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14. ABSTRACT Emerging evidence suggests that nuclear receptor (NR) coregulators have potential to act as master genes and their deregulation can promote oncogenesis. Proline-, glutamic acid-, and leucine-rich protein-1 (PELP1/MNAR) is a novel NR coregulator. IHC studies using human ovarian cancer tissue arrays (n=123) showed that PELP1/MNAR is 2 to 3 fold over expressed in 60% of ovarian tumors To examine the significance of PELP1/MNAR in ovarian cancer progression, we have generated model cells that over express PELP1/MNAR and ovarian cancer cells in which PELP1/MNAR expression is down regulated by stable expression of PELP1/MNAR-specific shRNA. Down regulation of PELP1/MNAR in cancerous ovarian model cells (OVCAR3) resulted in reduced proliferation, affected the magnitude of c-Src and AKT signaling and reduced tumorigenic potential of ovarian cancer cells in a nude mouse model. PELP1/MNAR over expression in non-tumorigenic immortalized surface epithelial cells (IOSE cells) promoted constitutive activation of c-Src and AKT signaling pathways and promoted anchorage independent growth. PELP knock down affected EGF mediated activation of cytoskeletal reorganization and increased sensitivity of chemotherapy drugs. Collectively these results suggest that PELP1/MNAR signaling plays a role in ovarian cancer cell proliferation and survival, and that its expression is deregulated in ovarian carcinomas.					
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Title: Role of PELP1 in EGFR-ER signaling Crosstalk in ovarian cancer cells

PI: Ratna K Vadlamudi

REPORT PERIOD: March 15, 2006-Mar 14, 2008

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 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 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. Initially using transient transfection assay, we have screened four shRNAs and identified two shRNAs (PELP1/MNAR-shRNA1 and PELP1/MNAR-shRNA2) that caused greater than 80% reduction in the endogenous PELP1/MNAR levels. These two shRNAs target two distinct regions of PELP1/MNAR thus serve as confirmatory controls for non-specific actions of shRNA. We then established OVCAR3 stable cell lines that express PELP1/MNAR-shRNA1 and -shRNA2. As a control, OVCAR3 cells were transfected with shRNA vector. Western blot analysis of total lysates revealed that the PELP1/MNAR-shRNA clones showed down regulation of PELP1/MNAR expression to ~80 % of the level seen in the parental and the vector-transfected clones (Fig. 1A). To further analyze the role of PELP1/MNAR on proliferation of OVCAR3 cells, we measured proliferation rate of these clones under low and high serum conditions. The results show that PELP1/MNAR shRNA cells had a

decrease in proliferation compared to the parental cells and that the effect of PELP1/MNAR down

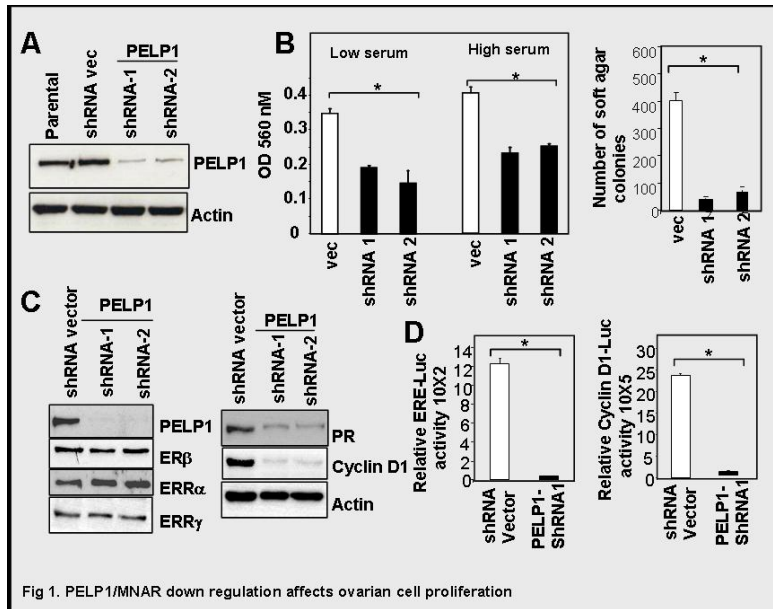


Fig 1. PELP1/MNAR down regulation affects ovarian cell proliferation

reporters also confirmed that PELP1 deregulation affect the expression of these genes (Fig. 1D). Collectively these results suggest that PELP1/MNAR play a critical role in the proliferation of ovarian cancer cells and PELP1/MNAR mediated NR-genomic functions may play role in the proliferation.

PELP1/MNAR down regulation affects non-genomic signaling in ovarian cancer cells. To

examine the possibility that PELP1/MNAR has a role in the activation of non-genomic signaling pathways in ovarian cells, we measured the activation of non-genomic signaling pathways that are shown to be modulated by PELP1/MNAR signaling including Src, AKT and MAPK. Total cell lysates from OVCAR3 stable cells expressing either vector or PELP1/MNAR-shRNA were analyzed using Western blotting with phospho-specific antibodies. PELP1/MNAR-shRNA-expressing cells had significantly less Src, AKT and MAPK

activation compared to shRNA vector transfected cells (Fig. 2A). However, PELP1 down regulation did not affect the status of Phospho-NF-kappa-B p65. (Fig. 2A) and we have used this as a control for phospho antibody blots in PELP1 clones.. To confirm that the signaling changes observed are due to PELP1/MNAR down regulation in OVCAR3 cells, we generated two additional ovarian model cells: BG1 cells stably expressing PELP1/MNAR (BG1-PELP1/MNAR) and SKOV3 cells expressing PELP1/MNAR shRNA. Down regulation of PELP1/MNAR in SKOV3 cells utilizing two distinct shRNAs that target PELP1/MNAR also resulted in substantial reduction of the Src and AKT signaling pathways (Fig. 2A, right panel) with no reduction in the phospho-NF-Kappa-B signaling. Similarly, BG1-PELP1/MNAR cells (pooled clones 1 and 2) showed 3-fold increase in PELP1/MNAR expression compared to the vector-transfected cells (Fig. 2B, left panel). Western analysis of the total

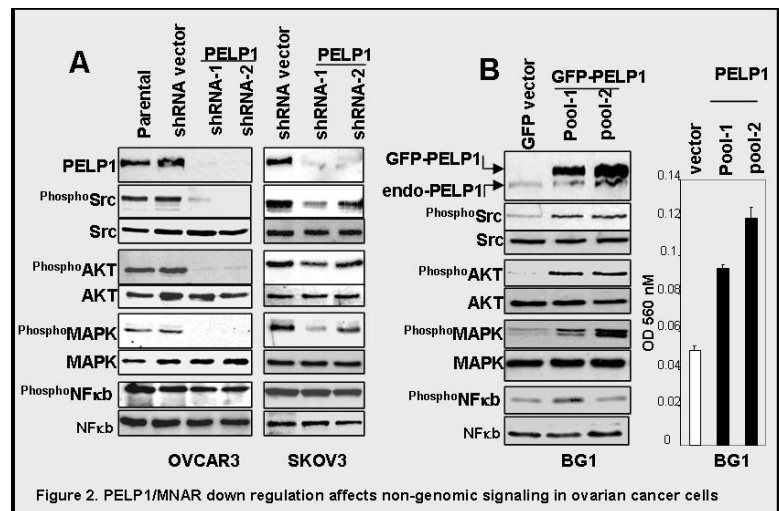
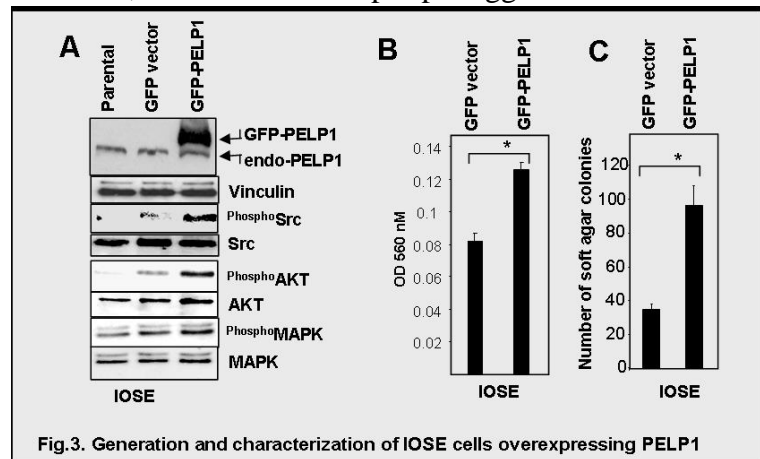


Figure 2. PELP1/MNAR down regulation affects non-genomic signaling in ovarian cancer cells

protein lysates showed that PELP1/MNAR over expression in BG1 cells promotes increased Src, AKT and MAPK signaling with no increase in phospho-NF-kappa-B signaling (Fig. 2B, *left panel*). PELP1 over expression in BG1 cells also proliferation potential of the cells (Fig. 2B, *right panel*). Collectively, the results from these three ovarian cancer model cells suggest that PELP1/MNAR signaling plays an essential role in Src, AKT, and MAPK signaling in ovarian cancer cells.

Deregulation of PELP1/MNAR expression in IOSE cells promote excessive non-genomic signaling and anchorage independent growth. Most ovarian neoplasms arise from the ovarian surface epithelium (OSE) (5). Dr. Aneursperg (collaborator of this study) developed immortalized OSE model cells (IOSE) from normal OSE by transfecting simian virus 40 large T antigen (6). The SV40T/t antigen inactivates both the p53 and pRb pathways and extends the life span of these cells to 10 passages while maintaining many of the properties of normal ovarian epithelium. To examine the putative function of PELP1/MNAR deregulation in ovarian tumors, we have used IOSE cells. Using Amaxa's Nucleofector transfection kit, we over expressed PELP1/MNAR in the IOSE model cells. To enable the monitoring of the transfected cells, we used GFP epitope-tagged PELP1/MNAR

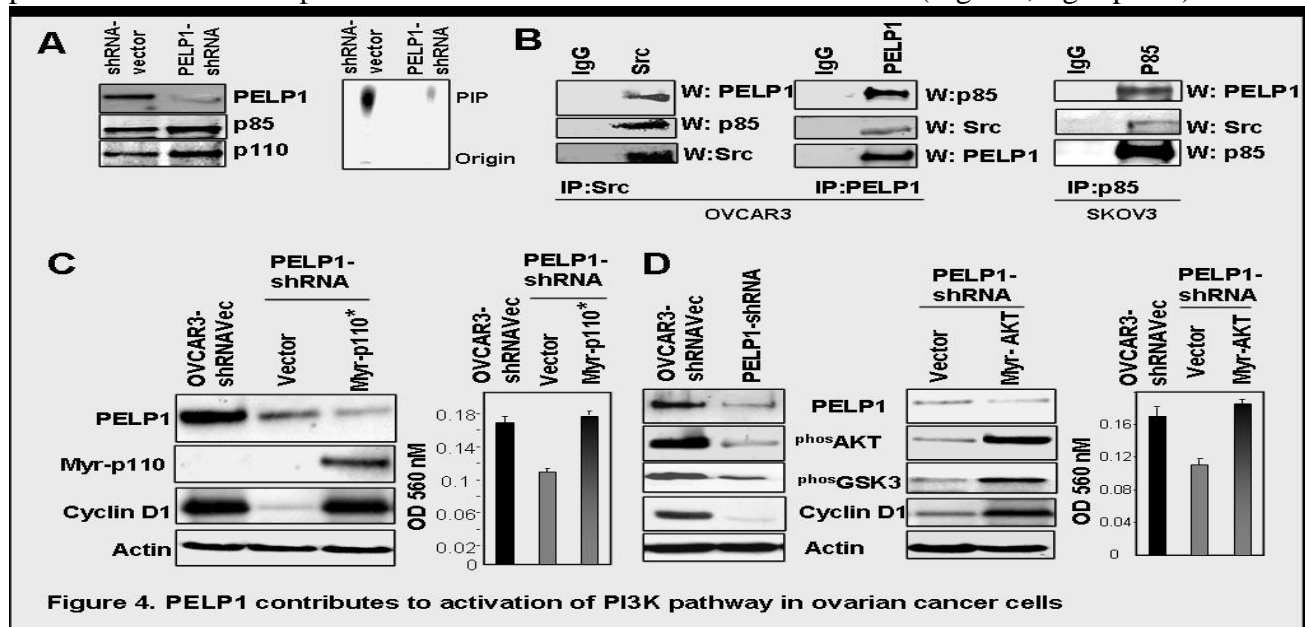
expression vector in these assays and GFP-vector was used as a control. The expression of PELP1/MNAR in transfected IOSE cells was analyzed by using Western analysis (Fig. 3A). This Amaxa's Nucleofector transfection typically resulted in transfection of >70% IOSE cells and generated IOSE model cells that over express 2-3 fold PELP1/MNAR compared to vector transfected IOSE cells. The status of non-genomic signaling in GFP-vector and GFP-PELP1/MNAR expressing cells was



then analyzed by using Western blot analysis using phospho-specific antibodies. The results showed that PELP1/MNAR over expression substantially increased Src, AKT and MAPK signaling in IOSE model cells (Fig. 3B). Similarly, PELP1/MNAR over expressing IOSE cells exhibited increased proliferation potential and showed increased anchorage independence in soft agar colony assays (Fig. 3C).

