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TITLE: Role of PELP1 in EGFR-ER Signaling Crosstalk in Ovarian Cancer Cells

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14. ABSTRACT Proline-, glutamic acid-, and leucine-rich protein (PELP)1, is a novel nuclear receptor (NR) coregulator. PELP1/MNAR serves as a scaffolding protein, participates in genomic and nongenomic functions of NRs and its expression is shown to be deregulated in hormonally responsive cancers. However little is known about PELP1 role in ovarian cancer progression. To examine the significance of PELP1 in ovarian cancer progression, we have generated model cells that overexpress PELP1 (IOSE-PELP1 and BG-1 PELP1) and ovarian cancer cells in which PELP1 expression is down regulated by stable expression of PELP1 specific shRNA (OVCAR3-PELP1-shRNA and SKOV3-shRNA). PELP1 overexpression in IOSE and BG1 model cells resulted in alterations in cell morphology with increased F-Actin containing structures including ruffles and filopodia. Analysis of cellular signalling pathways using phospho-specific antibodies revealed constitutive activation of c-Src kinase and increased phosphorylation of estrogen receptor. The expression of PELP1-shRNA in OVCAR3 cells dramatically decreased endogenous PELP1 expression and showed defects in cytoskeletal reorganization upon growth factor stimulation and exhibited low proliferation rate in invitro cell culture assays and invivo nude mice assays. Collectively these results suggest that PELP1 play a role in ovarian cancer cell proliferation and migration, and its expression is deregulated in ovarian carcinomas.					
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Award Number:W81XWH-06-1-0398

Project Period: March 15, 2006-April 14, 2008

Title: Role of PELP1 in EGFR-ER signaling Crosstalk in ovarian cancer cells

PI: Ratna K Vadlamudi

REPORT PERIOD: March 15, 2006-Mar 14, 2007

INTRODUCTION

A. Background: Ovarian cancer is one of the most common causes of death among gynecologic cancers and majority of the ovarian cancers are derived from the ovarian surface epithelium (1). Despite the epidemiological evidence supporting a role of estrogen in the ovarian cancer progression, the response to hormonal therapy using selective estrogen receptor modulators (SERMs) is only observed in 10- 15% of cases. In addition to hormonal signaling, deregulated epidermal growth factor receptor (EGFR) signaling and constitutive activation of cytosolic pathways (Src, PI3K, AKT) are both implicated in the development and progression of ovarian cancer(2). Emerging evidence suggests that complex interactions occur between ER and EGFR signaling components. Such crosstalk may contribute to the resistance to endocrine therapies. Recently our laboratory cloned a novel ER coactivator protein named proline glutamic acid and leucine-rich protein 1 (PELP1) (3). PELP1 is a novel scaffolding protein that has potential binding sites for several key molecules involved in the ovarian cancer progression including estrogen Receptor (ER), Epidermal growth factor receptor (EGFR), c-Src kinase , PI3K and therefore it is a potential candidate for the signaling cross talk in ovarian cancer cells (4). Our preliminary studies revealed deregulation of PELP1 in ovarian tumors. As PELP1 interacts with and activates proto-oncogenes such as ER, Src, PI3K, and EGFR, and because its expression is deregulated in ovarian cancers, **we hypothesize that PELP1 is a proto-oncogene, and that its over-expression and/or altered localization promotes excessive EGFR–ER signaling crosstalk, leading to proliferation and hormonal independence of ovarian cancer cells**

BODY

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

- Task 1.** To develop PELP1 model cell lines that overexpress or underexpress PELP1
- Task2.** To analyze the molecular mechanism of PELP1 crosstalk with ER-EGFR
- Task 3.** To analyze the role of PELP1 in ovarian tumorigenesis

Generation and characterization of OVCAR3-PELP1 shRNA cells. To examine the function of endogenous PELP1 in ovarian cancer cells we have utilized shRNA vectors. Initially using transient transfection assay, we have screened four shRNAs and identified two shRNAs that promote >80% reduction in the endogenous PELP1 levels. As a control, OVCAR3 cells were transfected with shRNA vector. OVCAR3 cells were transfected with control shRNA or PELP1 specific shRNA vectors. PELP1-shRNA cells showed distinct morphology compared to OVCAR3 parental and OVCAR3-vector expressing cells.

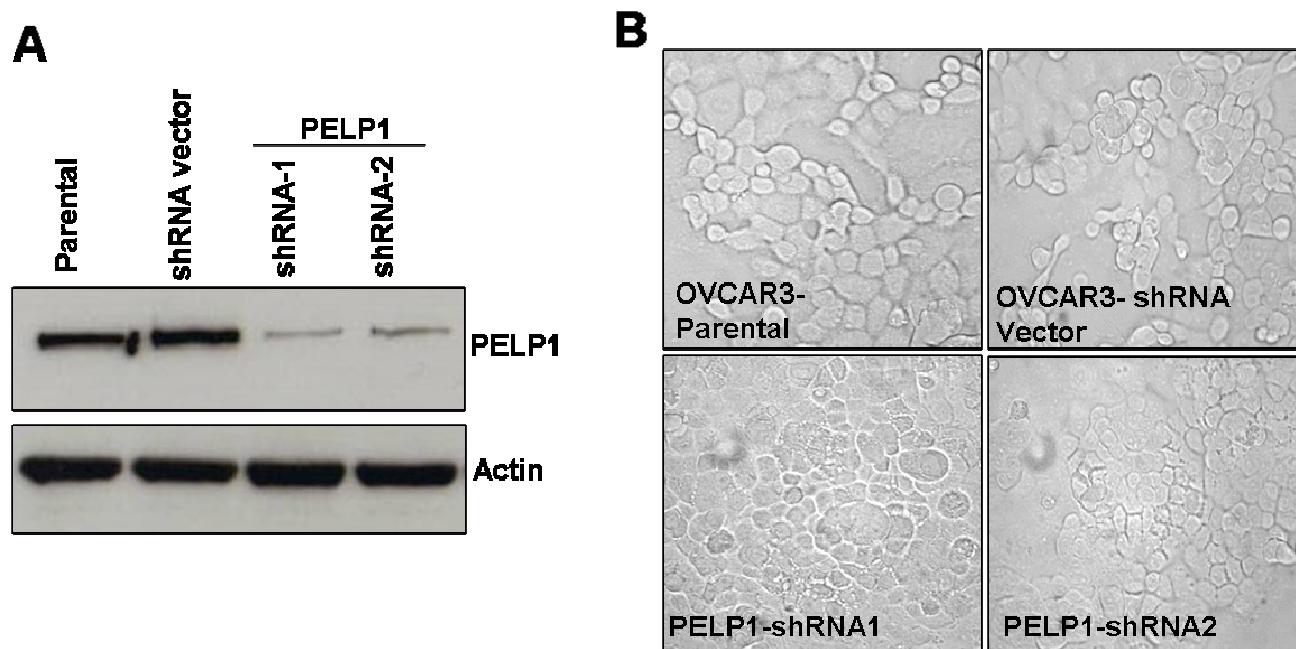


Figure 1. A. OVCAR3 cells, OVCAR3-vector and OVCAR3 cells stably expressing PELP1 shRNA Vectors (1 and 2) were lysed in RIPA buffer and PELP1 expression in these clones was analyzed by Western Blotting. **B.** Morphology of OVCAR3 cells expressing shRNA vector or PELP1 shRNA clones was analyzed by phase contrast microscopy.

Down regulation of PELP1 in OVCAR3 cells affects non-genomic signaling and cell proliferation in OVCAR3 cells. To examine the possibility that PELP1 has a role in the activation of nongenomic signaling pathways in ovarian cells, we measured activation of known nongenomic signaling pathways that are activated by PELP1. Total cell lysates from OVCAR3 stable cells expressing either vector or PELP1-shRNA were analyzed by western analysis using phospho specific antibodies. PELP1-shRNA expressing cells showed significantly less Src, AKT and MAPK activation compared to pcDNA transfected cells (Fig. 2A). To further analyze the role of PELP1 on proliferation of OVCAR3 cells, we have measured proliferation rate of these clones under low and high serum conditions. Results show that PELP1 shRNA cells showed decrease in proliferation

compared to control and PELP1 effect is more pronounced in low serum conditions compared to high serum (Fig. 2B).

