unique because it plays important roles in both genomic (15) and nongenomic actions of the ER (16, 17). In this review, we have summarized the current perspective on the significance of PELP1 signaling axis in ER biology and the novel possibility of including PELP1 as a therapeutic target in treatment regimens along with current endocrine therapies.

### PELP1 MOLECULAR ASPECTS

PELP1 contains several motifs and domains that are commonly present in many transcriptional coactivators, including 10 nuclear receptor (NR)-interacting boxes (LXXLL motifs), a zinc finger motif, a glutamic acid-rich domain, and two proline-rich domains (13). Accordingly, PELP1 is shown to interact with and modulate functions of several nuclear receptors and transcriptional activators including AR (18, 19), ERR (20), GR (21, 22), PR (13), RXR (23), FHL2 (19), AP1 (20, 24), and STAT3 (25). A distinctive feature of PELP1 is the presence of an unusual stretch of 70 acidic amino acids in the C-terminus that functions as a histone-binding region (15, 24). PELP1 is localized both in the nuclear and cytoplasmic compartments and had unique functions in both compartments. PELP1 directly interacts with several cytosolic kinases including the p85 subunit of PI3K (17), SH3 domain of c-Src via its N-terminal PXXP motif (26). PELP1 is shown to be phosphorylated by several kinases including PKA (27), HER2 (17), Src (26), CDKs (28), and its phosphorylation is modulated by estrogen and growth factors (25).

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## PELP1 IN ER GENOMIC SIGNALING

PELP1 functions as a coactivator of both ER $\alpha$  (13) and ER $\beta$  (29) and modulates their transactivation functions. PELP1 colocalizes and interacts with the acetyltransferases CBP and p300 (15). PELP1 promotes and maintains the hypoacetylated state of histones at the target chromatin, and ER binding reverses its role to hyperacetylate histones (24). PELP1 is also associated with deacetylases, including components of nucleosome remodeling and the histone deacetylation complex (30), and inhibition of deacetylase activity increases the PELP1 residency time at the target gene promoter (15). Researchers have found reduced levels of linker histone H1 in chromatin surrounding the region targeted by PELP1 (15). Furthermore, PELP1 was found to exist in a complex with histone modifying enzymes like histone methyltransferases and demethylases (31). Our ongoing studies shows that PELP1 interacts with the Histone H3 methylase (SETDB1/ESET) and lysine demethylase (KSD1) proteins and has potential to modulate enzymatic activities of SETDB1 and KSD1 (32). Taken together, these findings suggest that PELP1 contributes to and perhaps directs ER-mediated alterations in the chromatin that is required for the optimal transcriptional response by regulating the activities of chromatin-modifying enzymes and by facilitating optimal histone code at ER target genes.

## PELP1 IN ER NONGENOMIC SIGNALING

PELP1 participates in ER cytoplasmic and membrane-mediated signaling (MIS) events by coupling the ER with several cytosolic kinases (33). PELP1 modulates the interaction of ERs with cSrc, stimulating cSrc enzymatic activity, leading to the activation of the mitogen activated protein kinase (MAPK) pathway (14). PELP1 directly interacts with the p85 subunit of PI3K and enhances PI3K activity (17). Greger et al., reported a direct correlation between PELP1 expression levels and E2-induced activation of PI3 and Akt kinases. Mechanistic studies found that E2 treatment induced complex formation between PELP1, ER $\alpha$ , cSrc, and p85, the regulatory subunit of PI3K. The interaction between p85 and PELP1 requires activation of cSrc and PELP1 phosphorylation on Tyr 920 (26). These findings implicate that PELP1 acts as a scaffolding protein promoting ER interactions with intracellular kinases and this facilitate activation of ER-nongenomic signaling pathways.