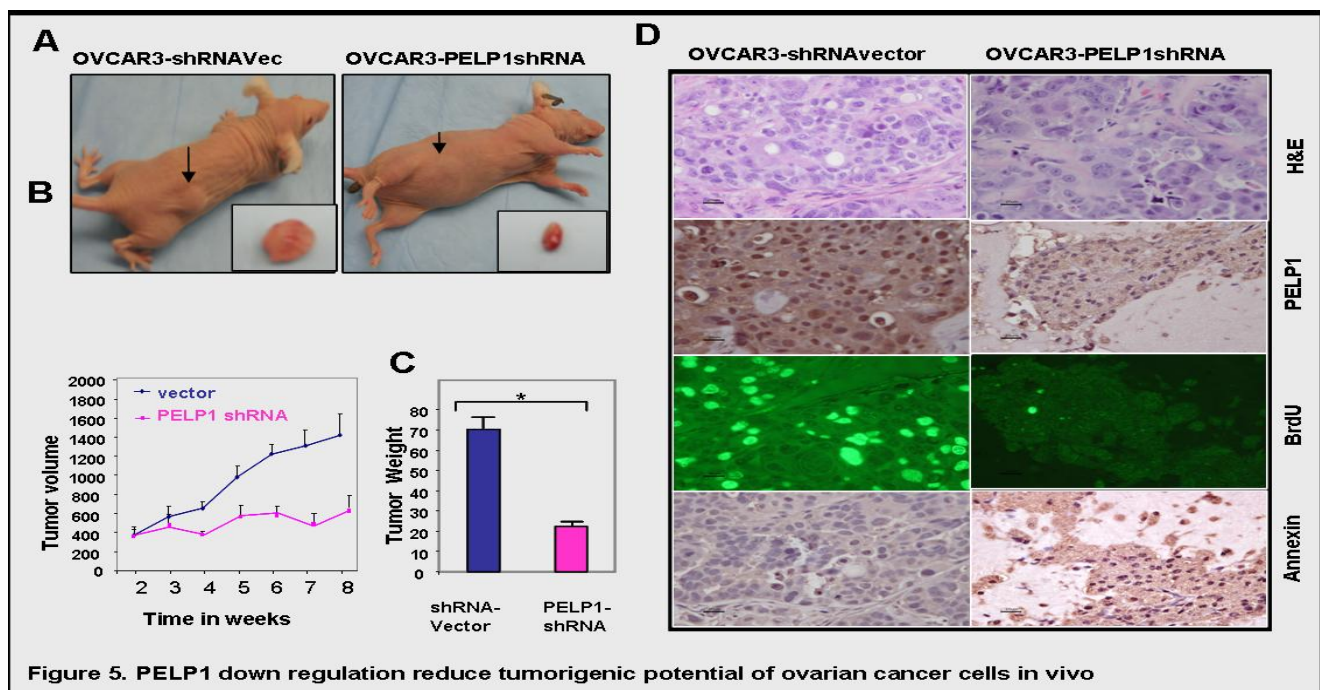
PELP1 modulates ovarian cancer cell proliferation via PI3K-AKT pathway. Since PELP1 shRNA clones exhibited decreased activation of Src and AKT kinases, we examined, whether PELP1 down regulation contributes to decreased PI3K activity. Results of PI3- kinase assay showed that PELP1 shRNA clones indeed have low PI3K activity (Fig. 4A). To examine whether functional PELP1-PI3K-Src complexes exists in ovarian cancer cells, we have performed immunoprecipitation of OVCAR3 cell lysates using antibodies that recognize endogenous Src and PELP1. The results showed that PELP1 interacts with p85 subunit of PI3K kinase and PELP1 form complexes with Src, and p85 subunit of PI3K (Fig. 4B). We also confirmed PELP1 interactions with p85 subunit of PI3K in SKOV3 cells (Fig. 4B, right panel). Since PELP1 interactions with Src kinase leads to activation of PI3K pathway, we hypothesized that over expression of activated form of PI3K will rescue the defects in PELP1 shRNA cells. To test this hypothesis, we have performed rescue experiments in PELP1 shRNA clones using activated p110 catalytic subunit of PI3K. We have transfected the OVCAR3-PELP1shRNA cells with vector alone or vector that express activated and membrane targeted PI3K catalytic subunit (myristolated p100*) using Nucleofector transfection methodology that facilitated

80-90% transfection efficiency. The results from this experiment showed that over expression of Myr-p110 α can restore the proliferation defect seen in PELP1shRNA cells (Fig. 4C, right panel). Further,

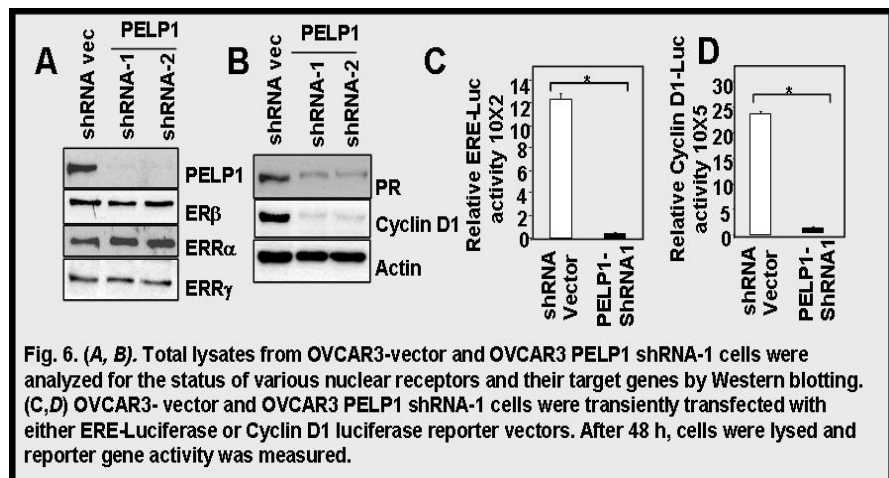


Myr-p110* over expression also restored the defect seen in the cyclin D1 levels in PELP1 shRNA clones (Fig. 4C, left panel). To further examine how defects in AKT activation leads to decreased proliferation in PELP1 shRNA clones, we examined the status of down stream effectors of AKT in PELP1 shRNA clones. We have observed a dramatic decrease in the phosphorylation of GSK3 β , a down stream target of AKT in the PELP1 shRNA clones. To further delineate the mechanism, we performed restoration experiments using activated form of AKT (Myr-AKT). Interestingly, over expression of Myr-AKT restored the defect seen in the GSK3 β phosphorylation, restored cyclin D1 levels and rescued the proliferation defect in PELP1 shRNA clones (Fig. 4D). Collectively these restoration experiments provide evidence that blockage of PI3K-AKT-GSK3 β -cyclin D1 pathway by PELP1 down regulation contributed the defect in the cells proliferation seen in the PELP1 shRNA clones.

PELP1/MNAR down regulation decreases tumorigenic potential of OVCAR3 cells *in vivo*. We then used a nude mouse xenograft model to examine whether PELP1/MNAR is required for tumorigenic potential of ovarian cancer cells *in vivo*. OVCAR3 cells stably expressing vector (n=7) or PELP1/MNAR-shRNA (n=7) were injected subcutaneously into mice and tumorigenic potential was monitored for 8 weeks (Fig. 5A). Under those conditions, OVCAR3 vector-transfected cells formed tumors and tumor grew linearly with time. However OVCAR3-PELP1/MNAR-shRNA injected sites showed tumors with substantial reduction in growth compared to control (Fig. 5B, C). These results suggested that PELP1/MNAR expression is essential for optimal growth of ovarian tumor cells *in vivo*. IHC examination of the tumors revealed that PELP1/MNAR-shRNA tumors retained the low expression of PELP and exhibited activation of Src and AKT to a lesser degree than the activation seen in OVCAR3 vector-transfected tumors (Fig. 5D). Further BrdU staining of the tumor sections revealed increased proliferation in the OVCAR3 vector transfected xenograft tumors compared to PELP1/MNAR shRNA tumors. Also, the PELP1/MNAR shRNA tumors showed increased apoptosis as revealed by annexin staining (Fig. 5D). These results suggest that PELP1/MNAR plays an essential role in the growth of ovarian tumor cells *in vivo*.

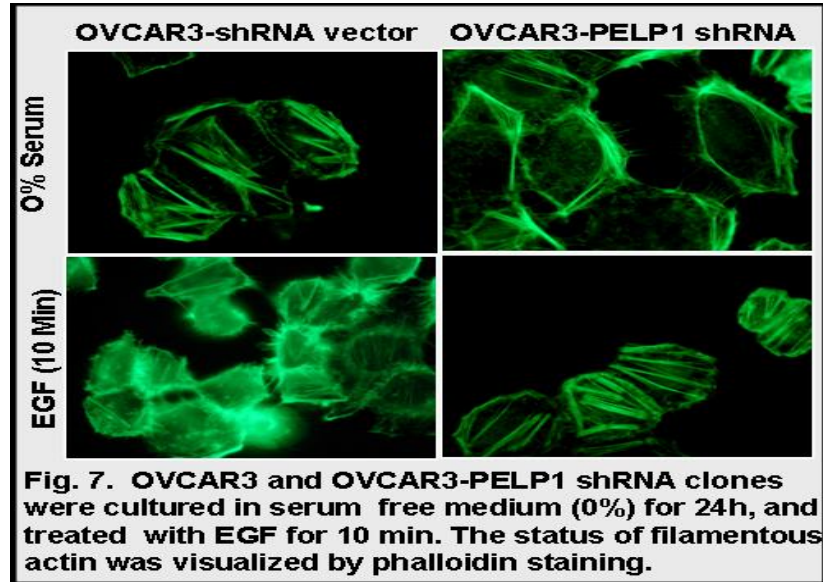


PELP1 Down regulation affects nuclear receptor (NR) genomic functions: Since earlier studies have shown that PELP1 functions as a coregulator of several NRs including ER, STAT3 and activates target genes such as PR and cyclinD1, we have examined the expression status of these genes in the PELP1 shRNA clones. Western analysis showed that PELP1 down regulation did not affect the expression levels of nuclear receptors in these cells (Fig. 6A, left panel). However, PELP1 down regulation affected the expression of PELP1 regulated NR target genes such as PR and CyclinD1 (Fig. 6B, right panel). Reporter gene assays using ERE- and Cyclin D1 luciferase reporters also confirmed that PELP1 deregulation affected the expression of these genes (Fig. 6C, D). Collectively these results suggest that PELP1 plays a critical role in the proliferation of ovarian cancer cells and PELP1 mediated NR-genomic functions may play a role in the proliferation.