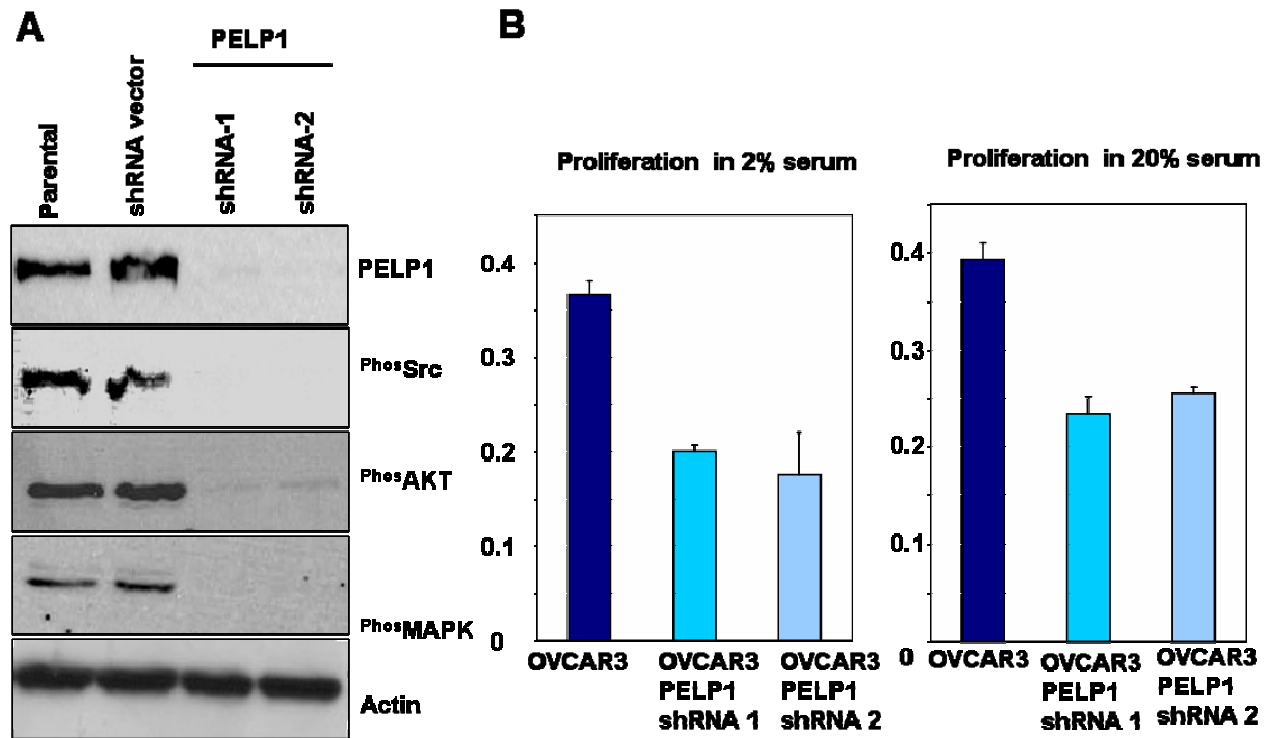


Figure 2. A. Activation of signaling pathways in OVCAR3 cells expressing PELP1-shRNA was analyzed by western analysis using phospho-specific antibodies. **B.** OVCAR3 and OVCAR3-PELP1 shRNA clones were cultured either in 2% or 20 % serum containing medium and cell proliferation was measured by MTT assay.

Generation and characterization of IOSE-PELP1 model cells. Our initial experiments to generate IOSE stable cells were failed because of the limitation on the cell passage in these primary cell lines. PELP1 transformation into IOSE cells did not extend their life span to more than 20-25 generations. To overcome these problems, we will have used Amaxa's Nucleofector transfection kit specifically designed for transfecting primary cells. To enable the monitoring of the transfected cells, we have used GFP epitope tagged PELP1 expression vector in these assays and GFP-vector was used as a control. The expression of PELP1 in transfected IOSE cells was analyzed by Western analysis (Fig. 3A) and by fluorescence microscopy (Fig. 3B). GFP vector showed localization primarily in the cytoplasm, however PELP1 showed cytoplasmic and nuclear localization. This Amaxa's Nucleofector transfection typically resulted in transfection of >80% IOSE cells and generated IOSE model cells that over express 2-3 fold PELP1. We have used Amaxa's Nucleofector transfection for testing effect of PELP1 deregulation on these primary cells in transient transfection assays. The status of nongenomic signaling in GFP and GFP-PELP1 expressing cells was then analyzed by western

analysis using phospho specific antibodies. The results showed that PELP1 over expression substantially increased Src, AKT and MAPK signaling in IOSE model cells (Fig. 3C). Similarly, PELP1 overexpressing IOSE cells exhibited increased anchorage independence in soft agar colony assays

(Fig.3D)

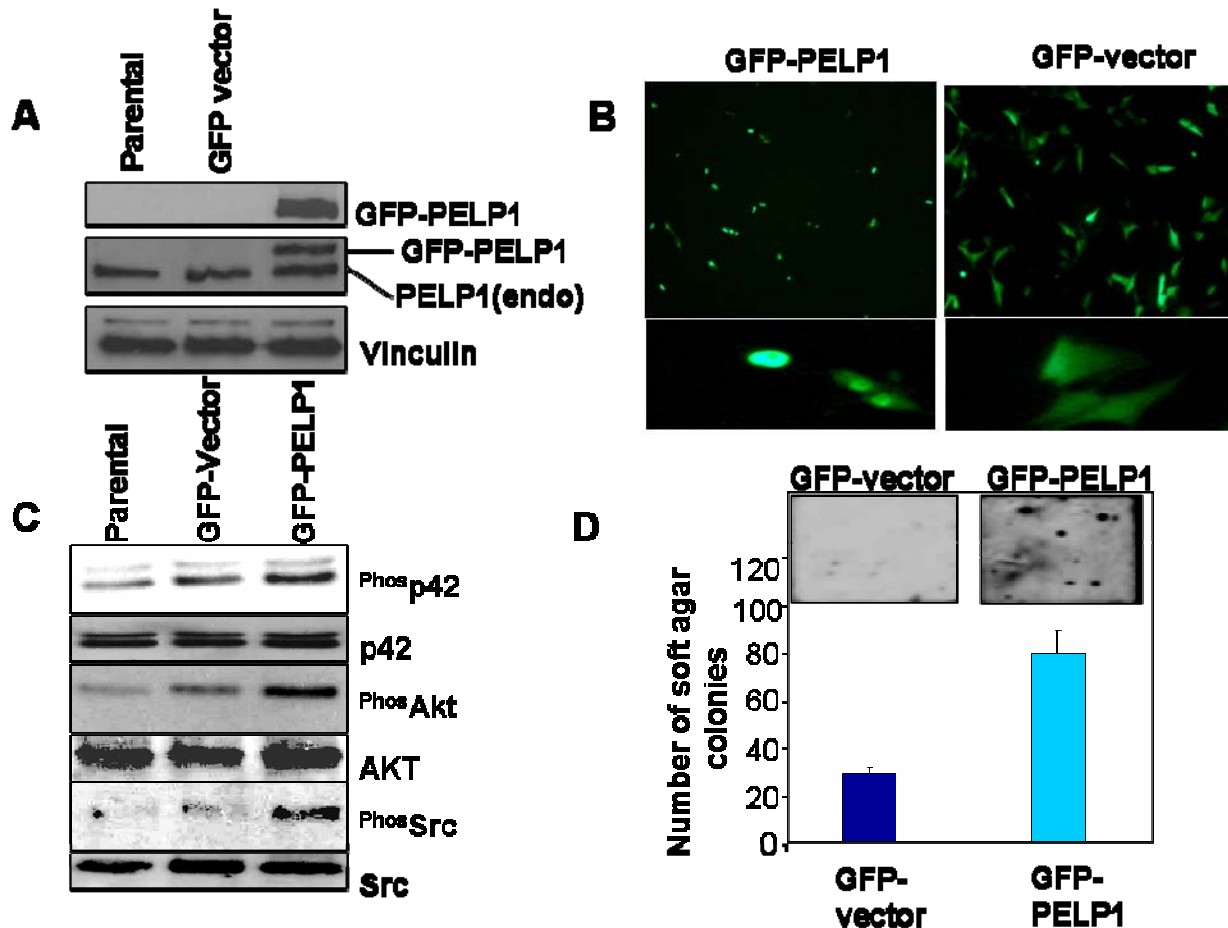


Figure 3. A, B. IOSE cells were transfected with GFP or GFP-PELP1 expression vector using Amaxa's Nucleofector kit. Expression of GFP-PELP1 protein was analyzed by (A) Western Blotting and (B) fluorescence microscopy. **C.** Activation of signaling pathways in IOSE cells-overexpressing PELP1 was analyzed by western analysis using phospho-specific antibodies. **D.** Anchorage Independence of PELP1 overexpressing IOSE cells were analyzed by softagar colony formation assays

Generation and characterization of BG1-PELP1 and SKOV3-PELP1-shRNA model cells. To confirm the signaling changes observed due to PELP1 upregulation and down regulation in IOSE and OVCAR3 cells respectively, we have generated two additional ovarian model cells: BG1 cells stably expressing PELP1 (BG1-PELP1) and SKOV3 cells expressing PELP1 shRNA. BG1-PELP1 cells

(pooled clones 1 and 2) showed three fold increases in PELP1 expression compared to vector transfected cells. Western analysis of the total protein lysates showed that PELP1 overexpression promotes increased Src, AKT and MAPK signaling. Similarly, down regulation of PELP1 in SKOV3 cells (SKOV3-shRNA 1, 2) resulted in substantial reduction of the nongenomic signaling pathways. Collectively the results from these four model cells suggest that PELP1 signaling play an essential role in Src-AKT signaling in ovarian cancer cells.