## PELP1 IN ER SIGNALING CROSS TALK

Growth factors promote tyrosine and serine phosphorylation of PELP1 (17, 34). PELP1 interacts with several growth factor receptors, including EGFR and HER2 (17, 35). Growth factor-induced phosphorylation of PELP1 is shown to affect subnuclear localization of PELP1 and also influence PELP1-mediated ER transactivation functions (27). PELP1 interacts with signal transducers and activators of transcription 3 (STAT3), and PELP1-STAT3 interactions play a mechanistic role in the

positive regulation of STAT3 transcription (36). Overexpression of PELP1 potentiates phosphorylation of STAT3 at Ser 727 and plays an important role in the growth factor mediated STAT3 transactivation functions. Such regulatory interactions of PELP1 have important functional implications in the cross-talk of estrogen receptor and growth factor signaling (36). PELP1 also interacts with hepatocyte growth factor receptor regulated tyrosine kinase substrate (HRS), and HRS was found to sequester PELP1 in the cytoplasm, leading to the activation of MAPK in a manner that is dependent on the epidermal growth factor receptor (37). Collectively, these findings suggest that growth factor signals promote phosphorylation of PELP1. As growth factor signals play key roles in hormonal therapy resistance, PELP1 interactions with growth factor axis may have functional implications in breast tumors with deregulated growth factor signaling.

## PELP1 IN CELL CYCLE PROGRESSION

PELP1 signaling plays an important role in E2-mediated cell cycle progression (26, 38). PELP1 is a Retinoblastoma (pRb)-interacting protein and PELP1 deregulation is shown to promote cyclin D1 expression (38). Mechanistic studies found that PELP1 plays a permissive role in E2-mediated cell cycle progression by enhancing E2 mediated G1-S progression. Increased PELP1 expression found in mammary glands during pregnancy also supports a physiological role for PELP1 in E2-mediated cell cycle progression in mammary glands (13). Evidence also indicates that PELP1 plays a role in meiosis by interacting with the androgen receptor (AR) and appears to mediate inhibition of meiosis via G $\beta\gamma$  signaling. Ligand activation and binding to the AR overcomes this inhibition, which allows maturation to occur (39). Recently completed work in our laboratory demonstrates that PELP1 is a novel substrate of both CDK4 and CDK2. PELP1 is recruited to several E2F target genes; functions as an E2F coregulator and mutation of CDK phosphorylation sites in PELP1 abolishes PELP1-mediated E2F transactivation functions (28). CDK phosphorylation of PELP1 may confer growth advantage to breast epithelial cells and thus contribute towards tumorigenesis by accelerating cell cycle progression.

## PELP1 DEREGLATION IN HORMONAL CANCERS

Several lines of evidence implicate PELP1 as a potential proto-oncogene and its expression is deregulated in a wide variety of hormone-driven tumors including breast (17, 40–42), endometrial (29), ovarian (43), and prostate cancer (19). Overexpression of PELP1 in fibroblasts and epithelial model cells results in cellular transformation and PELP1 over expression in breast cancer model cells potentiates rapid tumor growth in xenograft studies (42). PELP1 interacts with and modulates functions of several proto-oncogenes, including c-Src, STAT3, HER2, and EGFR (44). PELP1 has been reported to be widely expressed in breast cancer cells (13, 45) and is shown to be up