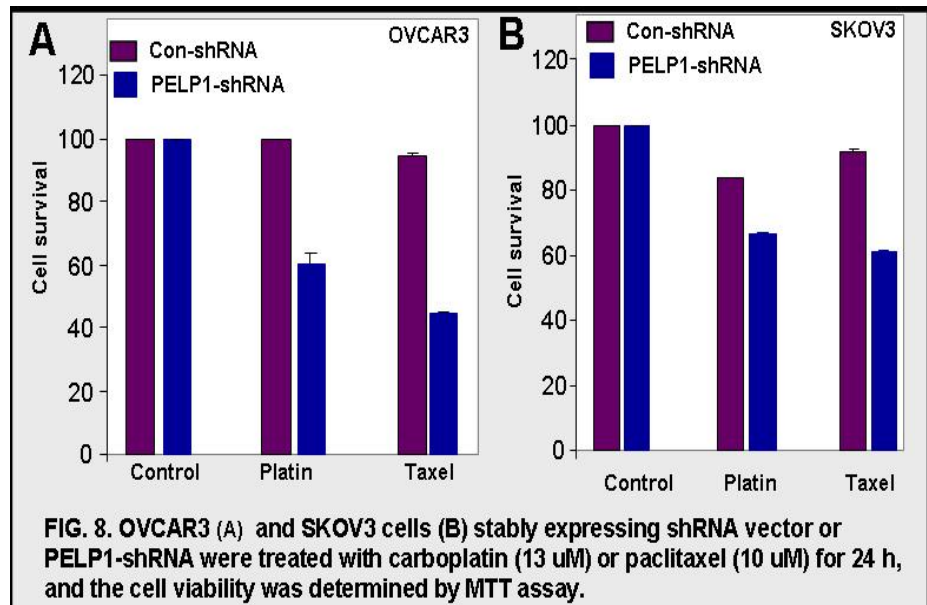


PELP1-shRNA clones exhibit defects in cytoskeleton reorganization. While culturing PELP1 shRNA cells, we have noticed that PELP1-shRNA cells showed distinct flat morphology compared to OVCAR3 parental and OVCAR3-vector cells (data not shown). Since PELP1 interacts with and

modulate activities of several proteins that are implicated in the cytoskeletal reorganization including Src, PI3K, FHL2, we examined whether lack of PELP1 expression contributes to cytoskeletal defects in ovarian cancer cells. OVCAR3 or OVCAR3 shRNA expressing cells were serum starved and treated with or without EGF and filamentous actin status was analyzed by phalloidin staining. Serum starved control OVCAR3 cells exhibited low F-actin structures with peripheral cortical actin, and EGF stimulation promote increased formation of ruffles and fillopodia. Interestingly in PELP1 knock down cells, EGF treatment did not promoted formation of fillopodia or ruffles (Fig. 7). However PELP1 shRNA cells showed increased accumulation of stress fibers which is also an indication of less motile cells. *These results suggest that PELP1 signaling may play a role in cytoskeleton reorganization and is required for optimal reorganization by growth factor signaling.*



PELP1 down regulation sensitizes ovarian cancer cells to chemotherapy. Since PELP1 interacting proteins Src, PI3K and STAT3 are implicated in the chemotherapy resistance, we hypothesized that PELP1 deregulation could contribute to chemotherapy resistance and down regulation of PELP1 sensitizes ovarian cancer cells to chemotherapy. To examine this possibility, we examined whether PELP1 down regulation by shRNA enhances chemotherapy sensitivity of carboplatin and paclitaxel in ovarian cancer cells. OVCAR3-PELP1 shRNA and SKOV3-PELP1-shRNA along with vector transfected control cells were treated with or with out carboplatin (13 μ M) and or paclitaxel (10 μ M) for 24 h and the cell viability was determined by MTT assay. Down regulation of PELP1 substantially enhanced the therapy sensitivity of OVCAR3 cells (Fig. 15A) and modestly enhanced drug effectiveness in SKOV3 cells (Fig. 8).



KEY RESEARCH ACCOMPLISHMENTS

- PELP1 function as a novel Proto-oncogene in ovarian cancer cells
- PELP1 play an essential role the activation of c-Src, and AKT pathways
- PELP1 play a critical role in cytoskeletal reorganization and cell morphology
- PELP1 down regulation by decreases the tumorigenic potential of cancer cells
- PELP1 down regulation enhances the potency of chemotherapy drugs

REPORTABLE OUTCOMES

This study has resulted in the following publications

1. Chakravarty D, Nair SS, Seetharaman B, Liu J, Tekmal RR, Ausperg N, Burrow M, Broaddus R, **Vadlamudi RK**. PELP1/MNAR in ovarian cancer: implications in tumorigenesis 2007. (American Association For Cancer Research, Annual Meeting 2007; vol. 4449).
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.
3. Chakravarty, D and **Vadlamudi, RK**. 2007. Nuclear Receptor Coregulators: Emerging Drug Targets for Treatment of Women Cancers. In: David K. Wong, eds. Tumorigenesis Research Advances. Nova Science Publishers, Hauppauge, NY, 2007. (Book Chapter).
4. Chakravarty D, Nair SS, Rajhans R, Piteshwara RP, Jinsong L, Seetharaman B, Le XF, Burrow ME, Nelly A, Tekmal RR, Broaddus RR, **Vadlamudi RK**. Role of PELP1/MNAR signaling in ovarian tumorigenesis. Cancer Research, April, 2008 (In press)

CONCLUSIONS

The findings from this study indicate that nuclear receptor coregulator PELP1/MNAR play a role in ovarian cancer progression and its expression is deregulated in ovarian tumors. Using normal and commonly used ovarian cancer cells and shRNA methodology, we have provided evidence that PELP1/MNAR deregulation contributes to excessive activation of genomic and non-genomic signaling pathways and PELP1 deregulation / functions play a role in the ovarian tumorigenesis. In addition, PELP1 appears to play a key role in cytoskeletal reorganization and participates in EGF–NR cross talk leading to cytoskeletal reorganization. Our ongoing experiments are testing the role of PELP1–EGF cross talk in enhancing ovarian cell migration. Analysis of cellular signaling pathways in PELP1/MNAR-overexpressing model cells revealed constitutive activation of c-Src kinase and AKT. The ability of PELP1/MNAR to modulate the c-Src-PI3K pathways and its potential deregulation in ovarian cancer cells suggest that the c-Src-PI3K pathway may represent one potential mechanism by which PELP1/MNAR promotes tumorigenesis in ovarian cancer cells via PI3K-AKT-GSK3 β -cyclin D1 pathway. We also found that down regulation of PELP1/MNAR decreased cell proliferation, and decreased ovarian tumor growth in a nude mouse model. Collectively these results suggest that PELP1 deregulation promotes ovarian cancer cell proliferation. Further, PELP1 down regulation enhances the sensitivity of chemotherapy drugs. Our ongoing experiments will test the potential of blocking PELP1 and or its associates Src-PI3K pathways in enhancing hormonal therapy in ovarian cancer cells. In summary, the results of this study provide the first evidence for the contribution of the PELP1/MNAR to the tumorigenic potential in ovarian cancer cells. Our findings also suggest that alterations in the levels or localization of PELP1/MNAR could occur during ovarian cancer progression and such alterations may provide survival advantage by excessively activating NR mediated genomic and non-genomic signaling. Collectively, these findings suggest a possibility that

the PELP1-Src-AKT axis could be used as a potential diagnostic and/or therapeutic target in ovarian cancer.

REFERENCES

1. Cannistra SA 1993 Cancer of the ovary. *N Engl J Med* 329:1550-1559
2. Ozols, R.F., Bookman, M.A., Connolly, D.C., Daly, M.B., Godwin, A.K., Schilder, R.J., Xu, X., and Hamilton, T.C. Focus on epithelial ovarian cancer. *Cancer Cell* 5:10-24, 2004.
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.
5. Auersperg,N., Wong,A.S., Choi,K.C., Kang,S.K. and Leung,P.C. Ovarian surface epithelium: biology, endocrinology, and pathology, *Endocr.Rev.*, 22: 255-288, 2001.
6. Maines-Bandiera,S.L., Kruk,P.A. and Auersperg,N. Simian virus 40-transformed human ovarian surface epithelial cells escape normal growth controls but retain morphogenetic responses to extracellular matrix, *Am.J.Obstet.Gynecol.*, 167: 729-735, 1992.

Appendix 1.

Chakravarty D, Nair SS, Seetharaman B, Liu J, Tekmal RR, Ausperg N, Burrow M, Broaddus R, **Vadlamudi RK**. PELP1/MNAR in ovarian cancer: implications in tumorigenesis 2007. (American Association For Cancer Research, Annual Meeting 2007; vol. 4449).

2007 AACR Annual Meeting**April 14-18, 2007****Los Angeles, CA**[Print this Page for Your Records](#)[Close Window](#)

Abstract Number: 4345

Presentation Title: PELP1/MNAR in ovarian cancer: implications in tumorigenesis

Presentation Start/End Time: Tuesday, Apr 17, 2007, 1:00 PM - 5:00 PM

Location: Exhibit Hall, Los Angeles Convention Center

Poster Section: 8

Poster Board Number: 22

Author Block: *Dimple Chakravarty, Sujit Nair, Seetharaman Balasenthil, Jinsong Liu, Rajeshwar Rao Tekmal, Nelly Auersperg, Matthew Burow, Russel Broaddus, Ratna Vadlamudi.* 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 overexpresion 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.

2007 AACR Annual Meeting

April 14-18, 2007

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Appendix 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.

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

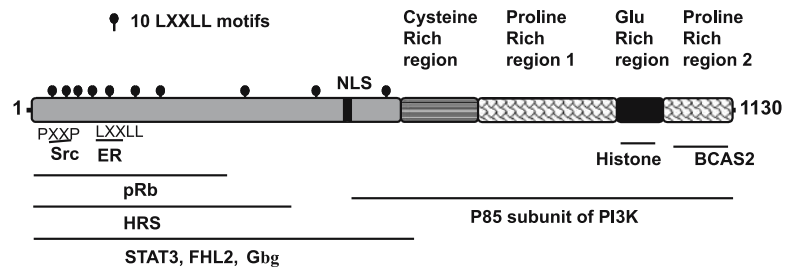
signaling complexes with ER. The primary structure of PELP1/MNAR contains several motifs/domains present in many transcriptional coactivators, including 10 nuclear receptor (NR) interacting boxes (LXXLL motifs), a zinc finger, a glutamic acid-rich domain, and 2 proline-rich domains [8, 9] (Fig. 1). Interestingly, 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

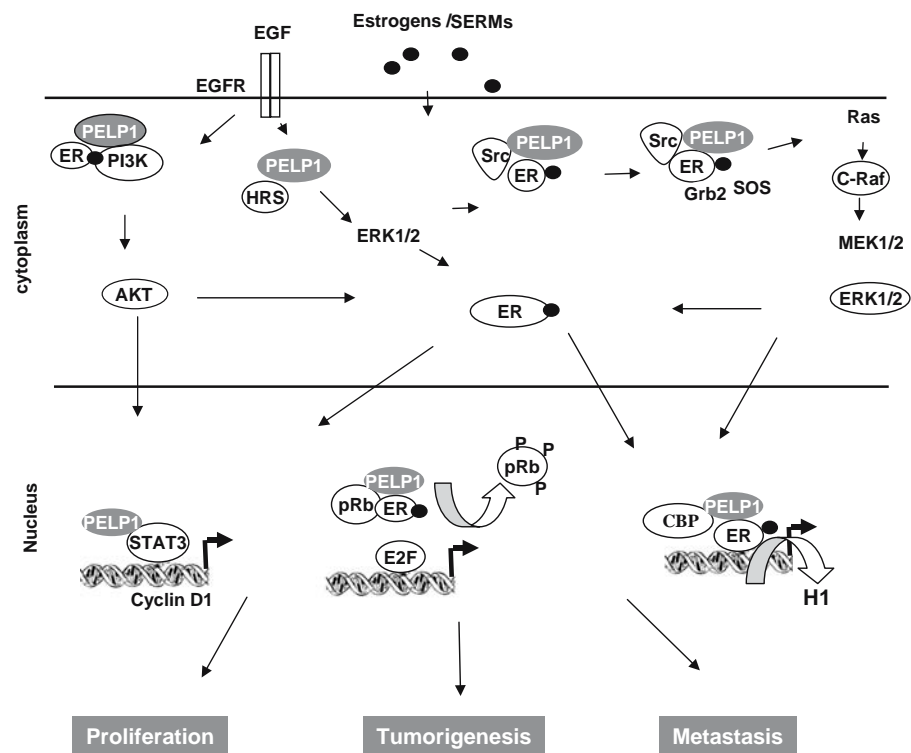
cytoplasmic localization in tumors (PELP1-cyto cells) were hypersensitive to E2 but resistant to tamoxifen. The altered localization of PELP1/MNAR to the cytoplasm was sufficient to trigger its interaction with the p85 subunit of PI3K, leading to PI3K activation [23]. Cytoplasmic PELP1/MNAR interacted 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- α -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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References