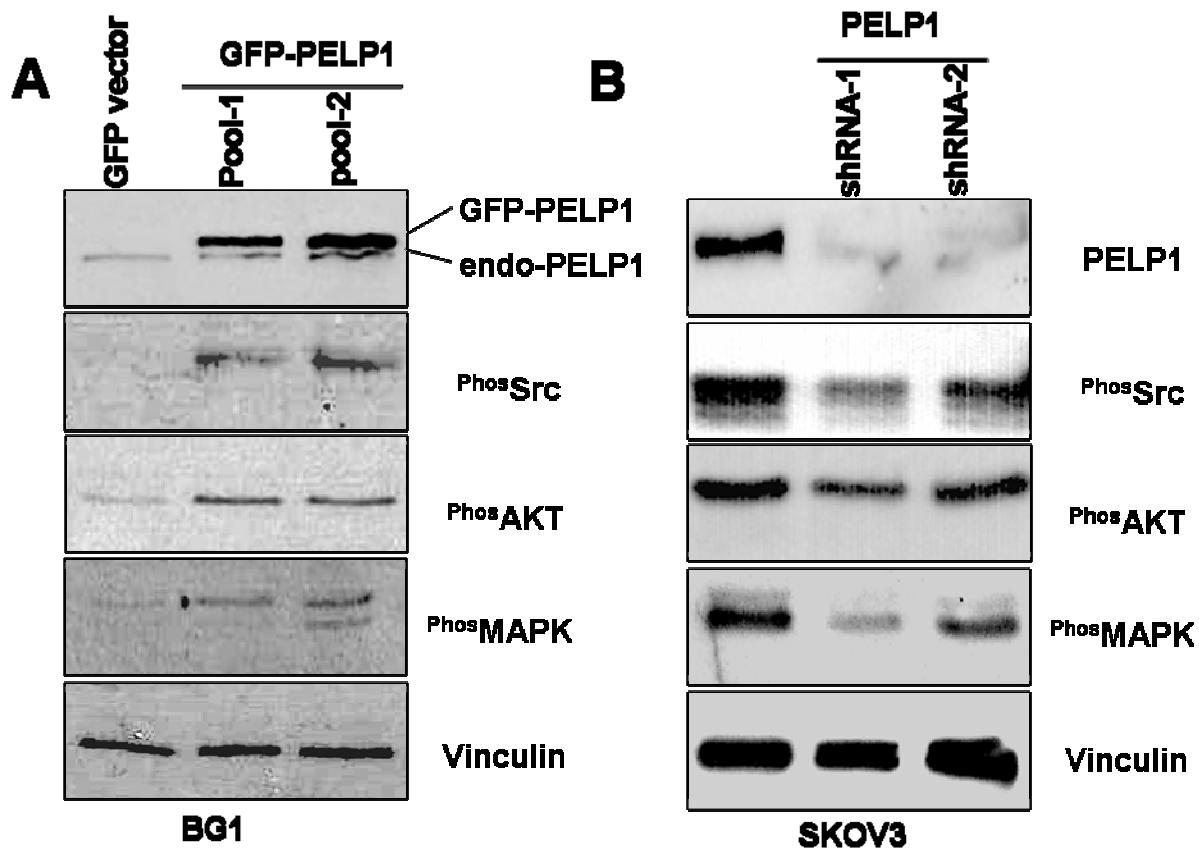


Figure 4. A, BG1 cells were transfected with GFP or GFP-PELP1 expression vector and stable cells (pooled clones) were selected. Expression of GFP-PELP1 protein and activation of signaling pathways were analyzed by western analysis using phospho-specific antibodies. **B,** SKOV3 cells expressing PELP1 shRNA1, 2 were generated by Amaxa's Nucleofector transfection and signaling was analyzed by western analysis using phospho-specific antibodies.

PELP1 down regulation affects cytoskeletal reorganization in ovarian cancer cells. Emerging studies strongly suggest that PELP1 participates in nongenomic actions by promoting ER interactions with c-Src kinase (12). Since PELP1 shRNA clones showed altered morphology and less activation of non genomic signaling pathways, we examined whether lack of PELP1 affects cytoskeleton rearrangements in ovarian cancer cells. We have used phalloidin staining and immunofluorescence microscopy to investigate whether PELP1 has a role in cytoskeletal changes induced by EGF and serum in the ovarian cells. We found that EGF treatment or serum treatment significantly increased

the formation of filamentous actin structures, including fillopodia, membrane ruffles and stress fibers in vector control cells. However, the reduction of endogenous PELP1 expression significantly affected ruffle and fillopodia formation with an increase in the formation of stress fibers/cortical actin (Fig. 5). These results suggest that PELP1 may have a role in growth factor mediated cytoskeletal changes in ovarian cancer cells.

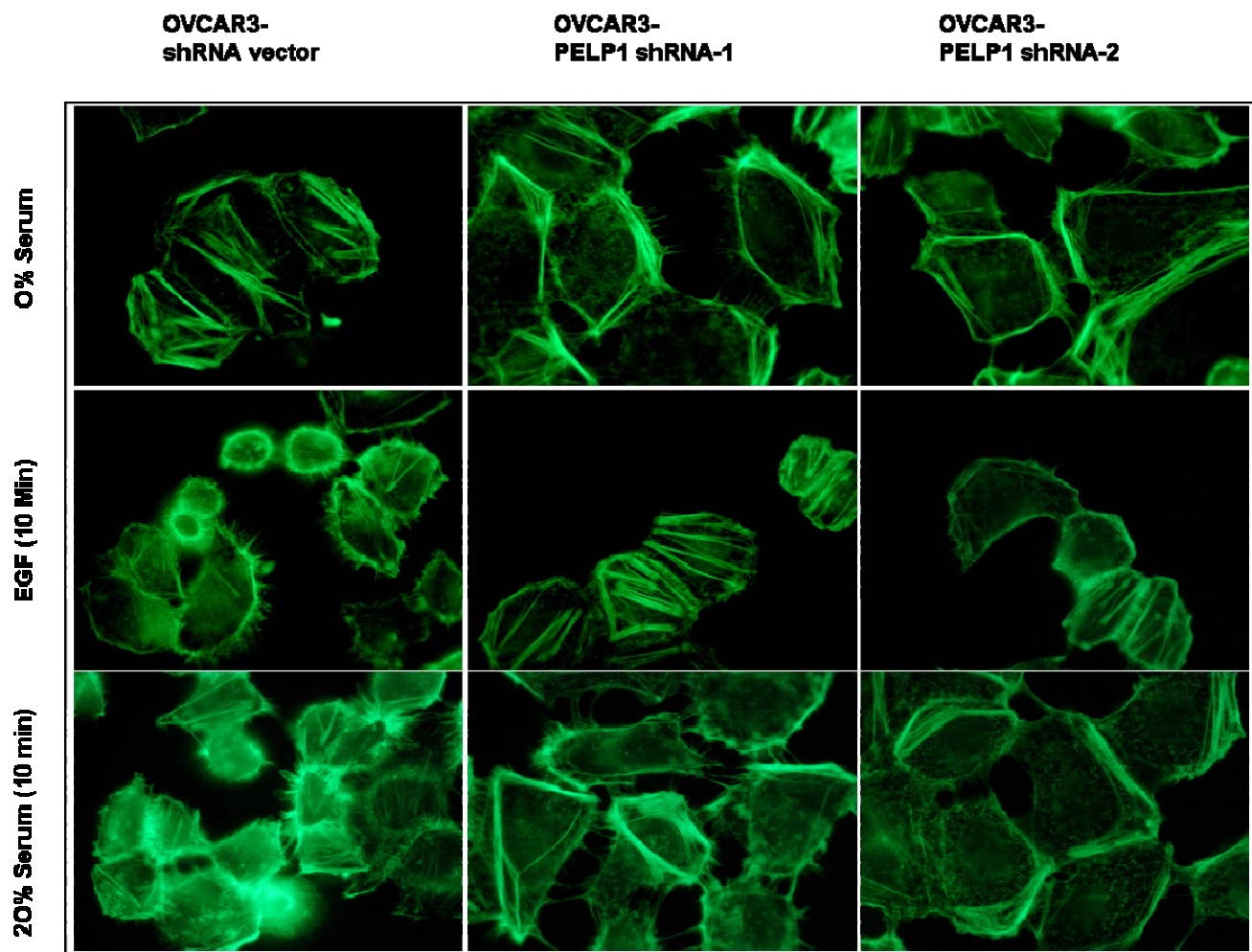


Figure 5. OVCAR3 and OVCAR3-PELP1 shRNA clones were cultured in serum free medium (0%) for 24h, after which they were treated with EGF or 20% serum for 10 minutes. The status of filamentous actin was visualized by phalloidin staining and was evaluated by fluorescence microscopy.