regulated in a subset of breast tumors (13, 46). A very recent study identified PELP1 as a cell fate determination factor (DACH1)-binding protein. DACH1 functions as an endogenous inhibitor of ER $\alpha$  transcriptional activity. Using 2,200 breast tumor samples, Popov et al. found that DACH1 expression is lost during breast cancer progression (47). Using mechanistic studies, they also demonstrated that DACH1 and PELP1 colocalize in the nucleus in ~80% of cells and that the relative balance of DACH1 and PELP1 in breast cancer cells has implications in ER $\alpha$  signaling as DACH1 loss can potentiate PELP1 coactivation functions of ER (47). Salivary duct carcinoma is a high-grade neoplasm with morphology similar to that of mammary duct carcinoma. Interestingly, these salivary tumors express PELP1 and ER, and PELP1 signaling may play a role in salivary tumorigenesis (48). Recently, Grivas et al. reported that PELP1 expression was higher in epithelial cells of colon carcinomas than in normal mucosa and that PELP1 overexpression in epithelial cells was found to be an independent favorable prognostic factor (49). PELP1 expression in myofibroblasts from normal mucosa as well as in carcinomas suggests that deregulated expression of coregulators in both epithelial cells and myofibroblasts may contribute to the initiation and progression of colorectal carcinoma (50). Marquez-Garban et al. reported deregulated PELP1 expression in lung tumors (51). These emerging findings suggest that PELP1 is a novel proto-oncogene and its deregulation can potentially contribute to oncogenesis in hormone-driven cancers.

### ROLE OF PELP1 IN METASTASIS

PELP1 interacts with several proteins involved in cytoskeleton remodeling, including Src kinase, PI3K, four and a half LIM protein 2, and ILK1 (17, 52–54). PELP1 overexpression uniquely enhances E2-mediated ruffles and filopodium-like structures and model cells that overexpress PELP1 exhibit increased cell motility (42). PELP1 modulates functions of metastasis-associated protein 1 (MTA1) via its interactions with MTA1-associated coactivator MICOA and promotes ER $\alpha$ -transactivation functions in a synergistic manner (30). Additionally, PELP1 modulates expression of MTA3, a gene implicated in the invasive growth of human breast cancers (55). Using breast tumor prognostic arrays, Rajhans et al. found that node-positive and metastatic tumors had higher PELP1 expression than the expression in node-negative specimens. Node-positive and metastatic tumors exhibited a greater expression of PELP1 than did node-negative tumors ( $P = 0.003$ ) (42). Our ongoing studies have also shown that PELP1-mediated ER-nongenotropic actions play a role in cell motility/metastasis (54). Habashy et al. investigated the clinical and biological relevance of PELP1 protein expression in 1,162 patients with invasive breast cancers and found that increased PELP1 expression is associated with tumor clinical parameters, shorter breast cancer-specific survival (BCSS), and disease-free incidence (DFI), implicating PELP1 protein expression as an independent prognostic

predictor of shorter BCSS and DFI in breast cancer (40). The ability of PELP1 to interact with various enzymes that modulate the cytoskeleton and its putative deregulation in metastatic breast tumors suggest that PELP1 signaling plays a role in tumor cell migration and metastasis.

### ROLE OF PELP1 IN LOCAL ESTROGEN SYNTHESIS

*In situ* estrogen synthesis has been implicated in tumor cell proliferation through autocrine or paracrine mechanisms especially in postmenopausal women. Several studies have reported a significant increase in activity of aromatase (Cyp19), a key enzyme involved in E2 synthesis in breast tumors (56). Recent studies from our laboratory suggested that PELP1 deregulation contributes to increased expression of aromatase and local E2 synthesis, and that PELP1 cooperates with growth factor signaling components in the activation of the *aromatase* gene. PELP1 deregulation uniquely contributed to this via activation of the *aromatase* promoter I.3/II (20). Mechanistic studies found that PELP1 interacts with LSD1 (32), that PELP1-LSD1-mediated histone epigenetic modifications play a role in the local aromatase expression and that PELP1 deregulation could play a role in modulating histone methylation at the *aromatase* promoter region (57). Immunohistochemistry (IHC) analysis of breast tumor arrays revealed that substantial number of PELP1 overexpressing breast tumors also overexpressed *aromatase* when compared with PELP1 low-expressing tumors (20), suggesting that PELP1 regulation of *aromatase* represents a novel mechanism for *in situ* estrogen synthesis, leading to tumor proliferation by an autocrine loop and thus opening a new avenue for ablating local aromatase activity in breast tumors.