1. Ariazi EA, Ariazi JL, Cordera F, Jordan VC (2006) Estrogen receptors as therapeutic targets in breast cancer. *Curr Top Med Chem* 6:195–216
2. Hall JM, McDonnell DP (2005) Coregulators in nuclear estrogen receptor action: from concept to therapeutic targeting. *Mol Interv* 5:343–357
3. Gururaj AE, Rayala SK, Vadlamudi RK, Kumar R (2006) Novel mechanisms of resistance to endocrine therapy: genomic and nongenomic considerations. *Clin Cancer Res* 12:1001s–1007s
4. Schiff R, Massarweh SA, Shou J, Bharwani L, Arpino G, Rimawi M et al (2005) Advanced concepts in estrogen receptor biology and breast cancer endocrine resistance: implicated role of growth factor signaling and estrogen receptor coregulators. *Cancer Chemother Pharmacol Suppl* 1:10–20
5. Kuang SQ, Liao L, Zhang H, Lee AV, O'Malley BW, Xu J (2004) AIB1/SRC-3 deficiency affects insulin-like growth factor I signaling pathway and suppresses v-Ha-ras-induced breast cancer initiation and progression in mice. *Cancer Res* 64:1875–1885

6. Torres-Arzuayus MI, Font de MJ, Yuan J, Vazquez F, Bronson R, Rue M et al (2004) High tumor incidence and activation of the PI3K/AKT pathway in transgenic mice define AIB1 as an oncogene. *Cancer Cell* 6:263–274
7. Balasenthil S, Vadlamudi RK (2003) Functional interactions between the estrogen receptor coactivator PELP1/MNAR and retinoblastoma protein. *J Biol Chem* 278:22119–22127
8. Vadlamudi RK, Wang RA, Mazumdar A, Kim Y, Shin J, Sahin A et al (2001) Molecular cloning and characterization of PELP1, a novel human coregulator of estrogen receptor alpha. *J Biol Chem* 276:38272–38279
9. Wong CW, McNally C, Nickbarg E, Komm BS, Cheskis BJ (2002) Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with Src/Erk phosphorylation cascade. *Proc Natl Acad Sci USA* 99:14783–14788
10. Khan MM, Hadman M, Wakade C, De Sevilla LM, Dhandapani KM, Mahesh VB et al (2005) Cloning, expression, and localization of MNAR/PELP1 in rodent brain: colocalization in estrogen receptor-alpha but not in gonadotropin-releasing hormone-positive neurons. *Endocrinology* 146:5215–5227
11. Pawlak J, Beyer C (2005) Developmental expression of MNAR mRNA in the mouse brain. *Cell Tissue Res* 320:545–549
12. Greger JG, Guo Y, Henderson R, Ross JF, Cheskis BJ (2006) Characterization of MNAR expression. *Steroids* 71:317–322
13. Mishra SK, Balasenthil S, Nguyen D, Vadlamudi RK (2004) Cloning and functional characterization of PELP1/MNAR promoter. *Gene* 330:115–122
14. Nagpal J, Nair S, Pothana S, Tekmal R, Kumar R, Vadlamudi RK (2006) Growth factor regulation of PELP1/MNAR functions: role of PKA-dependent phosphorylation. *Proc Amer Cancer Res* 47:2933
15. Ballif BA, Villen J, Beausoleil SA, Schwartz D, Gygi SP (2004) Phosphoproteomic analysis of the developing mouse brain. *Mol Cell Proteom* 3:1093–1101
16. Manavathi B, Nair SS, Wang RA, Kumar R, Vadlamudi RK (2005) 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* 65:5571–5577
17. Vadlamudi RK, Balasenthil S, Broaddus RR, Gustafsson JA, Kumar R (2004) 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* 89:6130–6138
18. Haas D, White SN, Lutz LB, Rasar M, Hammes SR (2005) The modulator of nongenomic actions of the estrogen receptor (MNAR) regulates transcription-independent androgen receptor-mediated signaling: evidence that MNAR participates in G protein-regulated meiosis in *Xenopus laevis* oocytes. *Mol Endocrinol* 19:2035–2046
19. Choi YB, Ko JK, Shin J (2004) The transcriptional corepressor, PELP1, recruits HDAC2 and masks histones using two separate domains. *J Biol Chem* 279:50930–50941
20. Nair SS, Mishra SK, Yang Z, Balasenthil S, Kumar R, Vadlamudi RK (2004) Potential role of a novel transcriptional coactivator PELP1 in histone H1 displacement in cancer cells. *Cancer Res* 64:6416–6423
21. Mishra SK, Mazumdar A, Vadlamudi RK, Li F, Wang RA, Yu W et al (2003) MICOA, a novel metastasis-associated protein 1 (MTA1) interacting protein coactivator, regulates estrogen receptor-alpha transactivation functions. *J Biol Chem* 278:19209–19219
22. Barletta F, Wong CW, McNally C, Komm BS, Katzenellenbogen B, Cheskis BJ (2004) Characterization of the interactions of estrogen receptor and MNAR in the activation of cSrc. *Mol Endocrinol* 18:1096–1108
23. Vadlamudi RK, Manavathi B, Balasenthil S, Nair SS, Yang Z, Sahin AA et al (2005) Functional implications of altered subcellular localization of PELP1 in breast cancer cells. *Cancer Res* 65:7724–7732
24. Nair S, Rajhans R, Nagpal J, Guider J, Kumar R, Vadlamudi RK (2006) PELP1/MNAR interacts with BCAS2: potential role in ER-mediated splicing. *Proc Amer Cancer Res* 47:2930
25. Rayala SK, Hollander P, Balasenthil S, Molli PR, Bean AJ, Vadlamudi RK et al (2006) Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) interacts with PELP1 and activates MAPK. *J Biol Chem* 281:4395–4403
26. Nair S, Seetharaman B, Mueller JMKS, Schule R, Kumar R, Vadlamudi RK (2006) PELP1/MNAR modulates LIM-only coactivator FHL2 transactivation functions. *Proc Amer Cancer Res* 46:1279
27. Unni E, Sun S, Nan B, McPhaul MJ, Cheskis B, Mancini MA et al (2004) Changes in androgen receptor nongenotropic signaling correlate with transition of LNCaP cells to androgen independence. *Cancer Res* 64:7156–7168
28. Singh R, Gururaj AE, Vadlamudi RK, Kumar R (2006) 9-*cis*-retinoic acid upregulates expression of transcriptional coregulator PELP1, a novel coactivator of RXR. *J Biol Chem* 281:10774–10783
29. Rajhans R, Nair S, Tekmal R, Kumar R, Vadlamudi RK (2006) Cellular transformation and oncogenesis by PELP1/MNAR, a major broad coactivator in mammalian cells. *Proc Amer Cancer Res* 47:4215
30. Heppner GH, Miller FR, Shekhar PM (2000) Nontransgenic models of breast cancer. *Breast Cancer Res* 2:331–334
31. Mishra SK, Talukder AH, Gururaj AE, Yang Z, Singh RR, Mahoney MG et al (2004) Upstream determinants of estrogen receptor-alpha regulation of metastatic tumor antigen 3 pathway. *J Biol Chem* 279:32709–32715
32. Rayala SK, Mascarenhas J, Vadlamudi RK, Kumar R (2006) Altered localization of a coactivator sensitizes breast cancer cells to tumor necrosis factor-induced apoptosis. *Mol Cancer Ther* 5:230–237
33. Vadlamudi RK, Balasenthil S, Sahin AA, Kies M, Weber RS, Kumar R et al (2005) Novel estrogen receptor coactivator PELP1/MNAR gene and ERbeta expression in salivary duct adenocarcinoma: potential therapeutic targets. *Hum Pathol* 36:670–675

Appendix 3.

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Nuclear Receptor Coregulators: Emerging Drug Targets for Treatment of Women Cancers

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ABSTRACT

Steroid hormones play pivotal roles in sex differentiation, reproductive functions and are implicated in the progression of endocrine-related cancers in women. The biological functions of steroidal hormones are mediated by nuclear receptors (NRs), a super family of ligand-regulated transcription factors that modulate a wide range of biological processes. NRs are implicated in the progression of a number of diseases, including women's cancers and are the second largest class of drug targets. Coregulators are an integral part of NR pathway and their composition in a given cell determine the magnitude and specificity of the NR signaling. NR-coregulator actions have expanded to chromatin modification and remodeling, initiation of transcription, RNA elongation and splicing, and protein degradation. Emerging evidence suggest that coregulators are likely to function as master genes sensing physiological signals and activating the appropriate set of genes using a wide variety of NRs. Alterations in both levels and functions of NR coregulators are reported to occur

during cancer progression. Deregulation of these coregulators could influence target gene expression and participate in the development of hormone-responsive cancers. With the enormous potential of coregulators acting as master regulators, their deregulation is likely to provide cancer cells an advantage in survival, growth and metastasis. In this chapter, we will summarize the findings on the nuclear receptor coregulators and explain why these molecules represent important therapeutic targets for treatment of women's cancers.

TEXT

1. General nuclear receptor signaling

Nuclear receptors (NRs) constitute a large family of transcription factors that regulate gene expression in a ligand-dependent manner (1;2). NRs play an important role in vertebrate development and have been implicated in a broad range of cellular responses, such as differentiation, proliferation, and homeostasis (3). Common structural features of all nuclear receptors include (i) an N-terminal ligand-independent transcriptional activation function domain-1 (AF1); (ii) a central DNA-

binding domain (DBD) responsible for targeting the receptor to highly specific DNA sequences comprising a response element and (iii) a C-terminal ligand-dependent transcriptional activation function domain, (AF2) (4). When a hormone binds to a NR, a conformational change is triggered that allows the NR to bind the responsive elements in the target gene promoters.

2. Estradiol and the estrogen receptor

The steroid hormone 17 β -estradiol (E2) plays an important role in controlling the expression of genes involved in a wide variety of biological processes, including development, differentiation, and homeostasis in various tissues, including bone, brain, breast, and uterus, and also in the cardiovascular system (1;2). The biological effects of E2 are mediated by its binding to the structurally and functionally distinct estrogen receptors (ERs) ER α and ER β (1), which belong to the nuclear receptor (NR) super family and which function as transcription factors to regulate target gene expression (5). Upon the binding of E2 to the ER, the ligand-activated ER translocates to the nucleus, binds to a responsive element in the target gene promoter, and stimulates gene transcription (called genomic/nuclear signaling) (1). Evidence also suggests that the ER can activate signaling at the membrane or within cytosol via its interactions with other signaling kinases/adaptors (called nongenomic/ extranuclear signaling) (6;7).

Mammary glands, ovaries and uterus are the main targets of sex steroid hormones (8). E2/ER signaling has been implicated in mammary gland development as well as in the initiation and progression of breast cancer (9). About 60-70% of breast tumors are ER-

positive(10). E2 also plays an important role in the normal development of the uterus and is involved in the pathogenesis of endometrial cancer (11). Ovarian cancer is common cause of death among gynecologic cancers with the majority of the ovarian cancers derived from the ovarian surface epithelium (12). Female sex hormones have a role in the progression of ovarian cancer, and the levels of estrogens in the ovary are at least 100-fold higher than circulating levels (13). Estrogens are synthesized from androgens by the aromatase enzyme and elevation of aromatase activity during tumorigenesis is suspected to contribute to local growth of ER-positive tumors (14;15). Abnormal expression or function of the ER has been shown in tumors of the reproductive organs (10). Furthermore, the development of resistance to hormonal replacement therapy for breast, ovarian or uterine cancers is related to aberrant expression and function of NR coregulators (16).

3. Coregulators modulate NR activity

NR coregulators are proteins that interact with nuclear receptors to modulate NR transactivation functions {Figure 1} (17;18). Recent evidence suggests that coregulators function as major regulators and coordinators of hormone receptor physiology, which includes the determination of tissue specificity of hormonal action, the integration of membrane and nuclear signaling, the integration of growth factor and physiological signaling to NRs, and the coordination of cell motility (18). Depending on their effect on outcomes of gene expression, coregulators are broadly divided into coactivators, which promote NR activation of transcription, and

corepressors, which suppress NR-dependent gene expression (19). Structural analysis of coactivators has identified the five-amino acid motif LXXLL, where X is any amino acid that is sufficient to mediate coregulator binding to the ligand-bound ERs (20). Coregulator complexes consist of multiple proteins (~10-20) with large subunits that contain diverse enzymatic activities needed by NRs to achieve diverse functions (21). The specific proteins in these complexes can bind to NRs via specific amino acid sequence motifs in a ligand-dependent or independent manner and can provide enzymatic or scaffolding functions. The transcriptional functions of the ER are influenced by several coactivators, including SRC1, GRIP1, AIB1, PELP1, CBP, p300, PGC1, E6AP, PCAF; and corepressors, including nuclear receptor corepressor (N-COR), silencing mediator for retinoic and thyroid receptor (SMRT), and metastasis tumor antigen 1 (MTA1) (1;5;17-19). During the past decade, it has become increasingly clear that recruitment of coregulatory proteins to ERs is required for ER-mediated optimal transcriptional and biological activities. More than 270 NR coregulators have been reported to date and current evidence implicates some of these molecules in pathogenesis (18).