PELP1 down regulation decreases tumorigenic potential of OVCAR3 cells *in vivo*. We used a nude mouse xenograft model to examine whether PELP1 contributes to tumorigenic potential of OVCAR3 cells *in vivo*. OVCAR3 cells stably expressing vector or PELP1-shRNA were injected subcutaneously into the left flank of the murine subjects. Tumorigenic potential was monitored for 8 weeks (Fig. 6). Under those conditions, OVCAR3 vector transfected formed tumors and tumor grow linearly with time . However OVCAR3-PELP1shRNA injected sites showed tumors with substantial

reduction in size compared to control (Fig. 6). These results suggest that PELP1 expression is essential for optimal growth of ovarian tumor cells *in vivo*.

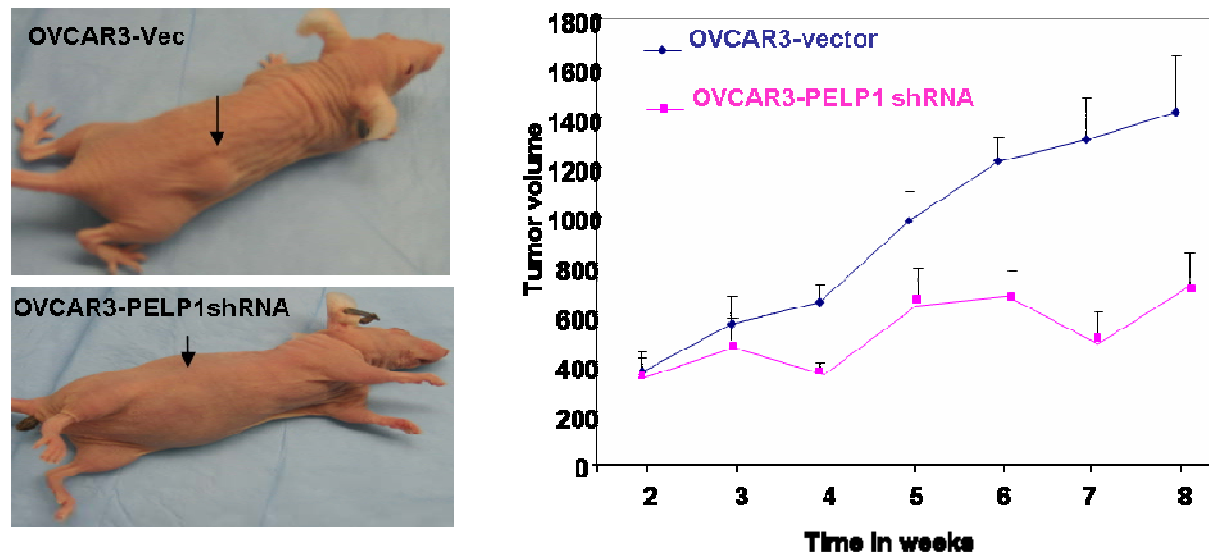


Figure 6. Nude mice were injected subcutaneously with 5×10^7 OVCAR3 (n=7) or OVCAR3-PELP1 shRNA (n=7) and tumor growth was measured at weekly intervals.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of Ovarian model cells with altered levels of PELP1 expression showed that PELP1 play an essential role the activation of c-Src, and AKT pathways in ovarian cancer model cells
- PELP1 siRNA studies suggested that PELP1 play a critical role in cytoskeletal reorganization and cell morphology
- PELP1 down regulation by shRNA decreases the tumorigenic potential of OVCAR3 cancer cells in nude mice model

REPORTABLE OUTCOMES

This study has resulted in the following publications

1. Chakravarty, D., Nair, S., Balasenthil, S., Liu, J., Tekmal, RR. Auersperg, N., Burow, M., Broaddus, R., Vadlamudi R. PELP1/MNAR in ovarian cancer: implications in tumorigenesis. American Association for Cancer Research Annual Meeting: Proceedings; 2007 Apr 14-18; Los Angeles, CA. Philadelphia (PA): AACR; 2007. Abstract # 4345.
2. Rajib R and Vadlamudi RK. Comprehensive analysis of recent biochemical and biologic findings regarding a newly discovered protein-PELP1/MNAR. Clin. Exp. Metastasis 2006;23:1-7.

CONCLUSIONS

In the first year of this study, we have generated *model* cells that overexpress PELP1 (IOSE-PELP1 and BG-1 PELP1) and ovarian cancer cells in which PELP1 expression is down regulated by stable expression of PELP1 specific shRNA (OVCAR3-PELP1-shRNA) and SKOV3-shRNA. Experimental results using these models revealed that PELP1 signaling play a role in the constitutive activation of Src-AKT pathways in ovarian cancer cells. Interestingly, PELP1 overexpressing in IOSE and BG1 model cells resulted in alterations in cell morphology with increased F-Actin containing structures including ruffles and fillopodia. The expression of PELP1-shRNA in OVCAR3 and SKOV3 cells substantially decreased endogenous PELP1 expression and showed defects in cytoskeletal reorganization upon growth factor stimulation and exhibited low proliferation rate *in vitro* and *in vivo* assays. Analysis of cellular signaling pathways using phospho-specific antibodies revealed constitutive activation of c-Src kinase and AKT. The ability of PELP1 to modulate the PI3K-AKT pathways and its potential deregulation in ovarian cancer cells suggest that PELP1-AKT pathway may represent one potential mechanism by which PELP1 promote tumorigenesis in ovarian cancer cells. Our ongoing studies in the second year will address the role of PELP1 in ovarian cell migration, cell proliferation *in vivo*, significance of PELP1 signaling on the biological functions, apoptosis, differentiation, role of PELP1 signaling in hormonal independence and mechanisms of PELP1 regulation of non genomic signaling.

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3. Vadlamudi, R.K., Wang, R., Mazumdar, A., Kim, Y., Shin, J., Sahin, A., and Kumar, R. Molecular cloning and characterization of PELP1, a novel human coregulator of Estrogen Receptor. *J. Biol. Chem.* 276: 38272-38279, 2001.
4. Rajib R and Vadlamudi RK. Comprehensive analysis of recent biochemical and biologic findings regarding a newly discovered protein-PELP1/MNAR. *Clin. Exp. Metastasis* 2006;23:1-7.

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Author Block:	<i>Dimple Chakravarty, Sujit Nair, Seetharaman Balasenthil, Jinsong Liu, Rajeshwar Rao Tekmal, Nelly Auersperg, Matthew Burow, Russel Broaddus, Ratna Vadlamudi.</i> UT Health Science Ct, San Antonio, TX, UT MD Anderson Cancer Center, San Antonio, TX, UT MD Anderson Cancer Center, Houston, TX, University of British Columbia, Vancouver, BC, Canada, Tulane University, New Orleans, LA

Emerging evidence suggests that Nuclear Receptor (NR) coregulators act as master genes and have potential to function as oncogenes. Proline-, glutamic acid-, and leucine-rich protein-1 (PELP1/MNAR) is a novel coregulator for several NRs including estrogen receptor and androgen receptor. PELP1/MNAR functions as a scaffolding protein, participates in genomic as well as nongenomic actions of NRs and couple NRs with several proteins that are implicated in oncogenesis including HER2, Src and PI3K kinases. PELP1 expression is deregulated in breast and endometrial cancers, however little is known about PELP1 role in ovarian cancer progression. Analysis of human genome databases and SAGE data suggested deregulation of PELP1 expression in ovarian cancer cells. Western analysis revealed ovarian cancer cells (SKOV3, OVCAR3) express 3 to 4 fold more PELP1 compared to normal immortalized ovarian surface epithelial cells (IOSE). To examine the significance of PELP1 in ovarian cancer progression, we have generated *model* cells that overexpress PELP1 (IOSE-PELP1 and BG-1 PELP1) and ovarian cancer cells in which PELP1 expression is down regulated by stable expression of PELP1 specific shRNA (OVCAR3-PELP1-shRNA). PELP1 overexpression in IOSE and BG1 model cells resulted in alterations in cell morphology with increased F-Actin containing structures including ruffles and filopodia. Analysis of cellular signalling pathways using phospho-specific antibodies revealed constitutive activation of c-Src kinase and increased phosphorylation of estrogen receptor. The expression of PELP1-shRNA in OVCAR3 cells dramatically decreased endogenous PELP1 expression and showed defects in cytoskeletal reorganization upon growth factor stimulation and exhibited low proliferation rate. Western analysis of PELP1 in normal and serous ovarian tumor tissues, showed 3 to 4 fold higher PELP1 expression in serous tumors compared to normal ovarian tissues. IHC studies using human ovarian

cancer tissue array (n=123), showed that PELP1 is 2 to 3 fold overexpressed in 60% of ovarian tumors. Further examination of different subtypes of ovarian tumors (including Serous, Endometrioid, Clear cell carcinoma and Mucinous tumors) suggested deregulation of PELP1 in all subtypes of ovarian cancer. Collectively these results suggest that PELP1 play a role in ovarian cancer cell proliferation and migration, and its expression is deregulated in ovarian carcinomas.

