AD_____

Award Number: W81XWH-08-1-0604

TITLE: Hormonal Resistance and Metastasis: ER-coregulator-Src Targeted Therapy

PRINCIPAL INVESTIGATOR: **Ratna K Vadlamudi, PhD**

CONTRACTING ORGANIZATION: University of Texas Health Sciences Center

San Antonio, TX 78229

REPORT DATE: September 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

√ Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-09-2009	Annual	09-01-08 to 08-31-09

4. TITLE AND SUBTITLE

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

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0604

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Ratna K Vadlamudi

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Univ. Teaxs Health Sciences
Center at San Antonio

San Antonio, TX 78229

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

US. Army Medical Research and
Material command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release

13. SUPPLEMENTARY NOTES

14. ABSTRACT

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. Despite the 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* to metastasis. In this study, we have generated *model* cells that have defects in coregulator PELP1-Src signaling axis. Using these models, we demonstrated that ER-nongenotropic actions play an important role in cell motility/invasion. 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. Pharmacological inhibition of Src kinase using dasatinib significantly inhibited E2-mediated nongenomic actions. 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.

15. SUBJECT TERMS
Estrogen receptor, coregulators, nongenomic actions, Src kinase, therapy resistance, metastasis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	39	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Annual Report

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, 2009

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/MNAR 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.*

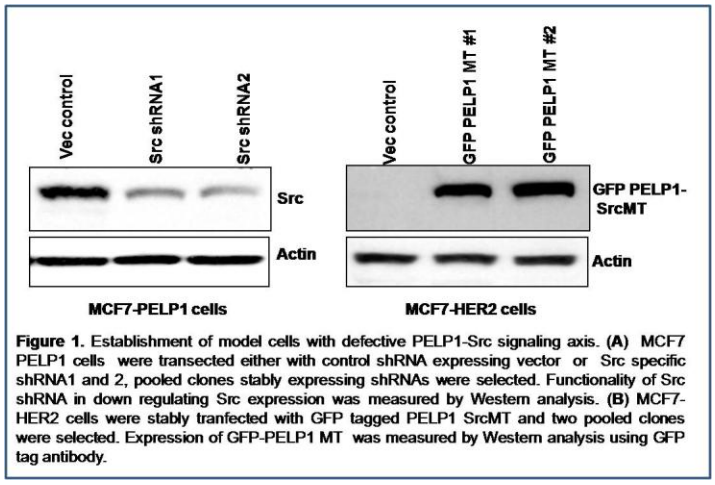


Figure 1. Establishment of model cells with defective PELP1-Src signaling axis. **(A)** MCF7 PELP1 cells were transected either with control shRNA expressing vector or Src specific shRNA1 and 2, pooled clones stably expressing shRNAs were selected. Functionality of Src shRNA in down regulating Src expression was measured by Western analysis. **(B)** MCF7-HER2 cells were stably tranfected with GFP tagged PELP1 SrcMT and two pooled clones were selected. Expression of GFP-PELP1 MT was measured by Western analysis using GFP tag antibody.

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

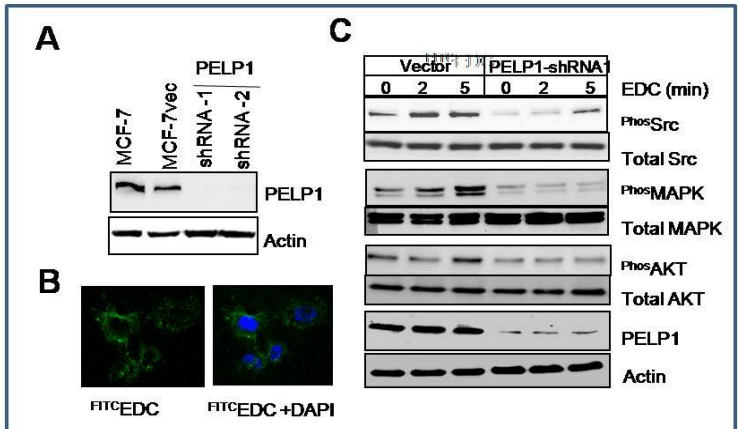


Fig 2. PELP1 Knockdown affects Src activation and E2 mediated non-genomic signaling. *A*, MCF7 or MCF7-PELP1-shRNA cells were lysed and expression of PELP1 was analyzed by Western blotting. *B*, MCF7 cells were treated with FITC-labeled EDC for 45 min and localization of EDC was analyzed by confocal microscopy and found outside the nucleus. *C*, MCF7 or MCF7-PELP1-shRNA cells were treated with EDC that uniquely activated estrogen (E2)-nongenomic signaling. Activation of signaling pathways was analyzed by Western blotting total protein lysates with phospho-specific antibodies.

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.

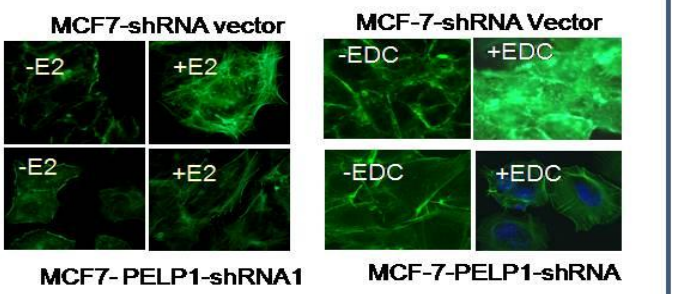


Fig. 3. Blockage of PELP1 signaling axis affects cytoskeleton reorganization. MCF7 or MCF7-PELP1-shRNA cells were treated either with E2 or estrogen dendrimers and the F-actin status was analyzed by phalloidin staining and visualized by confocal microscopy.

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. 4*A*). 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. 4*B*). Transient expression of PELP1SrcMT substantially interfered with E2-mediated activation of Src and MAPK (Fig. 4*C*). These results suggest that Src interactions with PELP1 is needed for optimal E2 mediated non genomic actions.