4. Coregulator mechanism of action

Coregulators appear to function as multitasking molecules in groups. Their actions include chromatin modifications, remodeling, initiation of transcription, RNA elongation and splicing, and protein degradation (5). Coactivators, like SRC1, AIB1 and CBP/p300, possess histone acetyltransferase activity, whereas corepressors, such as N-COR

and MTA1, are associated with histone deacetylases (19). Ligand binding to NRs appears to cause an exchange of coactivators for corepressors to facilitate transcription, and thus serve as the basic mechanism for switching gene repression to activation (7;19;22). Coregulators are often present within dynamic and heterogeneous steady-state complexes. Many coregulators are recruited at the promoter level as part of these preformed complexes(5). When present on target promoters, transcriptional coregulators play different roles either due to their specific enzymatic activities (e.g., kinase, acetyl- or methyltransferase; or ubiquitin- or sumo-ligase activities) or due to their ability to recruit other regulator proteins (19). Certain coregulators play a crucial role in remodeling chromatin structure by modifying histone tails and/or by promoting nucleosome remodeling, thus facilitating the access of other proteins to the promoter (4).

NRs appear to recruit multiprotein complexes that regulate higher-order chromatin domains into which nucleosomes are organized. For example, SW1/SNF, a complex that possesses ATPase activity, alters the nucleosomal structure and is involved in the transcriptional regulation of the ER (23). In addition to nucleosomal constraints, histone H1, which binds to the DNA flanking the nucleosome core, may repress ER-mediated transcription by selectively inhibiting ER-mediated transcriptional initiation (24). Coregulators also recruit and stabilize the basal transcriptional machinery, including RNA polymerase II, on the promoter, leading to the formation of transcriptional preinitiation and initiation complexes (25-29). A few reports also have suggested that activated NRs

modulate transcriptional elongation, although the potential coregulators involved are not well defined (5;19). Steroid hormone binding leads to the relocation of NRs to target promoters; the NRs serve as anchors for the subsequent sequential recruitment of different sets of coregulators, which are generally present in preformed but dynamic steady-state complexes. These coregulator complexes were shown to affect various rate-limiting transcriptional steps and lead to the assembly of the complete possessive transcriptional machinery (19).

Recent studies also suggest that coregulators are involved in ER-mediated splicing and some of these coregulators appear to be part of the spliceosome. A subset of NR coregulators acts as dual-functioning "coupling" proteins between transcription and splicing (30). Activated steroid hormone receptors recruit coregulators to the target promoter and participate in both the production and the splicing of the target gene transcripts, and thus may play a major role in regulating the nature of the spliced variants produced from certain target genes in response to steroid hormones (5).

In addition to its well-studied nuclear functions, NRs may also participate in cytoplasmic and membrane-mediated signaling events and ER coregulators also play a role in nongenomic ER signaling (31;32). Such nongenomic signaling has been linked to rapid responses of E2 through stimulation of the Src kinase, mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and protein kinase C pathways in the cytosol (7;22). Some evidence suggests that the nongenomic effects of estrogen

can regulate different cellular processes, such as proliferation, survival, apoptosis, and differentiation functions in diverse cell-types, including breast cancer cells (33).

5. Regulation of coregulators

Phosphorylation of coregulators appears to be the key regulatory mechanism that controls the localization, specificity and function of coregulators. For example, the coregulator SRC3/AIB1 has six phosphorylation sites, which are all required for coactivation of the ER. Differential phosphorylation of these sites on SRC3/AIB1 mediates different downstream events, for example activation of NF- κ B or oncogenic transformation of MEFs (34). Growth factor-mediated signaling pathways have been shown to modify coregulators by phosphorylation, resulting in the altered topology of coregulator proteins and eventually leading to ligand-independent activation or differential responses to selective estrogen receptor modulators (SERMs) (9,12). Ubiquitin (Ub) modification may also play an important role in the formation of both corepressor and coactivator complexes (19). Further, sumoylation (addition of a small ubiquitin-like modifier) of SRC-1 or GRIP1 enhances coactivation functions by retaining these cofactors in the nucleus (35;36). Recent evidence suggests that coregulator proteins are also subjected to acetylation- and methylation-specific modifications (19). For example, methylation of CBP by CARM1 is important for strong CBP coactivation of ER and GRIP1 complexes. Protein arginine methyl transferase (PRMT) recruitment and histone arginine methylation is a well established coactivation event of ligand-activated NRs (37)

6. Physiological functions of coregulators

Coregulators represent little molecules with big goals and are likely to function as a “master gene,” sensing physiological signals and activating the appropriate set of genes using a wide variety of NRs (38). By acting as sensors of environmental cues, coregulator molecules that move among hundreds of nuclear receptors have the potential to control the expression of various subsets of genes to produce desired goals, such as cell growth (39). Even though few studies to date have examined the role of coactivators in differential responses, some evidence suggests that the levels and the activities of coregulators can specify hormonal sensitivity of tumors. Within the past decade, it has become clear that recruitment of coregulatory proteins to ERs is required for ER-mediated optimal transcriptional and biological activities and that coregulators provide an additional level of complexity in ER action. Even though coregulators modulate NR functions, use of transgenic and KO mice models has demonstrated that each coregulator plays an important and overlapping function *in vivo* (40). SRC-1-null mice have a phenotype of generalized resistance to steroid hormones and displayed substantially compromised hormone-dependent proliferation of mammary ductal branches (41). Steroid receptor coactivator-3 (SRC-3) null mutant mice exhibited decreased development of mammary glands, suggesting that coactivators are critical for efficient proliferation and differentiation of mammary glands (42).

7. The role of coregulators in cancer

As a modulator of NR functions, coregulators are likely to play a role in

cancer progression. Even though few studies to date have examined the role of coactivators in hormone-dependent cancers, some data indicate that the levels and activities of coregulators can specify hormonal sensitivity of tumors (7;8;10;22). Many coregulatory proteins are present at rate-limiting levels and are shared by many NRs. Changes in the level of expression and/or activity of NR can lead to alterations in their signaling, which can result in a growth advantage for cancer cells (38). Recent data on several coregulator proteins, including SRC1, SRC2, SRC3, CBP300, support the concept of coregulators as master genes (43). With the enormous potential of coregulators as master regulators, their deregulation is likely to provide the cancer cells an advantage in survival, growth and metastasis. For example, the coregulator SRC3 is overexpressed or amplified in breast tumors (44;45) and now is considered a proto oncogene. Overexpression of SRC3/AIB1 promoted tumorigenesis in transgenic mice models (46;47), while SRC3 (AIB1) KO mice model showed that normal expression of SRC3/AIB1 is required for initiation of tumorigenesis by carcinogens and oncogenes (48;49). In addition, deregulation of the ER coregulator MTA1 promoted tumorigenesis via upregulation of the cyclin D1 pathway in transgenic mice model (50). A recent study reported that PELP1/MNAR is another ER coregulator that has tumorigenic potential. In this study, deregulation of PELP1 expression is shown to promote hormonal independent growth of breast cancer cells in nude mice model (51;52). Collectively, these findings suggest that, several ER-coregulatory proteins are differentially expressed in malignant tumors, and that their functions may be

altered. This coregulator dysregulation then influences target gene expression, leading to eventual development of hormone-resistant cancers.

8. Evidence for coregulator deregulation in various cancers

8.1 Breast cancer: Many members of the steroid receptor coregulator family, including SRC3/AIB1 are reported to be deregulated in women's cancers (53). A recent study found that PELP1/MNAR expression is deregulated in high-grade invasive tumors when compared with that in normal breast tissue and ductal carcinoma in situ tumors (51).

The expression level of E6-associated protein (E6-AP), an E3 ubiquitin-protein ligase decreases in tumors in comparison to the adjacent normal tissues (54). Since ER plays a major role in breast cancer development, changes in the expression level of E6-AP, a coactivator for ER, may interfere with the normal functioning of ER, and hence participate in the formation and progression of breast tumors. Elevated amounts of CREB-binding protein (CBP) have been reported in intraductal carcinomas as compared to those amounts found in normal mammary tissue (55). *BRCA1* is a breast cancer susceptibility gene, and functions as a ligand-independent corepressor for ER. Its inherited mutations are correlated with an increased risk of breast and ovarian cancers (56-59). Alterations in the expression of these corepressors, such as N-CoR and SMRT, have been reported and these alterations will have implications in hormonal therapy. Overexpression of the ER coregulator MTA1 in breast cancer cells is shown to promote aggressive phenotypes (50).

8.2 Endometrial cancer: Higher expression of AIB1 is reported in carcinoma-associated complex atypical

hyperplasia compared to normal endometrium. High AIB1 expression in endometrial carcinoma was associated with parameters of poor prognosis(53)., There was a significant correlation between the expression of ER and SRC-1 or p300/CBP(60) in endometrial hyperplasia. Higher expression of the co-activator AIB1 was reported in the endometrium of women with polycystic ovarian syndrome (PCOS) compared to normal endometrium (61). Another study reported that the nuclear receptor coregulators SRC-1, SRC-2, SRC-3, N-CoR, and SMRT were up-regulated in malignant endometrium, suggesting a role of these coregulators in the development of endometrial carcinoma (62). Expression and localization of the ER coregulator PELP1 is widely deregulated in endometrial cancers, and PELP1 and ER were localized predominantly in the cytoplasm of high-grade endometrial tumors (63). Furthermore, PELP1/MNAR exhibits phase-dependent localization and expression in the endometrium and enhances tamoxifen-mediated partial agonist signaling in endometrial cancer cells (63).

8.3 Ovarian cancer: Several recent studies demonstrated the presence of both ER α and ER β , the principle targets of E2, in both normal as well as in ovarian epithelial cancer cells (64). Among the various ovarian cancer subtypes, ER α immunostaining was reported in >70% of the cases of serous, endometrioid, mucinous adenocarcinimas while ER β immunopositivity was reported in 30-70% of ovarian cancer cases and is present in all ovarian cancer subtypes, including serous, endometrioid, mucinous and clear cell carcinomas(64). SRC-1, SRC-2, SRC-3, N-CoR and

SMRT expression has been reported in normal ovaries and ovarian carcinomas. (61;65). The levels of N-CoR and SMRT varied widely in primary ovarian epithelial cancers (65). The expression of these coregulator genes in ovarian tumours suggests that these factors may be available to interact with the ER or other NRs. Steroid receptor RNA activator (SRA) is an RNA that coactivates steroid hormone receptor-mediated transcription *in vitro*. SRA expression is strongly up-regulated in many human tumors of the breast, uterus, and ovary, suggesting a potential role in pathogenesis (66). Studies using human ovarian cancer tissue arrays revealed over expression of PELP1/MNAR in 60% of ovarian tumors. PELP1 is deregulated in several ovarian tumor subtypes, including serous tumors, endometrioid tumors, clear cell carcinomas, and mucous tumors, and predominantly localized in the cytoplasm in ovarian tumors (67).

9. Coregulators in endocrine therapy resistance

Endocrine therapy using tamoxifen, a selective estrogen receptor modulator (SERM), has been shown to improve relapse-free and overall survival (68). More recently, aromatase inhibitors, which deplete peripheral E2 synthesis, are shown to substantially improve disease-free survival in postmenopausal women (69). Furthermore, endocrine therapy has had a positive effect on the treatment of advanced metastatic disease. Despite these positive effects, initial or acquired resistance to endocrine therapies frequently occurs. Although the mechanisms for hormonal therapy resistance remains elusive, recent studies suggest the presence of alternative signaling pathways that

activate ER may account for hormonal therapy resistance (70-72).

It has been suggested that hormonal resistance to some extent could be explained by altered nuclear hormone receptor or coregulator levels or by inappropriately increased agonist activity of SERMs (73). ER coregulatory proteins appear to play an important role in the generally observed tissue-specific effects of tamoxifen (17). Recent evidence suggests that ER coactivators, in addition to ER, are targets of growth factor. In addition, growth factor-mediated phosphorylation of ER and ER-coregulatory proteins have been shown to have a role in tamoxifen resistance (74). Recent findings suggest that an increase in coactivator concentration and activity, through increased receptor tyrosine kinase activity, may enhance positive signal transduction of an antiestrogen-ER complex. High levels of coactivator AIB1 alone or together with high levels of HER-2/neu were associated with worse disease-free survival in tamoxifen-treated patients (75).