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PELP1/MNAR in Ovarian Cancer: Implications in Tumorigenesis

Dimple Chakravarty¹, Sujit S Nair¹, Seetharaman Balasenthil², Jinsong Liu², Rajeshwar Rao Tekmal¹, Nelly Auersperg³, Matthew Burow⁴, Russel Broaddus², Ratna Vadlamudi¹.
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Introduction

- Emerging evidence suggests that Nuclear Receptor (NR) coregulators can act as master genes and thus have potential to function as oncogenes
- Proline-, glutamic acid-, and leucine-rich protein-1 (PELP1/MNAR) is a novel coregulator for several NRs including estrogen receptor and androgen receptor.
- PELP1/MNAR functions as a scaffolding protein, participates in genomic as well as nongenomic actions of NRs
- PELP1 couple NRs with several proteins that are implicated in oncogenesis including HER2, Src and PI3K kinases
- PELP1 expression is deregulated in breast and endometrial cancers, however little is known about the role of PELP1 in ovarian cancer progression
- In this study, we report that PELP1 expression is deregulated in ovarian tumors and this provides evidence that PELP1 plays an important role in progression of ovarian cancer

Hypothesis

We hypothesize that PELP1 is a proto-oncogene, and that its over expression promotes excessive NR signaling crosstalk via activation of Src-MAPK, PI3K-Akt pathways, leading to proliferation and hormonal independence of ovarian cancer cells.

Results

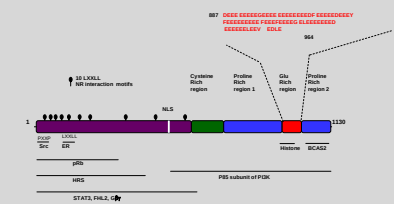


Figure 1. Schematic representation of PELP1/MNAR

PELP1 expression is upregulated in ovarian cancer cells and tumors

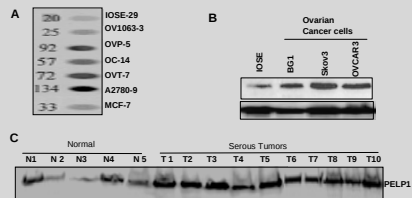


Figure 2. A. Serial analysis of gene expression (SAGE) of PELP1 in normal and ovarian cancer cells using publicly available data bases. B. Total lysates from normal and ovarian cancer cells were western blotted with PELP1 antibody. C. Total lysates from normal and Serous ovarian tumor tissues were western blotted with PELP1 specific antibody

PELP1 overexpression promotes nongenomic signaling in IOSE cells

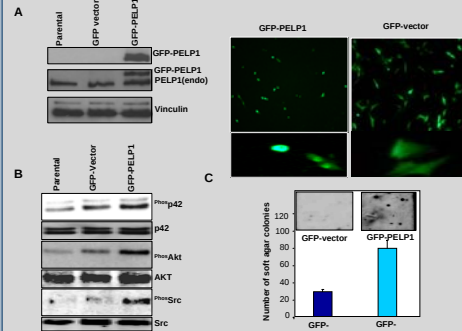


Figure 3. A. IOSE cells were transfected with GFP or GFP-PELP1 expression vector using Amara's Nucleofector kit. Expression of GFP-PELP1 protein was analyzed by Western Blotting and fluorescence microscopy. B. Activation of signaling pathways in IOSE cells-overexpressing PELP1 was analyzed by western analysis using phospho-specific antibodies. C. Anchorage independence of PELP1 overexpressing IOSE cells were analyzed by softagar colony formation assays

PELP1 down regulation alters cell morphology and nongenomic signaling

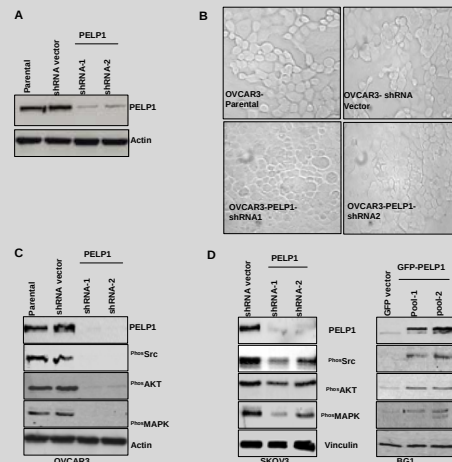


Figure 4. A. OVCAR3 cells or OVCAR3 cells stably expressing PELP1 shRNA Vectors (1 and 2) were lysed in RIPA buffer and PELP1 expression in these clones was analyzed by Western Blotting. B. Morphology of OVCAR3 cells expressing shRNA vector or PELP1 shRNA clones. C, D. Activation of signaling pathways in OVCAR3, SKOV3 and BG1 cells -overexpressing or -under expressing PELP1 was analyzed by western analysis using phospho-specific antibodies

PELP1 down regulation reduces tumorigenic potential in vivo

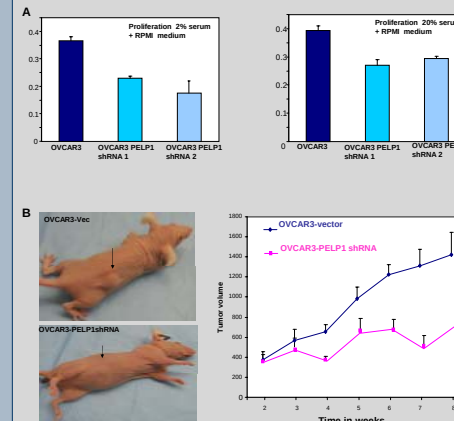


Figure 5. A. OVCAR3 and OVCAR3-PELP1 shRNA clones were cultured either in 2% or 20% serum containing medium and cell proliferation was measured by MTT assay. B. Nude mice were injected subcutaneously with 5×10^6 OVCAR3 (n=7) or OVCAR3-PELP1 shRNA (n=7) and tumor growth was measured at weekly intervals.

PELP1 expression is required for optimal cytoskeleton rearrangements

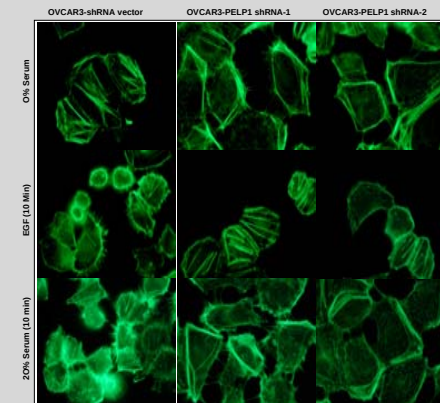


Figure 6. OVCAR3 and OVCAR3-PELP1 shRNA clones were cultured in serum free medium (0%) for 24h, after which they were treated with EGF or 20% serum for 10 minutes. The status of filamentous actin was visualized by phalloidin staining and was evaluated by fluorescence microscopy.

PELP1 expression is deregulated in human ovarian tumors

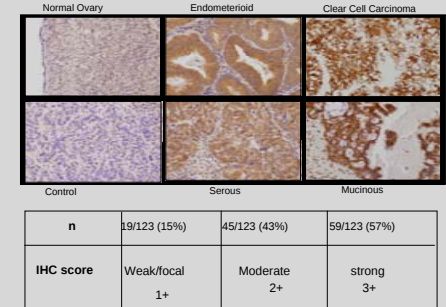
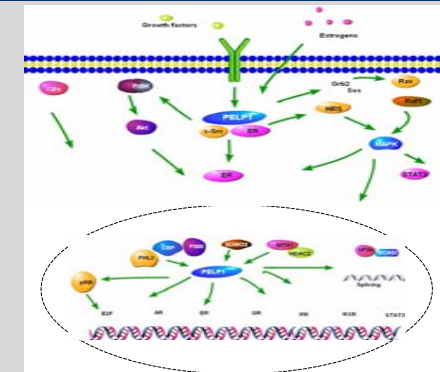


Figure 7. Summary of immunoreactive staining of PELP1 in the human ovarian array. 1-3 represents the intensity of staining: 1 weak, 2 moderate, 3 strong.