### ROLE OF PELP1 IN HORMONAL THERAPY RESISTANCE

Although the mechanisms for hormone therapy resistance remains elusive, recent studies suggest that the presence of alternative signaling pathways including ER nongenomic signaling and ER-cross talk with the growth factor components contributes to hormone therapy resistance (16, 53, 58). PELP1 deregulation appears to play an important role in the development of hormone therapy resistance (25). PELP1 plays an essential role in ER-nongenomic actions by coupling the ER with Src and PI3K pathways (16, 53). In addition, PELP1 interacts with growth factor signaling components and participates in the ligand-independent activation of ER (25). Although PELP1 is predominantly localized in the nucleus of hormonally responsive tissues (13, 40), it exhibits cytoplasmic localization in a subset of tumors from breast and endometrial tissues and salivary glands (17, 29, 34, 36, 48). Further, breast cancer model cells mimicking PELP1 cytoplasmic expression showed resistance to tamoxifen via excessive activation of the c-Src signaling axis (17). Recently, a transgenic (Tg) mouse model that uniquely expresses PELP1 in the cytoplasm (PELP1-cyto) was

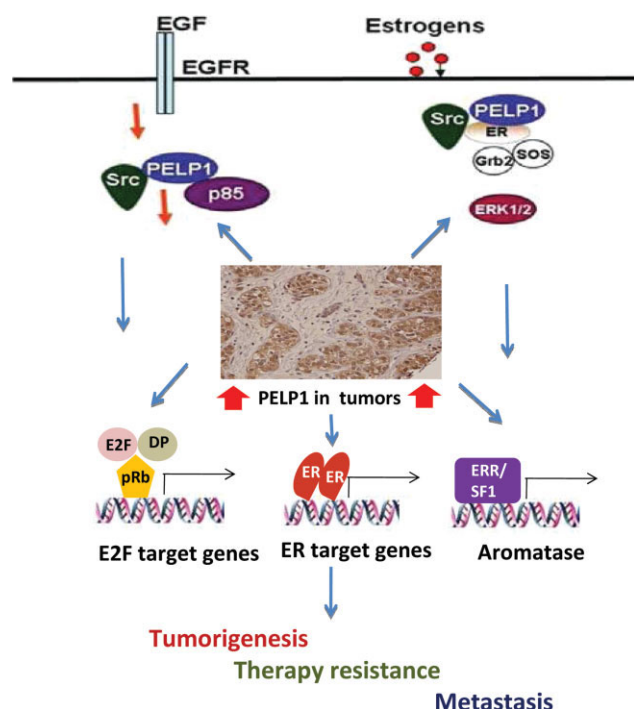


**Figure 1.** Schematic representation of the current understanding of PELP1 signaling pathway. PELP1 appears to function as a scaffolding protein that facilitates formation of various signaling complexes, and its expression is deregulated in hormonal cancers. PELP1 regulates multiple signaling pathways including ER-genomic signaling by facilitating epigenetic changes, ER-nongenomic signaling by activating Src-MAPK pathway, local estrogen synthesis via  $ERR\alpha$ -aromatase signaling, and cell cycle progression via pRb/E2F pathway. PELP1 deregulation in hormonal tumors is likely to contribute to the development of tumorigenesis, metastasis, and hormonal independence by activating multiple signaling pathways and by facilitating ER signaling cross talk. Insert in the middle shows PELP1 expression in advanced breast tumors as analyzed by IHC. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

developed (41). By 12 weeks of age, mammary glands of these Tg mice developed widespread hyperplasia with increased cell proliferation and exhibited resistance to tamoxifen therapy. In another study, IHC analysis of tumors from human patients indicated that cytoplasmic localization of PELP1 was an independent prognostic marker for determining response to tamoxifen (41). These findings suggest that PELP1 localization could be used as a determinant of hormone sensitivity or resistance. This evidence strongly suggests that PELP1 deregulation has potential to confer resistance of breast tumors to hormonal therapy.