Mislocalization of ER coregulators can also contribute to tamoxifen resistance. The MTA1s variant was reported to inhibit ER α transactivation by sequestering ER α in the cytoplasm via a direct interaction between the ER α and the MTA1s NR box (76). In another study, PELP1/MNAR was shown to be localized in the cytoplasm alone or in both the cytoplasm and nuclei in 58% of PELP1/MNAR-positive breast tumor cells (77). Altered localization of PELP1/MNAR to the cytoplasm was sufficient to trigger its interaction with the p85 subunit of PI3K, leading to activation of PI3K (77). Low mRNA levels of coactivator SRC-1 and

corepressor N-CoR before the onset of tamoxifen therapy have been shown to predict poor response (78;79). In addition, decreased N-CoR protein levels correlated with the acquisition of tamoxifen resistance in a transgenic mouse model system (80).

Recent studies also demonstrated that HER2 status plays an important role in the tumor-induced aromatase activity via the COX-2 pathway (81). Further, HER2 overexpression can promote ligand-independent recruitment of coactivator complexes to E2-responsive promoters, and thus may play a role in the development of Letrozole resistance (82). Deregulation of PELP1/MNAR expression increases the expression of aromatase, an enzyme that produces E2 locally (51).

Despite the epidemiological evidence supporting a role of estrogen in the ovarian cancer progression, the presence and the deregulation of ER coregulators in ovarian cancer, the response to hormonal therapy using SERMs is only observed in 10-15% of cases. It is speculated that relative balance and regulation of ER coregulatory proteins within a given target cell determine the sensitivity or resistance to SERMs (16). In addition to hormonal signaling, both deregulated epidermal growth factor receptor (EGFR) signaling and the constitutive activation of cytosolic pathways involving Src, PI3K, and AKT are implicated in the development and progression of ovarian cancer (83;84). Emerging evidence suggests that complex interactions occur between the ER and EGFR signaling components and such crosstalk may contribute to resistance to endocrine therapies (73).

10. Strategies to inhibit coregulators

Thus far, the general strategy used to block the action of NR hormones involves the use of antagonists or SERMs. However, the effectiveness of NR antagonists and SERMs can be compromised by cellular adaptations that seem to enable coactivators to bind to NR-antihormone/antagonist complexes, thus contributing to hormonal therapy resistance (85). Since many coregulators have intrinsic and associate enzymatic activities, targeting these activities in conjunction with hormonal therapy is a possible strategy. For example; inhibition of histone deacetylases (HDACs) has emerged as a potential strategy to reverse aberrant epigenetic changes associated with cancer (86). Since PRMT-mediated methylation appears to be important for some coregulator-mediated functions, methyltransferase inhibitors and/or drugs currently under development may also be useful in coregulator-driven tumors (87). Deregulation of HER2 oncogene expression/signaling has emerged as the most significant factor in the development of hormonal resistance. Since ER-coregulators are targeted by excessive ER-HER2 crosstalk leading to hormonal resistance in a subset of breast tumors (74), drugs targeting HER2 pathways in conjunction with endocrine therapies are useful in targeting coregulator-deregulated women cancers. Because multiple signaling pathways in addition to hormones are involved in activating ERs and ER coregulators, combination therapies using both endocrine and nonendocrine agents that block different pathways will have a better therapeutic effect and may delay development of hormonal resistance (10). Recent evidence implicates the ER coregulator PELP1 in playing an essential role in coupling the ER with

Src kinases and in leading to hormonal resistance. Since PELP1 is the only ER coregulator that is shown to couple the ER with Src kinase, and since expression of PELP1 and Src are commonly deregulated in breast cancer, blocking of the PELP1-Src pathway in combination with endocrine therapies may prevent hormonal therapy resistance and metastasis (52). Since many coregulators interact with the ligand-binding domain of the ER via the LXXLL motifs, an alternative approach to inhibit ER functions may be to develop compounds capable of blocking the ER-coactivator interaction itself. Indeed, recent studies show that peptides having the LXXLL sequence are able to block gene transcription induced by estrogen agonists working through the ER (88). Thus, small molecule analogues that perform the same function as these peptides acting as coactivator-binding inhibitors (CBIs) by blocking ER-

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13. References

- (1) McDonnell DP, Norris JD. Connections and regulation of the human estrogen receptor. *Science* 2002 May 31;296(5573):1642-4.
- (2) Bain DL, Heneghan AF, Connaghan-Jones KD, Miura MT. Nuclear receptor structure: implications for function. *Annu Rev Physiol* 2007;69:201-20.
- (3) Tsai MJ, O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* 1994;63:451-86.
- (4) Collingwood TN, Urnov FD, Wolffe AP. Nuclear receptors: coactivators, corepressors and chromatin remodeling in the control of transcription. *J Mol Endocrinol* 1999 Dec;23(3):255-75.
- (5) McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev* 1999 Jun;20(3):321-44.
- (6) Losel R, Wehling M. Nongenomic actions of steroid hormones. *Nat Rev Mol Cell Biol* 2003 Jan;4(1):46-56.
- (7) Manavathi B, Kumar R. Steering estrogen signals from the plasma membrane to the nucleus: Two sides of the coin. *J Cell Physiol* 2006 Jun;207(3):594-604

coregulator interactions may have a net antagonistic effect which is independent of ligand binding to the ER (21).

11. CONCLUSION

The role of coregulators as proto-oncogenes is an emerging area in the field of women's cancer research and thus represents a potential area for therapeutic targeting (Figure 1). A common theme is the occurrence of marked alterations in the levels and functions of coregulators during the progression of tumorigenesis. Endocrine therapy, including anti-estrogen therapy for ovarian cancers, has yielded very limited responses, thus re-evaluation of therapy with regard to receptor status, coregulator profile and tumor type may be warranted. A better knowledge of the critical coregulators involved in NR signaling pathways is essential to further exploit hormonal therapy and to identify new targets for prevention and treatment of women's cancers.

- (8) Wierman ME. Sex steroid effects at target tissues: mechanisms of action. *Adv Physiol Educ* 2007 Mar;31(1):26-33.
- (9) Osborne CK, Zhao H, Fuqua SA. Selective estrogen receptor modulators: structure, function, and clinical use. *J Clin Oncol* 2000 Sep;18(17):3172-86.
- (10) Ali S, Coombes RC. Endocrine-responsive breast cancer and strategies for combating resistance. *Nat Rev Cancer* 2002 Feb;2(2):101-12.
- (11) Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. *Clin Cancer Res* 2003 Jun;9(6):1980-9.
- (12) Cannistra SA. Cancer of the ovary. *N Engl J Med* 1993 Nov 18;329(21):1550-9.
- (13) Ho SM. Estrogen, progesterone and epithelial ovarian cancer. *Reprod Biol Endocrinol* 2003 Oct 7;1:73.
- (14) Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, et al. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocr Rev* 1994 Jun;15(3):342-55.
- (15) James VH, McNeill JM, Lai LC, Newton CJ, Ghilchik MW, Reed MJ. Aromatase activity in normal breast and breast tumor tissues: in vivo and in vitro studies. *Steroids* 1987 Jul;50(1-3):269-79.
- (16) Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocr Rev* 2004 Feb;25(1):45-71.
- (17) Lonard DM, O'Malley BW. Expanding functional diversity of the coactivators. *Trends Biochem Sci.* 2005 Mar;30(3):126-32
- (18) Lonard DM, O'Malley BW. The expanding cosmos of nuclear receptor coactivators. *Cell* 2006 May 5;125(3):411-4.
- (19) Rosenfeld MG, Lunyak VV, Glass CK. Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev* 2006 Jun 1;20(11):1405-28.
- (20) Heery DM, Kalkhoven E, Hoare S, Parker MG. A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature* 1997 Jun 12;387(6634):733-6.
- (21) Hall JM, McDonnell DP. Coregulators in nuclear estrogen receptor action: from concept to therapeutic targeting. *Mol Interv* 2005 Dec;5(6):343-57.
- (22) Barnes CJ, Vadlamudi RK, Kumar R. Novel estrogen receptor coregulators and signaling molecules in human diseases. *Cell Mol Life Sci* 2004 Feb;61(3):281-91.
- (23) Belandia B, Orford RL, Hurst HC, Parker MG. Targeting of SWI/SNF chromatin remodeling complexes to estrogen-responsive genes. *EMBO J* 2002 Aug 1;21(15):4094-103.
- (24) Cheung E, Zarifyan AS, Kraus WL. Histone H1 represses estrogen receptor alpha transcriptional activity by selectively inhibiting receptor-mediated transcription initiation. *Mol Cell Biol* 2002 Apr;22(8):2463-71.
- (25) Bastien J, Rochette-Egly C. Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene* 2004 Mar 17;328:1-16.
- (26) Dilworth FJ, Chambon P. Nuclear receptors coordinate the activities of chromatin remodeling complexes and coactivators to facilitate initiation of transcription. *Oncogene* 2001 May 28;20(24):3047-54.

- (27) Hermanson O, Glass CK, Rosenfeld MG. Nuclear receptor coregulators: multiple modes of modification. *Trends Endocrinol Metab* 2002 Mar;13(2):55-60.
- (28) Kinyamu HK, Archer TK. Modifying chromatin to permit steroid hormone receptor-dependent transcription. *Biochim Biophys Acta* 2004 Mar 15;1677(1-3):30-45.
- (29) McKenna NJ, O'Malley BW. Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* 2002 Feb 22;108(4):465-74.
- (30) Auboeuf D, Batsche E, Dutertre M, Muchardt C, O'Malley BW. Coregulators: transducing signal from transcription to alternative splicing. *Trends Endocrinol Metab* 2007 Feb; In press.
- (31) Levin ER. Integration of the extranuclear and nuclear actions of estrogen. *Mol Endocrinol* 2005 Aug;19(8):1951-9.
- (32) Song RX, Santen RJ. Membrane initiated estrogen signaling in breast cancer. *Biol Reprod* 2006 Jul;75(1):9-16.
- (33) Acconcia F, Kumar R. Signaling regulation of genomic and nongenomic functions of estrogen receptors. *Cancer Lett* 2006 Jul 8;238(1):1-14.
- (34) Wu RC, Qin J, Yi P, Wong J, Tsai SY, Tsai MJ, et al. Selective phosphorylations of the SRC-3/AIB1 coactivator integrate genomic responses to multiple cellular signaling pathways. *Mol Cell* 2004 Sep 24;15(6):937-49.
- (35) McKenna NJ, O'Malley BW. Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* 2002 Feb 22;108(4):465-74.
- (36) McKenna NJ, Xu J, Nawaz Z, Tsai SY, Tsai MJ, O'Malley BW. Nuclear receptor coactivators: multiple enzymes, multiple complexes, multiple functions. *J Steroid Biochem Mol Biol*. 1999 Apr-Jun;69(1-6):3-12
- (37) Chen D, Ma H, Hong H, Koh SS, Huang SM, Schurter BT, et al. Regulation of transcription by a protein methyltransferase. *Science* 1999 Jun 25;284(5423):2174-7.
- (38) O'Malley BW. Molecular biology. Little molecules with big goals. *Science* 2006 Sep 22;313(5794):1749-50.
- (39) Rosenfeld MG, Lunyak VV, Glass CK. Sensors and signals: coactivator/corepressor/ epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes and Development* 2006 Jun 1;20(11):1405-28.
- (40) Han SJ, DeMayo FJ, Xu J, Tsai SY, Tsai MJ, O'Malley BW. Steroid receptor coactivator (SRC)-1 and SRC-3 differentially modulate tissue-specific activation functions of the progesterone receptor. *Mol Endocrinol* 2006 Jan;20(1):45-55.
- (41) Xu J, Qiu Y, DeMayo FJ, Tsai SY, Tsai MJ, O'Malley BW. Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. *Science* 1998 Mar 20;279(5358):1922-5.
- (42) Xu J, Liao L, Ning G, Yoshida-Komiya H, Deng C, O'Malley BW. The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. *Proc Natl Acad Sci U S A* 2000 Jun 6;97(12):6379-84.
- (43) O'Malley BW. Coregulators: From whence came these master genes. *Mol Endocrinol* 2007 Feb 6 In press.