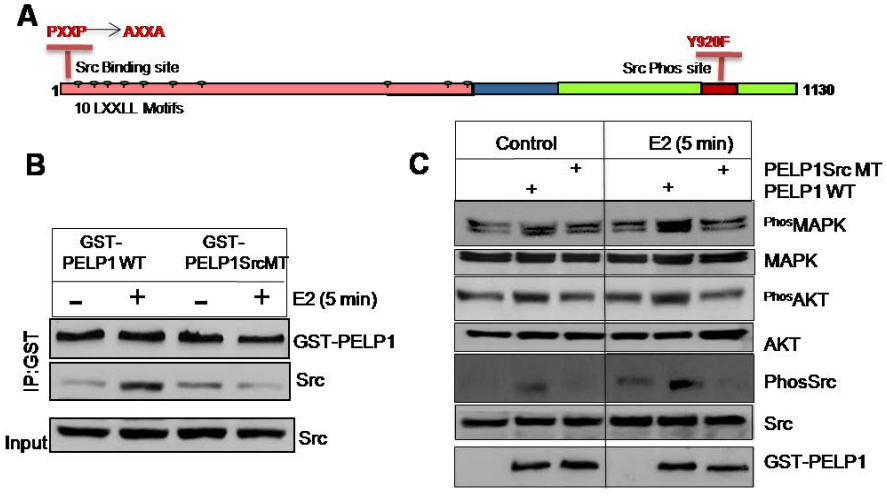


Figure 4. Src kinase is needed for optimal activation of PELP1-mediated E2 nongenomic actions. *A*, Schematic representation of PELP1 mutant that cannot bind or be phosphorylated by Src kinase. *B*, MCF7 cells were transfected with PELP1 WT or PELP1 SrcMT and treated with or without E2. Theability of the expressed proteins to interact with Src kinase was analyzed by immunoprecipitation. *C*, MCF7 cells were transfected with PELP1 WT or PELP1SrcMT using the Amaxa method and treated with or without E2. Activation of nongenomic signaling were measured by Western blotting with phospho-specific antibodies.

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.

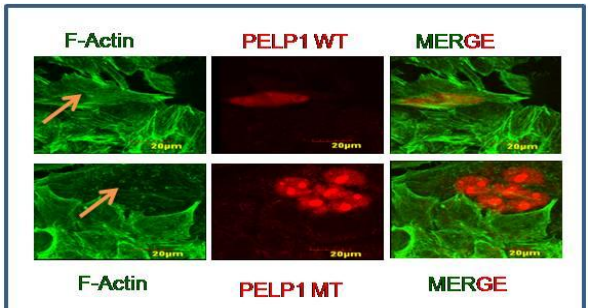


Figure 5. PELP1-Src kinase interactions are needed for E2 cytoskeletal reorganization. MCF7 cells were transiently transfected with either PELP1 WT or PELP1 SrcMT and treated with E2 for 5 min. The status of F-actin was analyzed by confocal microscopy

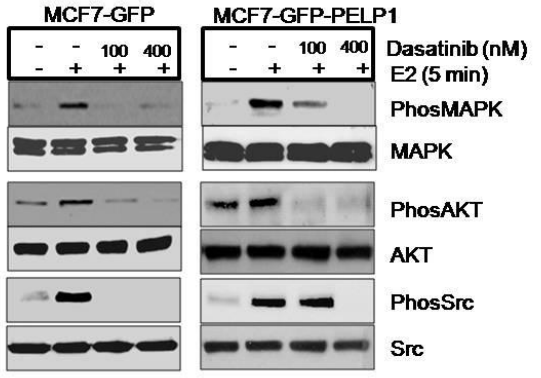


Figure 6. Src kinase inhibitor Dasatinib can block E2 mediated non genomic signaling. MCF7 or MCF7 cells that stably expressed PELP1 were treated with or without Src inhibitor dasatinib and with or without E2.Activation of nongenomic signaling was measured by Western blotting with phospho-specific antibodies.

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.

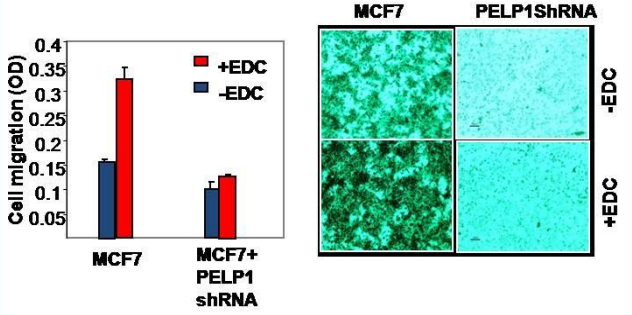


Figure 7. E2-mediated nongenomic actions promote cell migration. MCF7 cells, and MCF7 cells stably transfected with PELP1-shRNA were treated with or without EDC and the migratory potential was analyzed by using Boyden chamber assay. Photomicrographs of migrated cells in various treatments (right panel).

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 oveexpressing or therapy resistant cells such as LTLT that are resistant to letrozole (Fig. 8*A*). 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.*** Our ongoing experiments will test whether Src inhibitor can be used to down regulate aromatase expression in PELP1 and Her2 overexpressing cells.

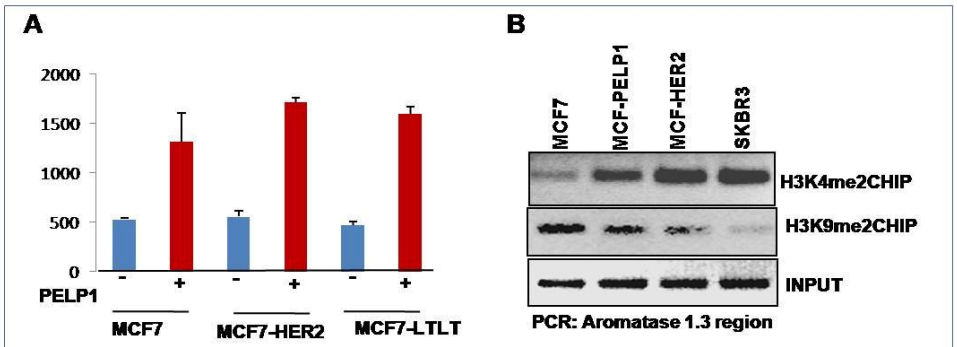


Figure 8. (A) MCF7, MCF-HER2 or letrozole resistant MCF7 (MCF-LTLT) cells were cotransfected with aromatase reporter gene along with or without PELP1 and the promoter activity was measured after 72 hours. **(B)** ChIP was performed using H3K9me2, K3K4me2 specific antibodies in indicated cells and the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