Schematic representation of PELP1/MNAR signaling pathway



Conclusions

- PELP1 expression is deregulated in ovarian cancer cells and tumors
- PELP1 plays an essential role in ovarian cancer cell proliferation
- PELP1 deregulation promotes constitutive activation of c-Src, AKT and MAPK pathways
- PELP1 siRNA studies suggested that PELP1 play a critical role in cytoskeletal reorganization
- PELP1 down regulation by shRNA decreases the tumorigenic potential of OVCAR3 cancer cells in nude mice model
- IHC studies using human ovarian cancer tissue array (n=123) showed that PELP1 is 2 to 3 fold over expressed in 60% of ovarian tumors
- The results from this study suggests that PELP1 has oncogenic potential and might play a role in ovarian cancer progression

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Comprehensive analysis of recent biochemical and biologic findings regarding a newly discovered protein-PELP1/MNAR

Rajib Rajhans · Ratna K. Vadlamudi

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Abstract Estradiol (E2) and estrogen receptor (ER) signaling have been implicated in the development and progression of several cancers. Emerging evidence suggests that the status of ER coregulators in tumor cells plays an important role in hormonal responsiveness and tumor progression. Proline, glutamic acid, and leucine-rich protein-1 (PELP1/MNAR)—a novel ER coactivator that plays an essential role in the ER's actions and its expression—is deregulated in several hormonal responsive cancers. The precise function of PELP1/MNAR in cancer progression remains unclear, but PELP1 appears to function as a scaffolding protein, coupling ER with several proteins that are implicated in oncogenesis. Emerging evidence suggests that PELP1/MNAR increases E2-mediated cell proliferation and participates in E2-mediated tumorigenesis and metastasis.

Keywords Estrogen receptor · Coregulators · PELP1/MNAR · Metastasis · Tumorigenesis

Abbreviations

AR	Androgen receptor
BCAS2	Breast Cancer amplified sequence 2
CBP	CREB binding protein
E2	Estradiol
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor

FHL2	Four and a half lim domain protein 2
HRS	Hepatocyte growth factor-regulated tyrosine kinase substrate
MAPK	Mitogen activated protein kinase
MNAR	Modulator of nongenomic actions of estrogen receptor
MTA1	Metastasis-associated protein 1
NR	Nuclear receptor
pRb	Retinoblastoma protein
PI3K	Phosphatidylinositol 3-kinase
STAT3	Signal transducer and activator of transcription
PELP1	Proline, glutamic acid, and leucine-rich protein-1

Introduction

The estrogen receptor (ER) has been implicated in the progression of several cancers, including breast, endometrium, and ovary. Although antiestrogens and aromatase inhibitors cause regression of ER-positive tumors, many patients with metastatic tumors eventually become resistant to this treatment [1]. The ER requires both a ligand and interactions with other proteins, such as coregulators, to achieve optimal transcriptional activation of target genes [2]. Several ER-coregulator proteins are differentially expressed in tumors [3]. Dysregulation of these coregulators could influence target gene expression and participate in the development of hormone-responsive cancers. ER and ER-coactivators are targets of growth factor signaling and their phosphorylation have a role in hormonal

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resistance [4]. ER-coregulators therefore play a role in hormonal therapy responsiveness, tumor progression, and metastasis [5, 6]. However, very little is known about the physiological role of ER coregulator proteins in the initiation and progression of cancer cells. The novel ER-coactivator, proline-, glutamic acid-, and leucine-rich protein-1 (PELP1, also known as modulator of nongenomic actions of estrogen receptor, MNAR) plays an important role in genomic and nongenomic ER activity, and its expression is deregulated in hormonal-dependent cancers [7–9]. In this review, we briefly summarize the emerging data on PELP1/MNAR, with particular emphasis on its role in tumorigenesis and metastasis.

PELP1/MNAR structure, expression and regulation

PELP1/MNAR encodes a protein of 1,130 amino acids, and is localized to chromosome 17p13.2. PELP1/MNAR has an unusually high number of proline, glutamic acid, and leucine residues. PELP1/MNAR has a predicted molecular weight of 120 kDa and an isoelectric point of 4.30, but because of the overall negative charge and excessive amount of prolines, the protein migrates on SDS-PAGE as a 160-kDa protein. PELP1/MNAR is normally expressed in a wide variety of tissues including brain, endometrium, mammary gland, ovary, prostate and testis [8, 10–12]. PELP1 expression is developmentally regulated in the mammary gland, is upregulated by E2-ER signaling, and differentially regulated by selective estrogen receptor modulators (SERMs) [13]. PELP1/MNAR is a phosphoprotein and bioinformatics analysis using motif scan program (http://scansite.mit.edu/cgi-bin/motif-scan_seq) revealed that PELP1/MNAR contains several potential sites for phosphorylation including 8 tyrosine kinase/phosphatase sites (recognized by EGFR, PDGFR, INSR, Src, Jak2, SHP1) and 207 serine/threonine kinase/phosphatase motifs (recognized by AKT, GSK, CDK, CK1, CK2, LKB1, MAPK, PKC, PKA, proline directed kinases,). A recent study indeed show that PELP1/MNAR is phosphorylated by protein kinase A (PKA) [14]. Another study using phosphoproteomic analysis revealed that PELP1/MNAR is phosphorylated at threonine 745 in developing brain [15]. PELP1/MNAR phosphorylation is modulated by hormones and growth factor signaling. PELP1/MNAR contains a central consensus nuclear localization site, and exhibits both cytoplasmic and nuclear localization [8]. No known enzymatic activity has been identified in PELP1/MNAR, but it may function as a scaffolding protein to couple various

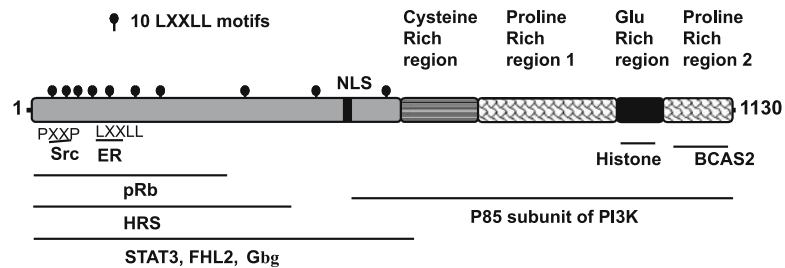
signaling complexes with ER. The primary structure of PELP1/MNAR contains several motifs/domains present in many transcriptional coactivators, including 10 nuclear receptor (NR) interacting boxes (LXXLL motifs), a zinc finger, a glutamic acid-rich domain, and 2 proline-rich domains [8, 9] (Fig. 1). Interestingly, proline-rich regions contain several consensus PXXP motifs that could interact with signaling proteins containing SH3 domains. Analysis of PELP1/MNAR primary sequence using ELM motif search program (<http://elm.eu.org>) revealed that PELP1 contained several conserved protein–protein interaction motifs including FHA, SH2, SH3, PDZ, WW, CDK, 14-3-3. Accordingly, emerging evidence suggest that PELP1/MNAR interacts with multiple signaling proteins including Src kinase, phosphatidyl inositol 3 kinase (PI3K), Signal transducer and activator of transcription-3 (STAT3), CREB-binding protein (CBP), Epidermal growth factor receptor (EGFR), Four- and half- lim-domain protein 2 (FHL2), G protein beta gamma, Retinoblastoma protein (pRb) [7–9, 16–18]. PELP1/MNAR also has an unusual stretch of 70 acidic amino acids in the C-terminus, that functions as a histone binding region [19, 20]. The proteins that interact with PELP1/MNAR (Table 1) indicate that PELP1/MNAR can couple ER with different signaling components and functions.

PELP1 cellular functions and targets

Genomic functions

PELP1/MNAR resides in the nuclear compartment of hormonally responsive tissues [8, 20]. Within the nucleus, PELP1/MNAR is present in several subcompartments, including chromatin, nucleoplasm, and nuclear matrix [20]. Hormonal stimulation promotes PELP1/MNAR recruitment to the ER target gene promoters in a dynamic manner, and PELP1/MNAR overexpression enhances ER transactivation functions in reporter gene assays [20]. Studies with siRNA targeted to PELP1/MNAR suggested that PELP1/MNAR is required for maximal activation of ER target genes [17]. PELP1 interacts with histones via the acidic C-terminal domain [19, 20]. PELP1/MNAR's affinity for histone H1 is higher than histone H3, and PELP1/MNAR plays a role in histone H1 displacement at the ER target gene promoters [20]. In addition, PELP1 interacts with the general transcriptional activator, CBP/P300, and colocalizes with acetylated histones [8, 20]. PELP1/MNAR has no intrinsic histone acetyltransferase (HAT) domain, but PELP1/MNAR

Fig. 1 Schematic representation of PELP1/MNAR domains and its interacting proteins



associated HAT activity was increased with E2 treatment. PELP1/MNAR also interacts with components of histone deacetylase complexes, including the NURD complex protein MTA1 [21], and HDAC2 [19]. Further, treatment with deacetylase inhibitors such as TSA, increases PELP1/MNAR residence time at the ER target gene promoter, suggesting that deacetylase complexes may have a role in PELP1/MNAR detainment in ER target promoters [20]. The ability to interact with histones, HAT enzymes, and histone deacetylase enzymes suggest that PELP1/MNAR may alter local chromatin structure in the vicinity of ER target promoters by coupling ER to chromatin-modifying enzymes.