- (44) List HJ, Reiter R, Singh B, Wellstein A, Riegel AT. Expression of the nuclear coactivator AIB1 in normal and malignant breast tissue. *Breast Cancer Res Treat* 2001 Jul;68(1):21-8.
- (45) Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, et al. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* 1997 Aug 15;277(5328):965-8.
- (46) Kuang SQ, Liao L, Wang S, Medina D, O'Malley BW, Xu J. AIB1/SRC-3 deficiency affects insulin-like growth factor I signaling pathway and suppresses v-Ha-ras-induced breast cancer initiation and progression in mice. *Cancer Res* 2005 Sep 1;65(17):7993-02.
- (47) Torres-Arzayus MI, Font de MJ, Yuan J, Vazquez F, Bronson R, Rue M, et al. High tumor incidence and activation of the PI3K/AKT pathway in transgenic mice define AIB1 as an oncogene. *Cancer Cell* 2004 Sep;6(3):263-74.
- (48) Kuang SQ, Liao L, Wang S, Medina D, O'Malley BW, Xu J. Mice lacking the amplified in breast cancer 1/steroid receptor coactivator-3 are resistant to chemical carcinogen-induced mammary tumorigenesis. *Cancer Res* 2005 Sep 1;65(17):7993-8002.
- (49) Kuang SQ, Liao L, Zhang H, Lee AV, O'Malley BW, Xu J. AIB1/SRC-3 deficiency affects insulin-like growth factor I signaling pathway and suppresses v-Ha-ras-induced breast cancer initiation and progression in mice. *Cancer Res* 2004 Mar 1;64(5):1875-85.
- (50) Bagheri-Yarmand R, Talukder AH, Wang RA, Vadlamudi RK, Kumar R. Metastasis-associated protein 1 deregulation causes inappropriate mammary gland development and tumorigenesis. *Development* 2004 Jul;131(14):3469-79.
- (51) Rajhans R, Nair S, tekmal R, Kumar R, Vadlamudi RK. Cellular transformation and oncogenesis by PELP1/NMAR, a major broad coactivator in mammalian cells. *Proc Amer Cancer Res* 2006, 47:4215.
- (52) Rajhans R, Vadlamudi RK. Comprehensive analysis of recent biochemical and biologic findings regarding a newly discovered NR coregulator PELP1/MNAR. *Journal of Clinical and experimental Metastasis*. 2006;23(1):1-7
- (53) Balmer NN, Richer JK, Spoelstra NS, Torkko KC, Lyle PL, Singh M. Steroid receptor coactivator AIB1 in endometrial carcinoma, hyperplasia and normal endometrium: Correlation with clinicopathologic parameters and biomarkers. *Mod Pathol* 2006 Dec;19(12):1593-605.
- (54) Gao X, Mohsin SK, Gatalica Z, Fu G, Sharma P, Nawaz Z. Decreased expression of e6-associated protein in breast and prostate carcinomas. *Endocrinology* 2005 Apr;146(4):1707-12.
- (55) Kurebayashi J, Otsuki T, Kunisue H, Tanaka K, Yamamoto S, Sonoo H. Expression levels of estrogen receptor-alpha, estrogen receptor-beta, coactivators, and corepressors in breast cancer. *Clin Cancer Res* 2000 Feb;6(2):512-8.
- (56) Martin AM, Blackwood MA, ntin-Ozerkis D, Shih HA, Calzone K, Colligon TA, et al. Germline mutations in BRCA1 and BRCA2 in breast-ovarian families from a breast cancer risk evaluation clinic. *J Clin Oncol* 2001 Apr 15;19(8):2247-53.
- (57) Fan S, Ma YX, Wang C, Yuan RQ, Meng Q, Wang JA, et al. p300 Modulates the BRCA1 inhibition of estrogen receptor activity. *Cancer Res* 2002 Jan 1 ;62(1):141-51.

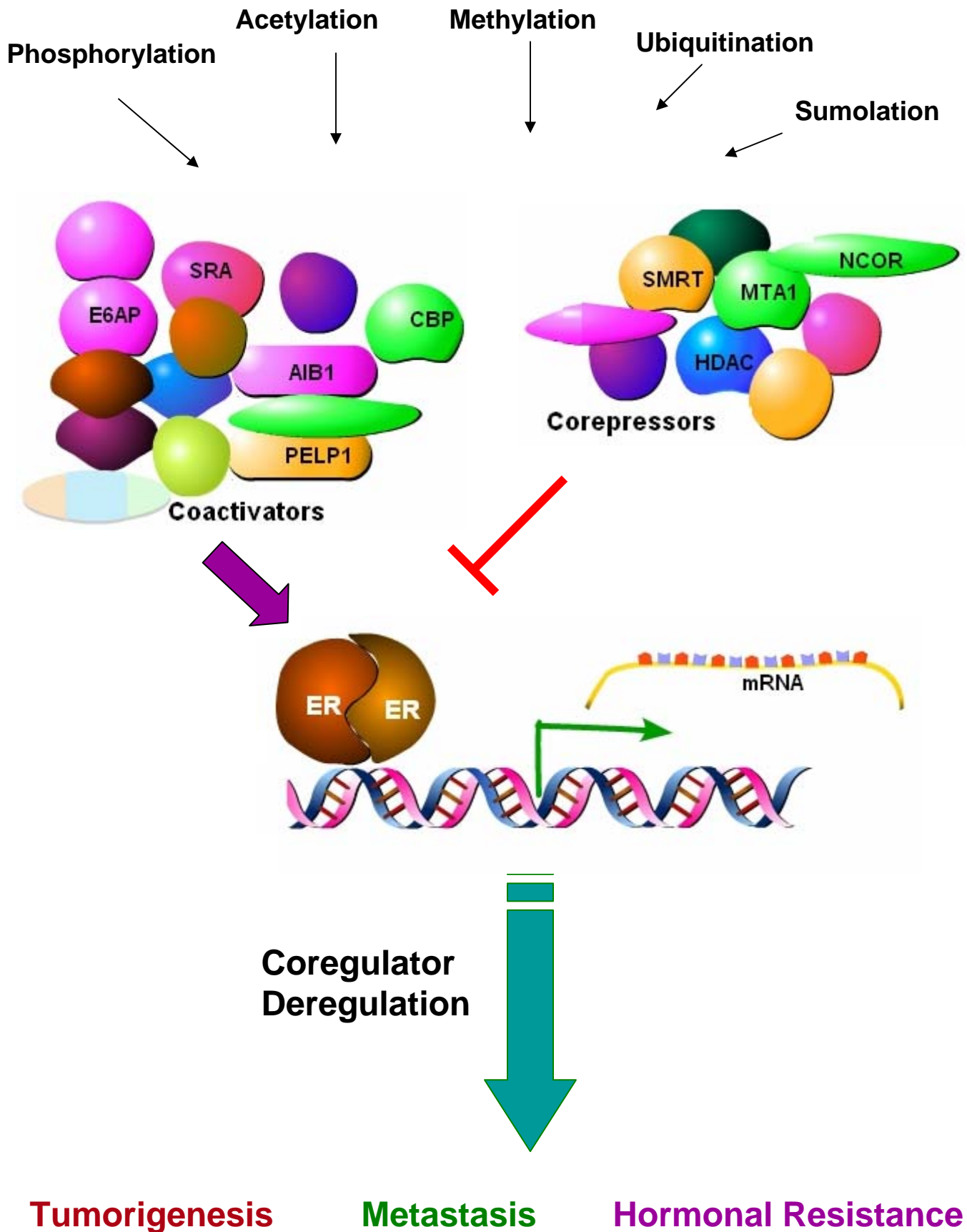
- (58) Ouchi T, Monteiro AN, August A, Aaronson SA, Hanafusa H. BRCA1 regulates p53-dependent gene expression. *Proc Natl Acad Sci U S A* 1998 Mar 3;95(5):2302-6.
- (59) Zheng L, Annab LA, Afshari CA, Lee WH, Boyer TG. BRCA1 mediates ligand-independent transcriptional repression of the estrogen receptor 1. *Proc Natl Acad Sci U S A* 2001 Aug 14;98(17):9587-92.
- (60) Uchikawa J, Shiozawa T, Shih HC, Miyamoto T, Feng YZ, Kashima H, et al. Expression of steroid receptor coactivators and corepressors in human endometrial hyperplasia and carcinoma with relevance to steroid receptors and Ki-67 expression. *Cancer* 2003 Nov 15;98(10):2207-13.
- (61) Villavicencio A, Bacallao K, Avellaira C, Gabler F, Fuentes A, Vega M. Androgen and estrogen receptors and co-regulators levels in endometria from patients with polycystic ovarian syndrome with and without endometrial hyperplasia. *Gynecol Oncol* 2006 Oct;103(1):307-14.
- (62) Kershah SM, Desouki MM, Koterba KL, Rowan BG. Expression of estrogen receptor coregulators in normal and malignant human endometrium. *Gynecol Oncol* 2004 Jan;92(1):304-13.
- (63) Vadlamudi RK, Balasenthil S, Broaddus RR, Gustafsson JA, Kumar R. 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* 2004 Dec;89(12):6130-8.
- (64) Fujimura M, Hidaka T, Kataoka K, Yamakawa Y, Akada S, Teranishi A, et al. Absence of estrogen receptor- α expression in human ovarian clear cell adenocarcinoma compared with ovarian serous, endometrioid, and mucinous adenocarcinoma. *Am J Surg Pathol* 2001 May;25(5):667-72.
- (65) Havrilesky LJ, McMahon CP, Lobenhofer EK, Whitaker R, Marks JR, Berchuck A. Relationship between expression of coactivators and corepressors of hormone receptors and resistance of ovarian cancers to growth regulation by steroid hormones. *J Soc Gynecol Investig* 2001 Mar;8(2):104-13.
- (66) Lanz RB, Chua SS, Barron N, Soder BM, DeMayo F, O'Malley BW. Steroid receptor RNA activator stimulates proliferation as well as apoptosis in vivo. *Mol Cell Biol* 2003 Oct;23(20):7163-76.
- (67) Chakravarty D, Nair S, Liu J, Balasenthil S, Tekmal RR, Burrow ME, Auersperg N, Broaddus RR, and Vadlamudi RK—PELP1/MNAR in Ovarian Cancer: Implications in Tumorigenesis. *AACR Proceedings* . 4345,2007.
- (68) Lewis-Wambi JS, Jordan VC. Treatment of Postmenopausal Breast Cancer with Selective Estrogen Receptor Modulators (SERMs). *Breast Dis* 2005;24:93-105.:93-105.
- (69) Leary A, Dowsett M. Combination therapy with aromatase inhibitors: the next era of breast cancer treatment? *Br J Cancer* 2006 Sep 18;95(6):661-6
- (70) Adjei AA, Hidalgo M. Intracellular signal transduction pathway proteins as targets for cancer therapy. *J Clin Oncol* 2005 Aug 10;23(23):5386-403.
- (71) Barletta F, Wong CW, McNally C, Komm BS, Katzenellenbogen B, Cheskis BJ. Characterization of the interactions of estrogen receptor and MNAR in the activation of cSrc. *Mol Endocrinol* 2004 May;18(5):1096-108.