KEY RESEARCH ACCOMPLISHMENTS:

- Establishment of breast model cells model cells with functional and defective PELP1-Src signaling axis
- Demonstration that endogenous PELP1 is needed for E2 mediated ER-nongenomic signaling
- Demonstration of the significance of ER-nongenomic signaling to the migratory potential of breast cancer cells
- Demonstration that dasatinib may have therapeutic utility in blocking ER-nongenomic actions

REPORTABLE OUTCOMES:

This study produced the following publications:

1. 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. 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.
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 *Journal of Steroid Biochemistry and Molecular Biology* 09/2009, In Press.

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-nongenotropic 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. 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. Our ongoing studies in the second year will address the role of PELP1 in breast cancer cell migration in vivo, therapeutic potential of Dasatinib in sensitizing therapy resistant cells and to lock local estrogen synthesis.

REFERENCES:

1. Ariazi, E. A., Ariazi, J. L., Cordera, F. & Jordan, V. C. Estrogen receptors as therapeutic targets in breast cancer. *Curr. Top. Med. Chem.* **6**, 195-216 (2006).

2. Lewis-Wambi, J. S. & Jordan, V. C. Treatment of Postmenopausal Breast Cancer with Selective Estrogen Receptor Modulators (SERMs). *Breast Dis.* **24:93-105.**, 93-105 (2005).

3. Leary, A. & Dowsett, M. Combination therapy with aromatase inhibitors: the next era of breast cancer treatment? *Br. J. Cancer.* **.**, (2006).

4. Acconcia, F., Barnes, C. J. & Kumar, R. Estrogen and tamoxifen induce cytoskeletal remodeling and migration in endometrial cancer cells. *Endocrinology.* **147**, 1203-1212 (2006).

5. Hall, J. M. & McDonnell, D. P. Coregulators in nuclear estrogen receptor action: from concept to therapeutic targeting. *Mol. Interv.* **5**, 343-357 (2005).

6. Smith, C. L. & O'Malley, B. W. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocr. Rev.* **25**, 45-71 (2004).

7. Vadlamudi, R. K. *et al.* Novel estrogen receptor coactivator PELP1/MNAR gene and ERbeta expression in salivary duct adenocarcinoma: potential therapeutic targets. *Hum. Pathol.* **36**, 670-675 (2005).

8. Vadlamudi, R. K. & Kumar, R. Functional and biological properties of the nuclear receptor coregulator PELP1/MNAR. *Nucl. Recept. Signal.* **5.** e004 (2007).

9. Harrington, W. R. *et al.* Estrogen dendrimer conjugates that preferentially activate extranuclear, nongenomic versus genomic pathways of estrogen action. *Mol. Endocrinol.* **20**, 491-502 (2006).

10. Summy, J. M. & Gallick, G. E. Treatment for advanced tumors: SRC reclaims center stage. *Clin. Cancer Res.* **12**, 1398-1401 (2006).

Appendix 1

2009 AACR Annual Meeting
April 18-22, 2009
Denver, CO

Print this Page for Your Records Close Window

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

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 through 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)

2009 AACR Annual Meeting
April 18-22, 2009
Denver, CO

Citation Format: {Authors}. {Abstract title} [abstract]. In: Proceedings of the 100th Annual Meeting of the American Association for Cancer Research; 2009 Apr 18-22; Denver, CO. Philadelphia (PA): AACR; 2009. Abstract nr {Abstract number}

Disclosure Information for CME-Designated Sessions

OASIS - Online Abstract Submission and Invitation System™ ©1996-2009, Coe-Truman Technologies, Inc.

*Manuscript
Click here to view linked References

Regulation of aromatase induction by nuclear receptor coregulator PELP1☆

Ratna K. Vadlamudi [a,]*, Rajib Rajhans[a], Dimple Chakravarty[a], Binoj C. Nair[a], Sujit S. Nair [a], Dean B. Evans [b], Shiuan Chen[c], Rajeshwar Rao Tekmal[a]

[a] Department of Obstetrics and Gynecology, and CTRC, San Antonio; [b]Novartis Pharma AG, Basel, Switzerland; [c]Division of Surgical Research, Beckman Research Institute, City of Hope, Durate, CA.

Key Words: Estrogen, Estrogen receptor, Coregulators, Aromatase, ERRα, PELP1, Breast cancer, epigenetics

☆ Presented at Ninth International Aromatase Conference: IAC 2008 (Shanghai, China, 13-16 October 2008)

*Corresponding author. Tel. 1 210 567 4930; Fax:1 210 567 4958

E-mail address: Vadlamudi@uthscsa.edu

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 upregulated 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 ERRα, 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.

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 (nongenomic 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 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 postmenopausal 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 postmenopausal 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 crosstalk [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 coactivator 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 nongenomic 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 (Figure 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-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/PII promoter and such deregulation could contribute to local E2 synthesis.

7. 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 crosstalk 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 1.3/II promoters under conditions of growth factor deregulation (Fig. 3).

8. 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].

9. 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.

10. 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 AroI.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 it expression may be deregulated in endometriosis.

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

12. 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]*.

13. 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 interacts

with histones and histone modifying enzymes, may play a role in chromatin remodeling at *aromatase* promoter (Fig. 3)

14. 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.

15. 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, 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).

16. 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, ERRα), 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 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.

Acknowledgments

Support for this research was provided by grants CA0095681 (R.K.V), W81XWH-08-1-0604 (R.K.V), CA75018 (R.R.T) and CTRC pilot grant P30CA54174.

Reference List

1. B.Moy, P.E.Goss, Estrogen receptor pathway: resistance to endocrine therapy and new therapeutic approaches, Clin. Cancer Res. 12 (2006) 4790-4793.