### THERAPEUTIC TARGETING OF PELP1

With several studies hinting toward the therapeutic benefits of targeting PELP1 in hormonal cancer initiation, progression,

and metastasis, it has become increasingly necessary to develop strategies to effectively interfere with PELP1 signaling or to silence the expression of PELP1 in cancers (Figure 1). Currently, there are no pharmacological inhibitors of PELP1. Therefore, targeting critical components of PELP1 signaling axis such as PELP1-Src axis, PELP1-CDK2, and PELP1-LSD1 axis could be used as a therapeutic option. Src kinase play an essential role in the activation of PELP1-mediated nongenomic signaling leading to cell migration, metastasis, and local E2 synthesis. Pharmacological inhibition of Src kinase using dasatinib (BMS-354825) significantly inhibits activation of PELP1-mediated tumorigenic functions (59). Therefore, the Src inhibitor dasatinib holds a therapeutic promise in blocking the PELP1 signaling axis. PELP1 is a novel substrate of CDKs and inhibition of CDK function by roscovitine is effective in reducing PELP1-mediated therapeutic potential (60). Tumor cells overexpressing PELP1 in the cytoplasm are distinctly sensitive to  $TNF\alpha$ -induced apoptosis. Therefore,  $TNF\alpha$ -based therapeutics could also be used as a strategy for tumors with cytoplasmic PELP1 expression (61). Pargyline is a selective monoamine oxidase inhibitor that blocks LSD1 activity (62) and is approved by FDA for treatment of moderate to severe hypertension. As PELP1 interacts with LSD1 and promotes epigenetic changes at ER target genes, inhibition of PELP1-LSD1 axis by pargyline could also be explored as an important therapy option. A recently conducted study in our laboratory indicated that treatment of breast cancer cell lines which overexpress PELP1 with pargyline substantially inhibited PELP1 tumorigenic functions, thus using pargyline may have therapeutic utility in PELP1 deregulated tumors (57). Ohshiro et al. found that resveratrol, a well-established phytoestrogen and chemopreventive agent, promotes PELP1 localization to autophagosomes. This association may be important in the process of cell death by autophagy in the cancer cells and suggests that resveratrol could be used to induce cell death via autophagy induction in PELP1-deregulated tumors (63). We recently developed PELP1 siRNA nanoparticles using chitosan (64), a biocompatible and biodegradable polymer to down regulate PELP1 expression. We tested and successfully confirmed the novel possibility of using PELP1siRNA nanoparticles to silence PELP1 expression in breast cancer cells. Our ongoing studies are using this approach to examine whether PELP1 downregulation sensitize therapy resistant breast cancer cells to hormone therapy (60, 65).

### CONCLUSIONS AND FUTURE DIRECTIONS

Accumulating evidence strongly suggests that PELP1 is a novel proto-oncogene, whose expression is deregulated in many hormonal cancers. Molecular studies implicate PELP1 as a molecular scaffold that allows the ER to form distinct complexes to facilitate signal transduction. Deregulation of PELP1 expression or functions will have wide implications in ER signaling and therapy response. For substantial improvement in cancer therapy, nonsingular approaches combining more than one

treatment agent into one delivery vehicle shows to be quite promising. Therefore, combining the chemotherapeutic drugs together with PELP1 axis targeting drugs appears to be promising for effective control of hormone therapy resistant tumors. Future studies elucidating the molecular mechanism of PELP1 actions and the molecular composition of PELP1-associated complexes in tumor cells compared to normal cells, characterizing the physiological function of PELP1 using Tg/knockout mouse models, and identifying genome-wide PELP1 target genes will assist in the development of novel therapeutic targets to block PELP1 signaling axis. As PELP1 expression is deregulated in hormonal tumors, understanding the mechanisms that contribute to PELP1 deregulation will provide novel avenues for therapeutic intervention. Future studies are also warranted to extend siRNA therapeutics to specifically down regulate PELP1 expression in hormonal cancers by using a targeted delivery approach. PELP1 appears to have a potential application in assessing the clinical outcome of patients with ER-positive breast cancer. As few published studies show that PELP1 expression correlates with therapy resistance and survival, future studies using a large number of advanced and therapy-resistant tumors are needed to establish PELP1 as a prognostic marker for hormonal tumors.