Nongenomic functions

PELP1/MNAR also plays a key role in nongenomic ER activity. PELP1/MNAR modulates ER's interaction with Src, stimulating Src enzymatic activity and activation of the mitogen activated protein kinase (MAPK) pathway [9]. Mutational analysis of ER α and c-Src mutants revealed that MNAR interacts with c-Src SH3 domain via its N-terminal PXXP motif. ER interacts with Src's SH2 domain at phosphotyrosine 537, and the MNAR–ER interaction further stabilizes

this complex [22]. PELP1/MNAR also directly interacts with the p85 subunit of PI3K and enhances PI3K activity [23]. Overexpression of PELP1/MNAR in the cytoplasm, which is common in tumors, promotes constitutive activation of AKT and ER, suggesting that deregulation of PELP1/MNAR promotes constitutive activation of the PI3K-AKT pathway and phosphorylation of the ER [23]. PELP1/MNAR also enhances phosphorylation of STAT3 at Ser727 in a src-MAPK-dependent manner [16]. PELP1/MNAR regulates STAT3 transcription from synthetic promoters and endogenous target genes such as cyclin D1, c-myc, and c-fos [16]. PELP1/MNAR can facilitate ER nongenomic signaling via Src kinase, PI3K, and STAT3 in the cytosol. PELP1/MNAR regulates meiosis via its interactions with heterotrimeric G $\beta\gamma$ protein, androgen receptor (AR), and by activating Src-MAPK pathway [18]. Thus, PELP1/MNAR can regulate multiple nongenomic signals depending on the cellular context.

Cell cycle progression

PELP1/MNAR sensitizes breast cancer cells to E2 signaling, and enhances E2-mediated progression to G1 > S phase. PELP1/MNAR promotes persistent

Table 1 PELP1/MNAR interacting proteins

#	Binding protein	Binding motif or region	Putative function	Reference
1	ER	LXXLL motifs	Coactivation of ER	[8, 9]
2	CBP/P300	Not determined	Increased HAT activity	[8]
3	c-Src	PXXP motifs	Activation of Src kinase	[9, 22]
4	pRb	Amino acids 1–330	Phosphorylation of pRb	[7]
5	Histones	Amino acids 884–1130	HistoneH1 displacement	[20]
6	HDAC2	Not determined	Repression of NRs	[19]
7	STAT3	Amino acids 1–600 (YXXQ, YXXV motifs)	Ser727 phosphorylation	[16]
8	PI3K	SH3 binding sites	Activation of PI3K	[23]
9	EGFR	Not determined	EGFR–ER cross-talk	[23]
10	G $\beta\gamma$	N-terminal half	Meiosis	[18]
11	AR	LXXLL motifs	Activation of Src kinase	[27]
12	GR	LXXLL motifs	Coregulation of GR	[9]
13	HRS	Amino acids 1–400	Activation of MAPK	[25]
14	FHL2	N-terminal half	Coactivator of FHL2	[26]
15	BCAS2	Amino acids 800–1130	ER-mediated splicing	[24]
16	RXR	Amino acids 1–400	Coactivator of RXR	[28]

hyperphosphorylation of the cell cycle switch protein retinoblastoma protein (pRb) in an E2-dependent manner [7]. PELP1/MNAR interacts with pRb via its C-terminal pocket domain, and the PELP1/pRb interactions have been found to play a role in the maximal activation of E2 target genes, such as cyclin D1 [7].

Splicing

Yeast 2-hybrid screens identified Breast Cancer Amplified Sequence-2 (BCAS2), a component of the spliceosome machinery, as a novel PELP1/MNAR interacting protein, and deletion analysis mapped the interaction site to the PELP1 C-terminal 800–1130 amino acids [24]. PELP1/MNAR interacts with BCAS2 in the nuclear compartment, and colocalizes with splicing factor SC35 at nuclear speckles. PELP1/MNAR interacts with RNA and enhances steroid hormone-mediated splicing [24]. The BCAS2-PELP1/MNAR interaction may regulate ER-mediated RNA splicing and have functional implications in ER-driven breast tumors.

Growth factor–ER signaling cross-talk

EGF promotes PELP1/MNAR association with the epidermal growth factor receptor (EGFR), resulting in the tyrosine phosphorylation of PELP1/MNAR [23]. PELP1/MNAR can enhance EGF-mediated ER transactivation, and mislocalization of PELP1/MNAR in the cytoplasm can increase ER basal activity via the EGFR-PI3K signaling pathways. In a yeast 2-hybrid screen, Rayala and coworkers demonstrated the physiological interaction of hepatocyte growth factor receptor substrate (HRS) and PELP1/MNAR [25]. Interestingly, HRS sequestered PELP1/MNAR in the cytoplasm, leading to the EGFR-dependent activation of MAPK. PELP1/MNAR can interact with several growth factor signaling components and may have important functional implications in ER/growth factor cross-talk.

Nuclear receptor (NR) signaling

PELP1/MNAR is a unique ER coregulator protein that contains 10 NR interaction boxes. PELP1 also interacts with several other NRs, including androgen receptors (AR), glucocorticoid receptors, and progesterone receptors [8, 9]. PELP1/MNAR modulates AR transactivation. PELP1/MNAR expression is deregulated in higher-grade prostate tumors. PELP1/MNAR forms a trimeric complex with FHL2, an AR coactivator, upon ligand stimulation to enhance

FHL2-mediated AR transactivation [26]. PELP1/MNAR participates in nongenomic AR activity by coupling AR with src kinase signaling in prostate cancer cells [27]. PELP1/MNAR also interacts with RXR alpha to enhance transactivation in response to 9-*cis*-retinoic acid [28].

Tumorigenic potential of PELP1

The ability of PELP1/MNAR to interact with and modulate several oncogenes (including c-Src, PI3K, STAT3, EGFR, cyclin D1) suggests that PELP1/MNAR might promote tumorigenesis. In NIH3T3-based foci formation assays, PELP1/MNAR deregulation promoted cell transformation [29]. PELP1/MNAR also enhances the transformation potential of c-src and other oncogenes in foci formation assays. PELP1/MNAR promoted anchorage-independent growth of breast cancer cells in soft agar assay, whereas reduction of endogenous PELP1/MNAR by siRNA substantially reduced E2-mediated growth in soft agar [23, 29]. MCF-7 cells stably expressing PELP1 showed tumor formation in 50% of injected sites when injected subcutaneously into the mammary fat pad, in the absence of any exogenous E2 treatment, via activation of MAPK and AKT [23, 29]. These results suggest that deregulation of PELP1/MNAR might be sufficient to promote tumorigenic phenotypes. Ability of PELP1/MNAR over expressing cells to promote tumorigenesis in the absence of exogenous E2 also suggests that PELP1/MNAR deregulation might contribute to hormonal independence by promoting local estrogen synthesis. Our ongoing studies indeed suggested that PELP1/MNAR deregulation increases the expression of aromatase (Cyp 19 I.3 promoter) an enzyme that produces local estrogen (Rajib et al., unpublished observations) leading to tumorigenesis at ultra low levels of estradiol. Thus, PELP1/MNAR deregulation has tumorigenic potential and may lead to the hormonal therapy resistance seen in hormonal-dependent cancers.