2. R.Losel, M.Wehling, Nongenomic actions of steroid hormones, Nat. Rev. Mol. Cell Biol. 4 (2003) 46-56.

3. L.Bjornstrom, M.Sjoberg, Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes, Mol. Endocrinol. 19 (2005) 833-842.

4. D.M.Lonard, B.W.O'Malley, The expanding cosmos of nuclear receptor coactivators, Cell. 125 (2006) 411-414.

5. T.N.Collingwood, F.D.Urnov, A.P.Wolffe, Nuclear receptors: coactivators, corepressors and chromatin remodeling in the control of transcription, J. Mol. Endocrinol. 23 (1999) 255-275.

6. N.J.McKenna, R.B.Lanz, B.W.O'Malley, Nuclear receptor coregulators: cellular and molecular biology, Endocr. Rev. 20 (1999) 321-344.

7. M.G.Rosenfeld, V.V.Lunyak, C.K.Glass, Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response, Genes Dev. 20 (2006) 1405-1428.

8. B.W.O'Malley, Molecular biology. Little molecules with big goals, Science. 313 (2006) 1749-1750.

9. B.W.O'Malley, Coregulators: from whence came these "master genes", Mol. Endocrinol. 21 (2007) 1009-13.

10. D.M.Lonard, R.B.Lanz, B.W.O'Malley, Nuclear receptor coregulators and human disease, Endocr. Rev. 28 (2007) 575-587.

11. E.R.Simpson, M.S.Mahendroo, G.D.Means, M.W.Kilgore, M.M.Hinshelwood, S.Graham-Lorence, B.Amarneh, Y.Ito, C.R.Fisher, M.D.Michael, Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis, Endocr. Rev. 15 (1994) 342-355.

12. Q.Lu, J.Nakmura, A.Savinov, W.Yue, J.Weisz, D.J.Dabbs, G.Wolz, A.Brodie, Expression of aromatase protein and messenger ribonucleic acid in tumor epithelial cells and evidence of functional significance of locally produced estrogen in human breast cancers, Endocrinology. 137 (1996) 3061-3068.

13. V.H.James, J.M.McNeill, L.C.Lai, C.J.Newton, M.W.Ghilchik, M.J.Reed, Aromatase activity in normal breast and breast tumor tissues: in vivo and in vitro studies, Steroids. 50 (1987) 269-279.

14. S.E.Bulun, S.Sebastian, K.Takayama, T.Suzuki, H.Sasano, M.Shozu, The human CYP19 (aromatase P450) gene: update on physiologic roles and genomic organization of promoters, J. Steroid Biochem. Mol. Biol. 86 (2003) 219-224.

15. T.Utsumi, N.Harada, M.Maruta, Y.Takagi, Presence of alternatively spliced transcripts of aromatase gene in human breast cancer, J. Clin. Endocrinol. Metab. 81 (1996) 2344-2349.

16. C.Zhou, D.Zhou, J.Esteban, J.Murai, P.K.Siiteri, S.Wilczynski, S.Chen, Aromatase gene expression and its exon I usage in human breast tumors. Detection of aromatase messenger RNA by reverse transcription-polymerase chain reaction, J. Steroid Biochem. Mol. Biol. 59 (1996) 163-171.

17. S.E.Bulun, D.Chen, M.Lu, H.Zhao, Y.Cheng, M.Demura, B.Yilmaz, R.Martin, H.Utsunomiya, S.Thung, E.Su, E.Marsh, A.Hakim, P.Yin, H.Ishikawa, S.Amin, G.Imir, B.Gurates, E.Attar, S.Reierstad, J.Innes, Z.Lin, Aromatase excess in cancers of breast, endometrium and ovary, J. Steroid Biochem. Mol. Biol. 106 (2007) 81-96.

18. V.G.Kaklamani, W.J.Gradishar, Adjuvant therapy of breast cancer, Cancer Invest. 23 (2005) 548-560.

19. A.E.Gururaj, S.K.Rayala, R.K.Vadlamudi, R.Kumar, Novel mechanisms of resistance to endocrine therapy: genomic and nongenomic considerations, Clin. Cancer Res. 12 (2006) 1001s-1007s.

20. R.Schiff, S.A.Massarweh, J.Shou, L.Bharwani, G.Arpino, M.Rimawi, C.K.Osborne, Advanced concepts in estrogen receptor biology and breast cancer endocrine resistance: implicated role of growth factor signaling and estrogen receptor coregulators, Cancer Chemother. Pharmacol. 56 Suppl 1:10-20. (2005) 10-20.

21. I.Shin, T.Miller, C.L.Arteaga, ErbB receptor signaling and therapeutic resistance to aromatase inhibitors, Clin. Cancer Res. 12 (2006) 1008s-1012s.

22. K.Subbaramaiah, L.R.Howe, E.R.Port, E.Brogi, J.Fishman, C.H.Liu, T.Hla, C.Hudis, A.J.Dannenberg, HER-2/neu status is a determinant of mammary aromatase activity in vivo: evidence for a cyclooxygenase-2-dependent mechanism, Cancer Res. 66 (2006) 5504-5511.

23. G.Sabnis, O.Goloubeva, D.Jelovac, A.Schayowitz, A.Brodie, Inhibition of the phosphatidylinositol 3-kinase/Akt pathway improves response of long-term estrogen-deprived breast cancer xenografts to antiestrogens, Clin. Cancer Res. 13 (2007) 2751-2757.

24. R.K.Vadlamudi, R.A.Wang, A.Mazumdar, Y.Kim, J.Shin, A.Sahin, R.Kumar, Molecular cloning and characterization of PELP1, a novel human coregulator of estrogen receptor alpha, J. Biol. Chem. 276 (2001) 38272-38279.

25. C.W.Wong, C.McNally, E.Nickbarg, B.S.Komm, B.J.Cheskis, Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with Src/Erk phosphorylation cascade, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 14783-14788.

26. Y.B.Choi, J.K.Ko, J.Shin, The transcriptional corepressor, PELP1, recruits HDAC2 and masks histones using two separate domains, J. Biol. Chem. 279 (2004) 50930-50941.