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## 2009 AACR Annual Meeting

April 18-22, 2009

Denver, CO

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**Abstract Number:** 2387

**Session Title:** Endocrinology 2

**Presentation Title:** **Therapeutic potential of blocking ER non-genomic actions on ER-positive metastasis**

**Presentation Start/End Time:** Monday, Apr 20, 2009, 1:00 PM - 5:00 PM

**Location:** Hall B-F, Poster Section 12

**Poster Section:** 12, 12

**Poster Board Number:** 11

**Author Block:** *Dimple Chakravarty, Sujit Nair, Binoj Chandrasekhar Nair, Long Wang, Abhik Bandyopadhyay, Joseph K. Agyin, Frank Lee, Lu-Zhe Sun, I-Tien Yeh, Rajeshwar Rao Tekmal, Ratna K. Vadlamudi.* UT Health Science Ctr., San Antonio, TX, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ

Estrogen receptor (ER) is implicated in breast cancer progression and the majority of the human breast cancers start out as hormone-dependent. A large portion of metastases retain their ER when the primary tumors are ER+ve. Several recent studies detected the presence of ER, ER-coregulator Proline Glutamic acid Leucine rich Protein 1 (PELP1), and aromatase in metastatic breast tumors. Even though substantial information is available on the mechanism of ER-ve metastasis, the role of ER signaling in ER+ve breast metastasis is an *understudied* area. Emerging evidence suggests that in addition to well-studied nuclear functions, ER also participates in non-genomic (cytoplasmic and membrane-mediated) signaling. In this study we examined whether ER non-genomic signaling play a role in ER+ve metastasis. To dissect the mechanism of ER non-genomic signaling on cell migration and metastasis, we used ligands (estrogen, estrogen-dendrimers), shRNA (ER, PELP1, Src), dominant active or negative constructs of PELP1 and Src and ER positive breast cancer cells (MCF7, ZR75) that over express GFP vector or GFP-PELP1. Our studies revealed that PELP1 and Src kinase play an essential role in the activation of ER non-genomic signaling leading to cell migration. Blockage of ER-PELP1-Src axis using dominant negative mutants significantly affected ER non-genomic signaling. PELP1 mutant that cannot bind Src kinase functioned as dominant negative and substantially affected ER non-genomic signaling leading to defects in cytoskeleton. Using dominant negative and active constructs of PELP1 and Src, we have identified that E2 non-genomic signaling promotes cytoskeleton reorganization via ER-PELP1-Src pathway. Utilizing Boyden chamber assays, we have demonstrated that deregulation of PELP1 contribute to increased cell migratory function via excessive activation of Src kinase and by promotion of local estrogen synthesis. Nude mice injected (tail route) with ZR75-GFP control cells showed 0-1 metastatic nodules while ZR75-PELP1 cells showed increased propensity to metastasize with 8-12 nodules in lungs and 6-8 nodules in liver. Nude mice injected (cardiac route), ZR75-PELP1GFP, but not control GFP cells, showed metastases to bone. Pharmacological inhibition of Src kinase using Dasatinib (BMS-354825) significantly inhibited activation E2 mediated non-genomic actions. Dasatinib also inhibited the migratory potential of PELP1 over expressing breast cancer cells and also affected PELP1's ability to promote local estrogen synthesis. Collectively, our results suggest that ER non-genomic actions play a role in ER+ve cell motility/metastasis. ER-PELP1-Src axis represents a novel target for preventing the emergence of ER+ve metastatic cells and pharmacological inhibitor Dasatinib may have therapeutic utility in blocking ER positive metastasis. (This study is supported by DOD BCRP grant#W81XWH-08-1-0604)