Role of PELP1 in metastasis

The fact that PELP1/MNAR interacts with proteins involved in cytoskeleton remodeling (including Src kinase, PI3K, FHL2) and participates in E2-mediated nongenomic signaling pathways [3, 9, 23] suggests that it may regulate E2-mediated cell migration and have a role in metastasis. MCF10A cells, developed at the Karmanos Cancer Institute, are useful in vitro models to

examine altered gene expression during tumorigenesis. This model contains a spectrum of cell lines for examining gene expression during progression of breast malignancy [30]. Western blot analysis of cell lysates from these models cells showed increased expression of PELP1/MNAR as a function of tumorigenesis, i.e., higher expression in cells with increased metastasis potential, suggesting that PELP1/MNAR may play a role in metastasis [29]. PELP1/MNAR overexpression uniquely enhanced E2-mediated ruffles and filopodia-like structures, which regulates attachment and movement of cytoplasmic components that are responsible for cell migration [29]. Reduction of endogenous PELP1/MNAR affected E2-mediated ruffle formation and increased stress fibers in MCF-7 cells. In Boyden chamber assays, PELP1/MNAR-overexpressing MCF-7 cells increased cell motility upon E2 treatment, whereas knockdown of PELP1/MNAR by siRNA substantially reduced E2-mediated cell motility compared with control MCF-7 cells [29]. PELP1 modulates functions of metastasis tumor antigen 1 (MTA1), a protein implicated in metastasis. PELP1/MNAR also interacts with the MTA1-associated coactivator (MICOA) and promotes ER-transactivation functions in a synergistic manner [21]. The expression of metastasis tumor antigen 3 (MTA3) regulates the invasive growth of human breast cancers. The ability of PELP1/MNAR to modulate MTA3 expression and its interaction with various MTA family members suggests that its deregulation could promote metastasis [31]. Immunohistochemical analysis of PELP1/MNAR expression and localization using a tumor progression array (252 breast carcinomas and normal breast specimens) revealed that PELP1/MNAR expression is deregulated in higher-grade invasive tumors compared to normal breast and DCIS tumors [29]. Node-positive, metastatic tumors have 2- to 3-fold higher expression of PELP1/MNAR compared to node-negative breast tumors, suggesting that PELP1 expression is altered in metastatic tumors.

Expression of PELP1 in tumors

PELP1 expression and localization is deregulated in tumors. PELP1/MNAR is widely expressed in breast cancer cells [8, 12]. A comparison of PELP1/MNAR expression with 16 samples of paired normal breast and breast tumor samples revealed that breast tumors have 3- to 5-fold higher expression than normal tissues [8]. Although PELP1/MNAR is predominantly localized in the nucleus of hormonally responsive tissues, PELP1/MNAR was localized in the cytoplasm either alone or with nuclear localization in 58% of PELP1/MNAR-positive tumors [23]. Cells that mimic PELP1/MNAR

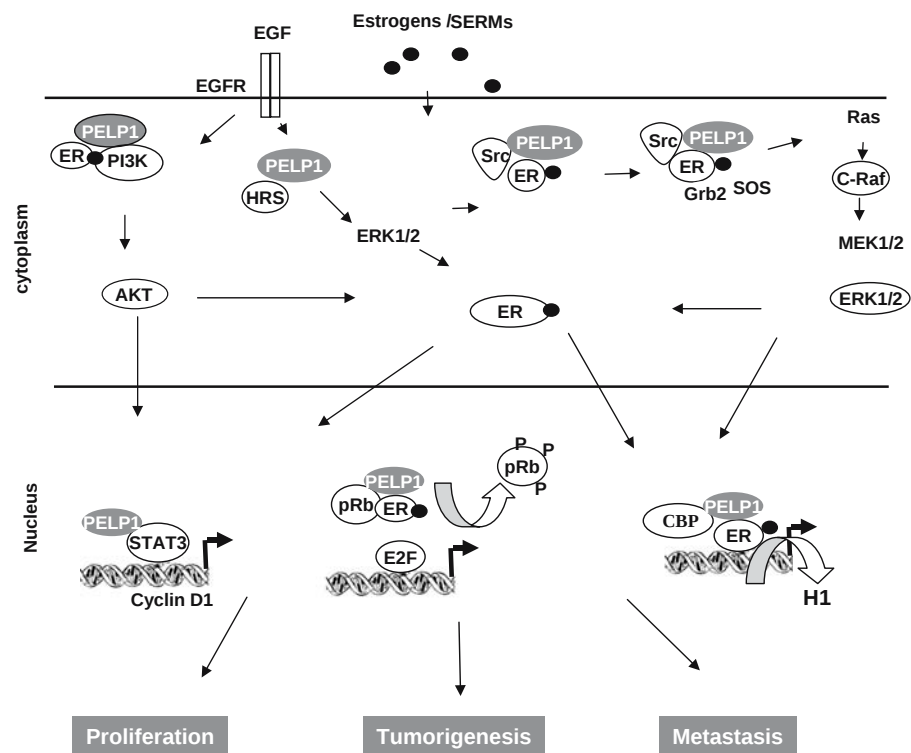
cytoplasmic localization in tumors (PELP1-cyto cells) were hypersensitive to E2 but resistant to tamoxifen. The altered localization of PELP1/MNAR to the cytoplasm was sufficient to trigger its interaction with the p85 subunit of PI3K, leading to PI3K activation [23]. Cytoplasmic PELP1/MNAR interacted with the trafficking molecule, HRS, to activate MAPK in the presence of EGFR [25]. Clones of MCF-7 human breast cancer cells overexpressing cytoplasmic PELP1/MNAR were more sensitive to TNF-alpha-induced apoptosis than wild-type nuclear PELP1- and pcDNA vector-expressing clones [32]. The results from this study suggest that altered localization of PELP1/MNAR modulates hormonal sensitivity of breast cancer cells, thus paving the way for developing new treatment strategies for tumors with cytoplasmic PELP1 expression.

PELP1/MNAR exhibits phase-dependent localization and expression in the endometrium [17]. PELP1/MNAR enhances both ER α - and ER β -mediated transcription, as well as tamoxifen-mediated partial agonist signaling, in endometrial cancer cells [17]. PELP1/MNAR expression and localization are widely deregulated in endometrial cancers. In addition, PELP1/MNAR and ER α are localized predominantly in the cytoplasm of high-grade endometrial tumors, suggesting that PELP1/MNAR deregulation may play a role in endometrial cancer progression.

Analysis of serial analysis of gene expression (SAGE) data in the human genome databases suggest that ovarian cancer cells express 3- to 4-fold more PELP1/MNAR transcripts than immortalized normal ovarian surface epithelial cells (<http://www.ncbi.nlm.nih.gov/sage>). Western analysis of PELP1 in normal and Serous ovarian cancer tissues showed that PELP1/MNAR expression is 3- to 4-fold higher in Serous tumors compared to normal ovarian tissues. Ongoing studies in our laboratory using human ovarian cancer tissue arrays revealed that PELP1 is 2- to 3-fold overexpressed in 60% of ovarian tumors. PELP1 is deregulated in several ovarian tumor subtypes, including Serous, Endometroid, Clear cell carcinoma, and Mucinous tumors, and predominantly localized in the cytoplasm in ovarian tumors (Vadlamudi et al., unpublished observations).

Salivary duct carcinoma is a high-grade neoplasm with similar morphology to mammary duct carcinoma. Interestingly, these tumors express PELP1/MNAR and ER α [33]. Immunohistochemical staining performed on 70 salivary duct carcinomas revealed strong PELP1/MNAR expression in 51 (73%) and ER β in 52 (74%) tumors. PELP1/MNAR and ER β were coexpressed in 35 (50%) tumors [33]. PELP1/MNAR staining was

Fig. 2 Schematic representation of the current understanding of PELP1/MNAR signaling. PELP1 play a role in ER genomic and nongenomic functions and is depicted by placing PELP1/MNAR in the both cytoplasmic and nuclear compartments along with the identified complexes. Deregulation of PELP1/MNAR expression or localization may alter the ratio of genomic to nongenomic signaling, thus might contribute to the hypersensitivity of tumor cells to low levels of estradiol. This would promote, tumorigenesis, hormonal independence and metastasis



predominantly cytoplasmic, whereas ER β staining was nuclear and occasionally cytoplasmic in tumor cells.

Conclusions

PELP1/MNAR appears to play an essential role in both ER genomic and nongenomic activity, as well as in ER/growth factor signaling cross-talk (Fig. 2). The abnormal expression and function of PELP1/MNAR in human tumors, as well as its ability to cooperate with other oncogenes, promote adhesion-independent proliferation, and enhance tumorigenesis in nude mice model suggests that PELP1/MNAR plays a role in the initiation and/or progression of tumor growth. The increased expression of PELP1/MNAR in metastatic model cells and node-positive tumors, as well as its ability to modulate E2-mediated cytoskeleton changes and cell migration, suggests that PELP1/MNAR plays a role in cancer cell metastasis. However, the mechanism by which PELP1/MNAR promotes these functions is not clear. PELP1/MNAR expression/localization is altered in a subset of tumors, promotes local estrogen synthesis, and promotes resistance to tamoxifen in cancer model cells, suggesting that deregulation of PELP1/MNAR may have implications in hormonal therapy. Future studies elucidating the molecular mechanism of action of PELP1/MNAR in normal and

tumor cells and profiling the expression of PELP1/MNAR in large numbers of tumor samples would allow use of this novel ER-coregulator protein as a diagnostic marker and as a target for novel therapies.

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