27. S.S.Nair, S.K.Mishra, Z.Yang, S.Balasenthil, R.Kumar, R.K.Vadlamudi, Potential role of a novel transcriptional coactivator PELP1 in histone H1 displacement in cancer cells, Cancer Res. 64 (2004) 6416-6423.

28. R.K.Vadlamudi, R.Kumar, Functional and biological properties of the nuclear receptor coregulator PELP1/MNAR, Nucl. Recept. Signal. 5:e004. (2007) e004.

29. A.Rosendorff, S.Sakakibara, S.Lu, E.Kieff, Y.Xuan, A.DiBacco, Y.Shi, Y.Shi, G.Gill, NXP-2 association with SUMO-2 depends on lysines required for transcriptional repression, Proc Natl. Acad. Sci. U. S. A. 103 (2006) 5308-5313.

30. R.K.Vadlamudi, B.Manavathi, S.Balasenthil, S.S.Nair, Z.Yang, A.A.Sahin, R.Kumar, Functional implications of altered subcellular localization of PELP1 in breast cancer cells, Cancer Res. 65 (2005) 7724-7732.

31. J.G.Greger, N.Fursov, N.Cooch, S.McLarney, L.P.Freedman, D.P.Edwards, B.J.Cheskis, Phosphorylation of MNAR promotes estrogen activation of phosphatidylinositol 3-kinase, Mol. Cell Biol. 27 (2007) 1904-1913.

32. S.Balasenthil, R.K.Vadlamudi, Functional interactions between the estrogen receptor coactivator PELP1/MNAR and retinoblastoma protein, J. Biol. Chem. 278 (2003) 22119-22127.

33. R.K.Vadlamudi, R.Bagheri-Yarmand, Z.Yang, S.Balasenthil, D.Nguyen, A.A.Sahin, H.P.den, R.Kumar, Dynein light chain 1, a p21-activated kinase 1-interacting substrate, promotes cancerous phenotypes, Cancer Cell. 5 (2004) 575-585.

34. R.K.Vadlamudi, S.Balasenthil, R.R.Broaddus, J.A.Gustafsson, R.Kumar, Deregulation of estrogen receptor coactivator proline-, glutamic acid-, and leucine-rich protein-1/modulator of nongenomic activity of estrogen receptor in human endometrial tumors, J. Clin. Endocrinol. Metab 89 (2004) 6130-6138.

35. C.Dimple, S.S.Nair, R.Rajhans, P.R.Pitcheswara, J.Liu, S.Balasenthil, X.F.Le, M.E.Burow, N.Auersperg, R.R.Tekmal, R.R.Broaddus, R.K.Vadlamudi, Role of PELP1/MNAR signaling in ovarian tumorigenesis, Cancer Res. 68 (2008) 4902-4909.

36. R.Rajhans, S.Nair, A.H.Holden, R.Kumar, R.R.Tekmal, R.K.Vadlamudi, Oncogenic Potential of the Nuclear Receptor Coregulator Proline-, Glutamic Acid-, Leucine-Rich Protein 1/Modulator of the Nongenomic Actions of the Estrogen Receptor, Cancer Res. 67 (2007) 5505-5512.

37. P.K.Marcom, C.Isaacs, L.Harris, Z.W.Wong, A.Kommarreddy, N.Novielli, G.Mann, Y.Tao, M.J.Ellis, The combination of letrozole and trastuzumab as first or second-line biological therapy produces durable responses in a subset of HER2 positive and ER positive advanced breast cancers, Breast Cancer Res Treat. 102 (2007) 43-49.

38. J.Shou, S.Massarweh, C.K.Osborne, A.E.Wakeling, S.Ali, H.Weiss, R.Schiff, Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer, J. Natl. Cancer Inst. 96 (2004) 926-935.

39. B.Manavathi, S.S.Nair, R.A.Wang, R.Kumar, R.K.Vadlamudi, Proline-, glutamic acid-, and leucine-rich protein-1 is essential in growth factor regulation of signal transducers and activators of transcription 3 activation, Cancer Res. 65 (2005) 5571-5577.

40. R.Rajhans, H.B.Nair, S.S.Nair, V.Cortez, K.Ikuko, N.B.Kirma, D.Zhou, A.E.Holden, D.W.Brann, S.Chen, R.R.Tekmal, R.K.Vadlamudi, Modulation of in situ Estrogen Synthesis by PELP1: Potential ER Autocrine Signaling Loop in Breast Cancer Cells, Mol. Endocrinol. 22 (2008) 649-64

41. J.Kitawaki, T.Noguchi, T.Amatsu, K.Maeda, K.Tsukamoto, T.Yamamoto, S.Fushiki, Y.Osawa, H.Honjo, Expression of aromatase cytochrome P450 protein and messenger ribonucleic acid in human endometriotic and adenomyotic tissues but not in normal endometrium, Biol. Reprod. 57 (1997) 514-519.

42. S.Matsuzaki, M.Canis, J.L.Pouly, P.J.Dechelotte, G.Mage, Analysis of aromatase and 17beta-hydroxysteroid dehydrogenase type 2 messenger ribonucleic acid expression in deep endometriosis and eutopic endometrium using laser capture microdissection, Fertil. Steril. 85 (2006) 308-313.

43. H.Utsunomiya, Y.H.Cheng, Z.Lin, S.Reierstad, P.Yin, E.Attar, Q.Xue, G.Imir, S.Thung, E.Trukhacheva, T.Suzuki, H.Sasano, J.J.Kim, N.Yaegashi, S.E.Bulun, Upstream stimulatory factor-2 regulates steroidogenic factor-1 expression in endometriosis, Mol. Endocrinol. 22 (2008) 904-914.

44. K.Zeitoun, K.Takayama, M.D.Michael, S.E.Bulun, Stimulation of aromatase P450 promoter (II) activity in endometriosis and its inhibition in endometrium are regulated by competitive binding of steroidogenic factor-1 and chicken ovalbumin upstream promoter transcription factor to the same cis-acting element, Mol. Endocrinol. 13 (1999) 239-253.

45. C.Yang, B.Yu, D.Zhou, S.Chen, Regulation of aromatase promoter activity in human breast tissue by nuclear receptors, Oncogene. 21 (2002) 2854-2863.