**[3127] Pathological Significance and Therapeutic Implications of ER Non-Genomic Actions.**

**Chakravarty D, Nair SS, Cortez V, Dandamudi R, Challa RB, Tekmal RR, Kumar R, Vadlamudi RK, Lee FY**  
**University of Texas Health Sciences Center at San Antonio, San Antonio, TX; Bristol-Myers Squibb,**  
**Lawrenceville, NJ; The George Washington University Medical Center, Washington, DC**

**Background:** Emerging evidence suggests that in addition to genomic functions, estrogen receptor (ER) participates in extra-nuclear rapid signaling, commonly termed as nongenomic signaling, via the formation of signaling complexes in the cytoplasm. Although much is known about ER genomic actions, the mechanism and pathobiology of ER-nongenomic actions remains poorly understood. Proline glutamic acid rich protein 1(PELP1), an established ER coregulator that participates both in ER-genomic and nongenomic actions. Recent findings implicate PELP1 as a novel proto-oncogene and its expression correlates well with a poor prognosis and therapeutic resistance. In the present study, we examined pathological significance of ER-PELP1 nongenomic signaling in the development of mammary tumorigenesis.

**Methods:** To evaluate the role of ER-PELP1 nongenomic signaling in tumorigenesis and therapeutic resistance, we have generated the following breast cancer model cells with functional or defective ER-PELP1-Src axis: (1) MCF-7 cells; (2) MCF-7 HER2 that overexpress HER2; (3) MCF7-Tam that acquired resistance to tamoxifen and (4) MCF-7Ca-LTLT that acquired resistance to Letrozole. These model cells were searched for evidence of functional nongenomic signaling using phosphospecific antibodies, and for hormonal responsiveness in the proliferation assays. To establish the physiologic significance of ER-nongenomic action, we have used xenograft- and transgenic-mice (TG) models that express ER coregulator PELP1 in the cytoplasm (PELP1cyto). Combinatorial therapy of endocrine agents along with drugs that block nongenomic signaling including Src inhibitor (Dasatinib, BMS-354825) were tested in various biologic assays.

**Results:** Mechanistic studies using PELP1- or Src-defective models revealed that knockdown of either PELP1 or Src kinase abolishes ER-nongenomic signaling. Pharmacological inhibition of the Src kinase using Dasatinib significantly inhibited activation ER non-genomic actions. Dasatinib also inhibited the tumorigenic potential of breast cancer cells with PELP1 overexpression in xenograft studies, as well as sensitized resistant model cells to hormonal therapy. Cytoplasmic expression of PELP1 promoted excessive activation of Src kinase in the mammary gland of PELP cyto Tg mice. Aging of the PELP1cyto Tg mice (n=60) for 24 months revealed the presence of an excessive activation of ER-nongenomic signaling in the mammary gland, and promoted hyperplasia at the age of 10 weeks with formation of mammary carcinomas beginning at ~12 months of age. These results suggest that ER-nongenomic signaling has the potential to promote formation of ER-positive mammary carcinomas.

**Conclusions:** Our results suggest that non-genomic signaling components have potential to promote ER-positive breast tumorigenesis and confer therapeutic resistance. Our findings suggest that ER-PELP1-Src axis represents a novel target for blocking ER-nongenomic actions. As ER genomic and nongenomic signaling are involved in tumorigenesis and resistance, combination therapies using tamoxifen or letrozole with Dasatinib that block different pathways may have a better therapeutic efficacy and could delay the on-set of hormonal resistant in the advanced breast tumors. This research was supported by grants CA0095681 and W81XWH-08-1-0604.

Friday, December 11, 2009 5:30 PM

**Poster Session 3: Tumor Biology: Novel/Emerging Therapeutic Targets (5:30 PM-7:30 PM)**

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