46. S.Chen, T.Itoh, K.Wu, D.Zhou, C.Yang, Transcriptional regulation of aromatase expression in human breast tissue, J. Steroid Biochem. Mol. Biol. 83 (2002) 93-99.

47. T.Kouzarides, Chromatin modifications and their function, Cell. 128 (2007) 693-705.

48. I.Garcia-Bassets, Y.S.Kwon, F.Telese, G.G.Prefontaine, K.R.Hutt, C.S.Cheng, B.G.Ju, K.A.Ohgi, J.Wang, L.Escoubet-Lozach, D.W.Rose, C.K.Glass, X.D.Fu, M.G.Rosenfeld, Histone methylation-dependent mechanisms impose ligand dependency for gene activation by nuclear receptors, Cell. 128 (2007) 505-518.

49. S.S.Nair, B.C. Nair, D.Chakravarty, R.R.Tekmal, R.K.Vadlamudi, Regulation of histone H3 methylation by ER coregulator PELP1: A novel paradigm in coregulator function. Proceedings of AACR Meeting 2009, Abstract #09-AB-3856-AACR.

50. F.Lan, A.C.Nottke, Y.Shi, Mechanisms involved in the regulation of histone lysine demethylases, Curr. Opin. Cell Biol. 20 (2008) 316-325.

Figure legends

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.

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 48h, 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 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 hours. 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 72h, reporter activity was measured. J, Total cell lysates from BG1 cells stably expressing vector or PELP1 were analyzed for aromatase expression by Western.

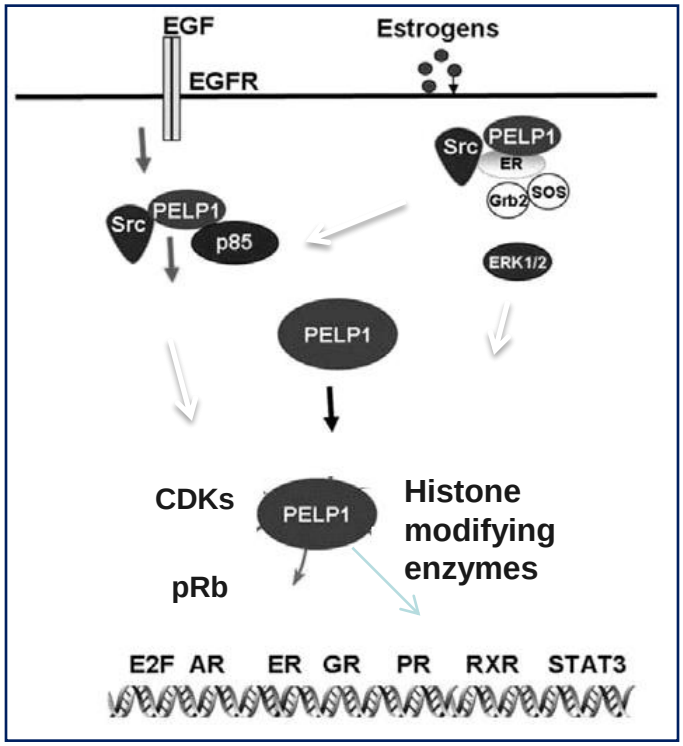
Fig. 3. Schematic representation of PELP1 regulation of *aromatase* promoter. Growth factor signals or PELP1 deregulation via overexpression promotes PELP1 recruitment to aromatase promoter (-213 to +38 region). At the aromatase promoter, PELP1 interactions with orphan receptor ERRα in conjunction with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

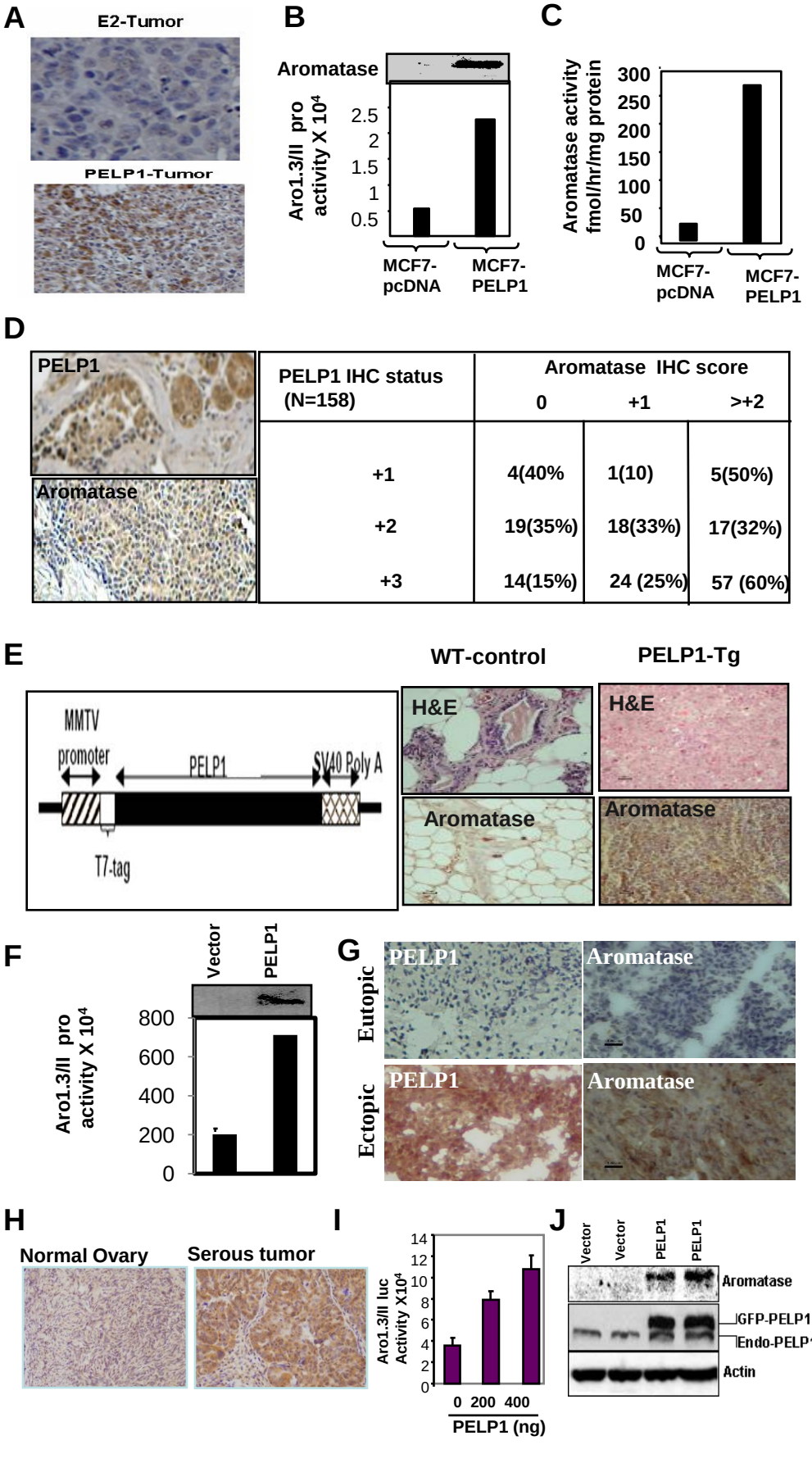
Fig. 4. PELP1 promotes epigenetic modifications at the aromatase promoter. A, Chromatin immune precipitation (ChIP) was performed using H3K9me2, K3K4me2 specific antibodies in indicated cells and the status of H3 methylation was analyzed by PCR using aromatase 1.3/PII promoter specific primers**.** B, MCF7–PELP1, MCF7-HER2 cells were cultured in a 94 well plate and treated with 3 mM of Pargyline for 72 hours. Cell viability was measured by ATP assay (Promega, Cell Titer Glo ATP assay).

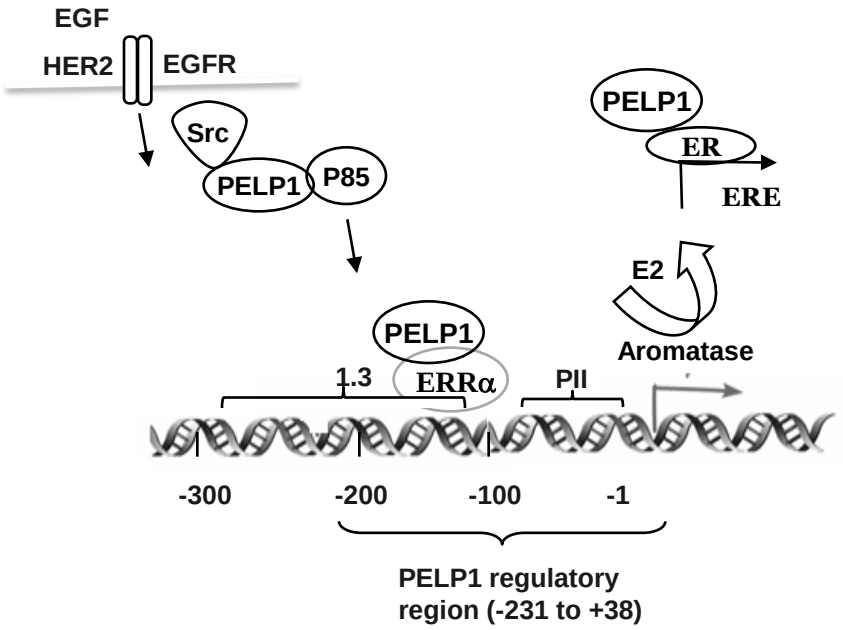
Fig. 5. Schematic representation of potential PELP1 autocrine signaling loop in cancer cells. Growth factor signaling and / or activation of non-genomic signaling pathways promotes ER coactivator PELP1 to form a complex with ERRα and PNRC2, leading to activation of

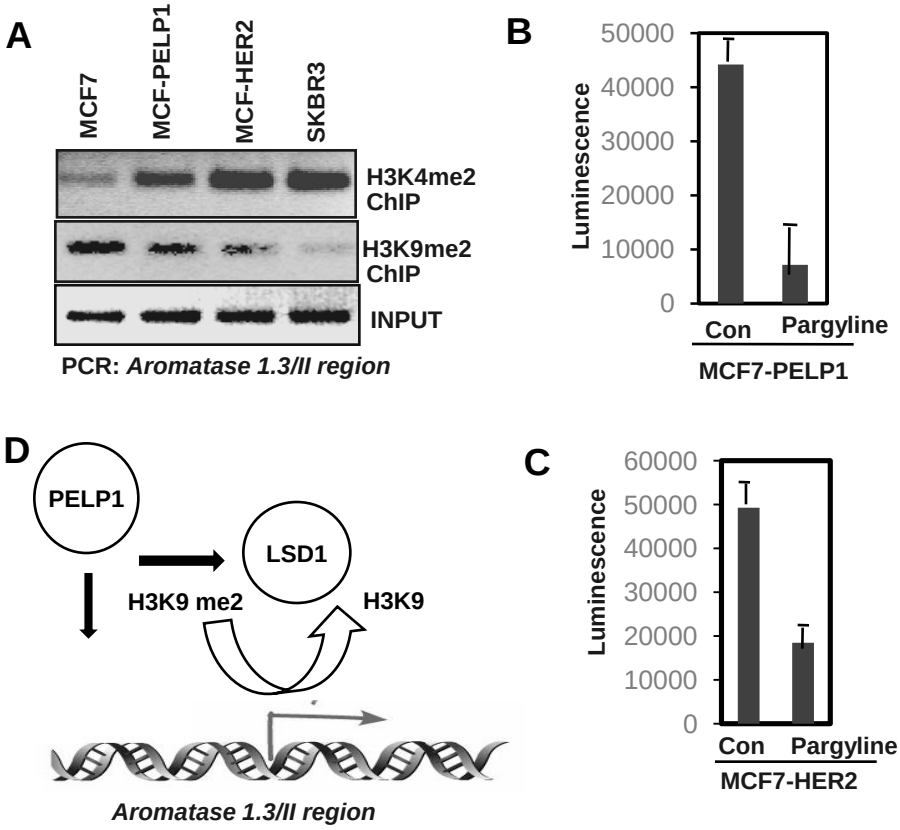
aromatase expression promoting formation of a positive feedback loop locally synthesizing E2, results in the activation of E2-ER-PELP1 signaling in breast cancer cells .

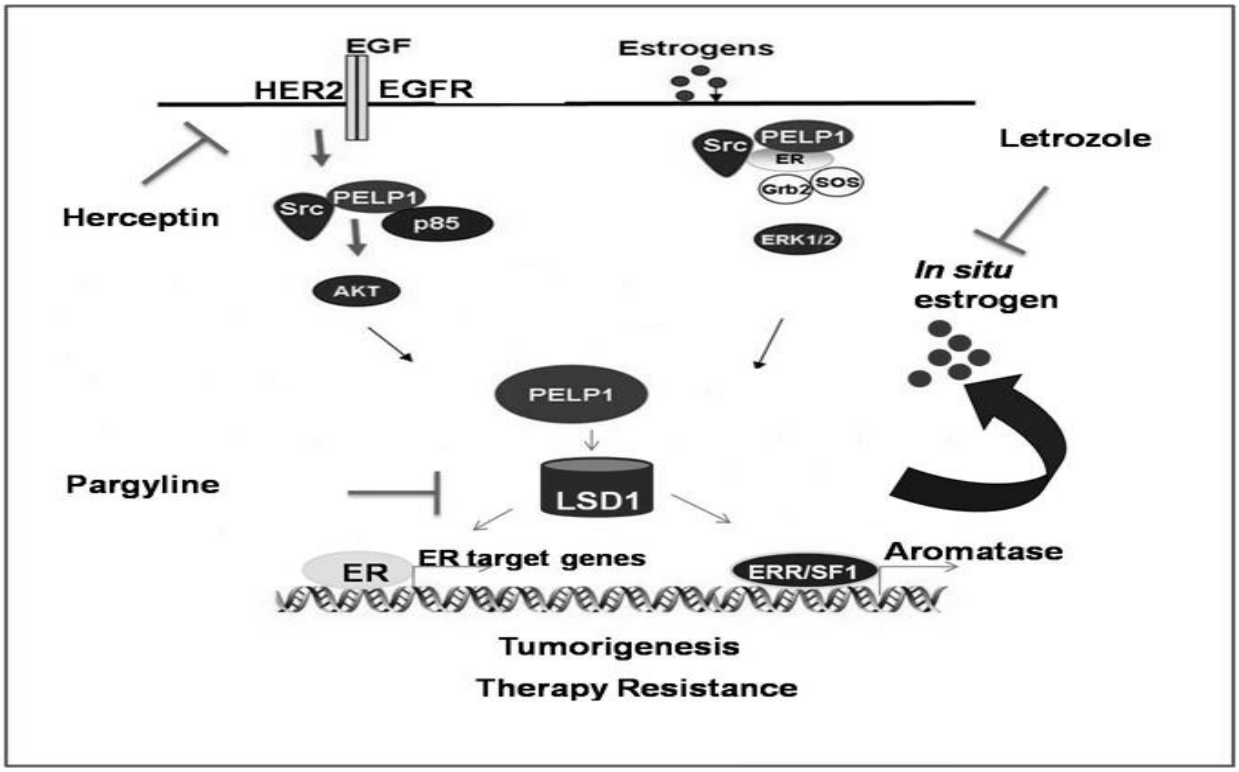
.


EGF
Estrogens
EGFR
Src
PELP1
ER
Grb2
SOS
Src
PELP1
p85
ERK1/2
PELP1
CDKs
PELP1
Histone modifying enzymes
pRb
E2F AR ER GR PR RXR STAT3


A
E2-Tumor
PELP1-Tumor
B
Aromatase
Aro1.3/II pro activity X 10^4
2.5
2
1.5
1
0.5
MCF7-pcDNA
MCF7-PELP1
C
Aromatase activity fmol/hr/mg protein
300
250
200
150
100
50
0
MCF7-pcDNA
MCF7-PELP1
D
PELP1
Aromatase
PELP1 IHC status (N=158)
Aromatase IHC score
0
+1
>+2
+1
4(40%
1(10)
5(50%)
+2
19(35%)
18(33%)
17(32%)
+3
14(15%)
24 (25%)
57 (60%)
E
WT-control
PELP1-Tg
MMTV
promoter
PELP1
SV40 Poly A
T7-tag
H&E
H&E
Aromatase
Aromatase
F
Vector
PELP1
Aro1.3/II pro activity X 10^4
800
600
400
200
0
G
Eutopic
Ectopic
PELP1
Aromatase
PELP1
Aromatase
H
Normal Ovary
Serous tumor
I
Aro1.3/II luc Activity X10^4
14
12
10
8
6
4
2
0
0 200 400
PELP1 (ng)
J
Vector
Vector
PELP1
PELP1
Aromatase
GFP-PELP1
Endo-PELP1
Actin


EGF
HER2
EGFR
Src
PELP1
P85
PELP1
ER
ERE
E2
Aromatase
PELP1
1.3
ERRα
PII
-300
-200
-100
-1
PELP1 regulatory
region (-231 to +38)


A
MCF7
MCF-PELP1
MCF-HER2
SKBR3
H3K4me2 ChIP
H3K9me2 ChIP
INPUT
PCR: Aromatase 1.3/II region
B
Luminescence
50000
40000
30000
20000
10000
0
Con
Pargyline
MCF7-PELP1
D
PELP1
LSD1
H3K9 me2
H3K9
Aromatase 1.3/II region
C
Luminescence
60000
50000
40000
30000
20000
10000
0
Con
Pargyline
MCF7-HER2


EGF
HER2
EGFR
Estrogens
Herceptin
Src
PELP1
p85
AKT
Src
PELP1
ER
Grb2
SOS
ERK1/2
Letrozole
In situ
estrogen
PELP1
Pargyline
LSD1
ER
ER target genes
ERR/SF1
Aromatase
Tumorigenesis
Therapy Resistance