- (72) Wong CW, McNally C, Nickbarg E, Komm BS, Cheskis BJ. Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with Src/Erk phosphorylation cascade. *Proc Natl Acad Sci U S A* 2002 Nov 12;99(23):14783-8.
- (73) Schiff R, Massarweh S, Shou J, Osborne CK. Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregulators modulate response. *Clin Cancer Res* 2003 Jan;9(1 Pt 2):447S-54S.
- (74) Shou J, Massarweh S, Osborne CK, Wakeling AE, Ali S, Weiss H, et al. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. *J Natl Cancer Inst* 2004 Jun 16;96(12):926-35.
- (75) Osborne CK, Bardou V, Hopp TA, Chamness GC, Hilsenbeck SG, Fuqua SA, et al. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *J Natl Cancer Inst* 2003 Mar 5;95(5):353-61.
- (76) Kumar R, Wang RA, Mazumdar A, Talukder AH, Mandal M, Yang Z, et al. A naturally occurring MTA1 variant sequesters oestrogen receptor-alpha in the cytoplasm. *Nature* 2002 Aug 8;418(6898):654-7.
- (77) Vadlamudi RK, Manavathi B, Balasenthil S, Nair SS, Yang Z, Sahin AA, et al. Functional implications of altered subcellular localization of PELP1 in breast cancer cells. *Cancer Res* 2005 Sep 1;65(17):7724-32.
- (78) Berns EM, van S, I, Klijn JG, Foekens JA. Predictive value of SRC-1 for tamoxifen response of recurrent breast cancer. *Breast Cancer Res Treat* 1998 Mar;48(1):87-92.
- (79) Girault I, Lerebours F, Amarir S, Tozlu S, Tubiana-Hulin M, Lidereau R, et al. Expression analysis of estrogen receptor alpha coregulators in breast carcinoma: evidence that NCOR1 expression is predictive of the response to tamoxifen. *Clin Cancer Res* 2003 Apr;9(4):1259-66.
- (80) Lavinsky RM, Jepsen K, Heinzl T, Torchia J, Mullen TM, Schiff R, et al. Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proc Natl Acad Sci U S A* 1998 Mar 17;95(6):2920-5.
- (81) Subbaramaiah K, Howe LR, Port ER, Brogi E, Fishman J, Liu CH, et al. HER-2/neu status is a determinant of mammary aromatase activity in vivo: evidence for a cyclooxygenase-2-dependent mechanism. *Cancer Res* 2006 May 15;66(10):5504-11.
- (82) Shin I, Miller T, Arteaga CL. ErbB receptor signaling and therapeutic resistance to aromatase inhibitors. *Clin Cancer Res* 2006 Feb 1;12(3 Pt 2):1008s-12s.
- (83) Altomare DA, Wang HQ, Skele KL, De RA, Klein-Szanto AJ, Godwin AK, et al. AKT and mTOR phosphorylation is frequently detected in ovarian cancer and can be targeted to disrupt ovarian tumor cell growth. *Oncogene* 2004 Jul 29;23(34):5853-7.
- (84) Cannistra SA. Cancer of the ovary. *N Engl J Med* 1993 Nov 18;329(21):1550-9.
- (85) Shang Y, Brown M. Molecular determinants for the tissue specificity of SERMs. *Science* 2002 Mar 29;295(5564):2465-8.
- (86) Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. *Nat Rev Drug Discov* 2006 Sep;5(9):769-84.

- (87) Krause CD, Yang ZH, Kim YS, Lee JH, Cook JR, Pestka S. Protein arginine methyltransferases: evolution and assessment of their pharmacological and therapeutic potential. *Pharmacol Ther* 2007 Jan;113(1):50-87.
- (88) Norris JD, Paige LA, Christensen DJ, Chang CY, Huacani MR, Fan D, et al. Peptide antagonists of the human estrogen receptor. *Science* 1999 Jul 30;285(5428):744-6.

Figure legend

Figure 1. Schematic representation of estrogen receptor signaling in the nuclear compartment. In addition to estrogen binding, multiple coregulator complexes are required for optimal activation of the ER-mediated transcription. Coregulators appear to function in groups and their functions are regulated by posttranslational modifications including phosphorylation, acetylation, methylation and ubiquitination. Emerging evidence suggest that deregulation of coregulator proteins can promote tumorigenesis and hormonal independence. Few coregulators that are shown to be deregulated in cancer are depicted in the figure.



Appendix 4.

Chakravarty D, Nair SS, Rajhans R, Piteshwara RP, Jinsong L, Seetharaman B, Le XF, Burrow ME, Nelly A, Tekmal RR, Broaddus RR, **Vadlamudi RK**. Role of PELP1/MNAR signaling in ovarian tumorigenesis. Cancer Research, April, 2008 (In press)

Role of PELP1/MNAR signaling in ovarian tumorigenesis

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Abstract

Emerging evidence suggests that nuclear receptor (NR) coregulators have potential to act as master genes and their deregulation can promote oncogenesis. Proline-, glutamic acid-, and leucine-rich protein-1 (PELP1/MNAR) is a novel NR coregulator. Its expression is deregulated in hormone-driven cancers. However, the role of PELP1/MNAR in ovarian cancer progression remains unknown. Analysis of SAGE data suggested deregulation of PELP1/MNAR expression in ovarian tumors. Western analysis of PELP1/MNAR in normal and serous ovarian tumor tissues, showed 3 to 4 fold higher PELP1/MNAR expression in serous tumors compared to normal ovarian tissues. To examine the significance of PELP1/MNAR in ovarian cancer progression, we have generated *model* cells that overexpress PELP1/MNAR and ovarian cancer cells in which PELP1/MNAR expression is down regulated by stable expression of PELP1/MNAR-specific shRNA. Down regulation of PELP1/MNAR in cancerous ovarian model cells (OVCAR3) resulted in reduced proliferation, affected the magnitude of c-Src and AKT signaling and reduced tumorigenic potential of ovarian cancer cells in a nude mouse model. PELP1/MNAR overexpression in non-tumorigenic immortalized surface epithelial cells (IOSE cells) promoted constitutive activation of c-Src and AKT signaling pathways and promoted anchorage independent growth. IHC studies using human ovarian cancer tissue arrays (n=123) showed that PELP1/MNAR is 2 to 3 fold overexpressed in 60% of ovarian tumors and PELP1/MNAR deregulation occurs in all different types of ovarian cancer. Collectively these results suggest that PELP1/MNAR signaling plays a role in ovarian cancer cell proliferation and survival, and that its expression is deregulated in ovarian carcinomas.

Introduction

Amongst the gynecological malignancies in Western countries, ovarian cancer has the highest mortality rate and is the most common female malignancies (1). Despite modest improvements in response rates, overall survival rates remain disappointing for patients with advanced ovarian cancer (2). Ovarian cancer is an endocrine-related cancer, but it is still unclear which hormone-regulated mechanisms are critical in the pathogenesis of ovarian cancer (3). The biological functions of steroid hormones are mediated by nuclear receptors (NRs), a super family of ligand-regulated transcription factors that modulate a wide range of biological processes (4). In addition to exerting their well-studied nuclear functions, the NRs also participates in cytoplasmic and membrane-mediated signaling events through the stimulation of the Src kinase, mitogen-activated protein kinase (MAPK), and protein kinase B (AKT) pathways (5-7). Emerging evidence implicates NR signaling in the progression of ovarian cancer and several recent studies demonstrated the presence of NRs, including ER α , ER β , GR, PR, AR, ERR α , ERR β in ovarian epithelial cancer cells and tumors (8-12).

In the past decade it has become increasingly clear that the sole existence of NRs is not sufficient to account for the diverse biological roles of NRs. Coregulators that interact with NRs appear to provide an additional level of complexity in NR action and coregulator composition in a given cell determines the magnitude and specificity of the NR signaling (13;14). Many coregulatory proteins are present at rate-limiting levels, shared by many NRs and thus have the potential to coordinately regulate cell processes such as proliferation, translation, energy generation, and motility (4;14;15). With the enormous potential of coregulators as master regulators, their deregulation is likely to provide the cancer cells an advantage in proliferation, survival, and metastasis (15;16). A few recent studies examined the status of NR coregulators in

ovarian cancer cells and tumors and found deregulation of few coregulators including AIB1, SRA, and ARA70 (17-19). Collectively, these emerging findings suggest that several NR-coregulatory proteins have potential to be differentially expressed in malignant tumors, and that their functions may be altered, leading to tumor progression.

Proline-, glutamic acid-, leucine-rich protein-1 (PELP1/MNAR) (20), also known as modulator of the nongenomic actions of the estrogen receptor, MNAR (21), is unique because it plays important roles both in the genomic (22) and the non-genomic actions of the NRs (5;23). PELP1/MNAR promotes cell proliferation by sensitizing cells to G1>S progression via its interactions with the pRb pathway (24). In the nuclear compartment PELP1/MNAR interacts with histones and histone-modifying enzymes, and thus plays a role in chromatin remodeling for ligand-bound NRs (25). Recent evidence also suggests that PELP1/MNAR couples NRs to several signaling pathways such as Src-MAPK, PI3K-AKT, and EGFR-STAT3 (5;26) and that PELP1/MNAR expression is deregulated in human breast and endometrial cancers (25;27). Although these studies suggested that PELP1/MNAR has tumorigenic potential, whether PELP1/MNAR plays a role in ovarian cancer has not yet been defined.

In this study, we investigated whether PELP1/MNAR signaling and expression play roles in ovarian tumorigenesis. Using cell line models, we provided evidence that PELP1/MNAR signaling plays a critical role in ovarian cancer cell proliferation and signaling. Using PELP1/MNAR-specific shRNA and a nude mouse model, we provided evidence that PELP1/MNAR play a significant role in the survival and progression of ovarian tumors. Using human tumor tissue arrays, we demonstrate for the first time that PELP1/MNAR expression is deregulated in several ovarian tumor subtypes, including serous tumors, endometrioid tumors,

clear cell carcinomas, and mucinous tumors, and thus may prove to be a useful tool as a diagnostic or prognostic marker.

Materials and Methods

Cell cultures and reagents. OVCAR3 cells were purchased from American-type culture collection (ATCC) and maintained in RPMI 1640 supplemented with 20% serum. SKOV3 cells obtained from ATCC were maintained in McCoy's 5A supplemented with 10% serum. BG1 cells were earlier described (28) and were maintained in RPMI 1640 supplemented with 10% fetal calf serum. Non-tumorigenic SV40 Tag-immortalized ovarian surface epithelial derived cells (IOSE-80) were earlier described (29) and cultured in medium 199:MCDB 105 (1:1; Sigma-Aldrich Corp., St Louis, MO) containing 15% fetal bovine serum (FBS; Hyclone Laboratories Ltd, Logan, UT), 100 U/ml penicillin G and 100 mg/ml streptomycin (Life Technologies, Inc., Rockville, MD). Antibodies against vinculin were purchased from Sigma Chemical Co (St. Louis, MO). GFP-epitope antibody was purchased from Clontech (Mountain View, CA). PELP1/MNAR antibody was purchased from Bethyl laboratories (Montgomery, TX). Antibodies against phospho-AKT, phospho-MAPK, Phospho-GSK3, phospho-NF-Kappa-B, and phospho-Src were purchased from Cell Signaling (Beverly, MA).

Generation of PELP1/MNAR model cells. OVCAR3 cells stably expressing PELP1/MNAR-shRNA and BG1 cells stably expressing PELP1/MNAR were generated using FuGENE-6 transfection (Roche, Indianapolis, IN) and were selected using G418 selection (1 mg/mL). For IOSE, and SKOV3 model cell generation, we used electroporation (Nucleofection) to transfect PELP1/MNAR plasmids or shRNAs according to the manufacturer's instructions (Amaxa Biosystems, Hannover, Germany). With this method we achieved 80-90% transfection

efficiency as monitored by GFP expression. Briefly, 10^7 cells at 70% confluence were transfected with either 5 μ g of GFP-PELP1/MNAR or PELP1/MNAR-ShRNA DNA or appropriate vectors. The cells were initially plated in a six-well plate on poly-D-lysine-coated plates. After 24 h the cells were transferred to regular tissue culture plates. The effects of PELP1/MNAR overexpression or down regulation was measured after 72 h by using Western blot analysis. ERE and Cyclin D1 luciferase were performed as described earlier (24). For rescue experiments, 5 μ g of activated p110 (pBJ-Myr-p110*) or activated AKT (pcDNA-Myr-AKT) plasmids were transfected in OVCAR3-PELP1shRNA clones using Nucleofection.

RNA interference. Four PELP1/MNAR-specific shRNA (SureSilencing shRNA plasmids, catalogue # KH19454N) and control shRNA vector were purchased from SuperArray (Frederick, MD). The targeted sequences are shRNA1:GGACCAAGGTGTATGCGATAT; shRNA2; AAGGAGGAGCCTGAAGAACTT; shRNA3:ATGCTGCTGTCCTCAGAAGAT; shRNA-4 AGACCAGCCTTTGTCCACTAT:. PELP1/MNAR-shRNA design was based on the GenBank accession number NM_014389. PELP1/MNAR shRNA transfections were performed using Fugene6.

Proliferation, soft-agar and PI3K assays. Proliferation of PELP1 shRNA clones was measured by a modified 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay using the MTT assay kit as per the manufacturer's instructions (Sigma). Briefly, the cells were plated on 24-well plates at a density of $\sim 10,000$ cells per well, and growth was measured after 72 h. Each assay was done in quadruplicate, and the mean and the SD were calculated. Soft-agar colony-growth assays were performed as previously described (5). Total protein lysates from OVCAR3 or OVCAR3 shRNA clones were immunoprecipitated with p85 antibody and PI3K kinase assay was performed as described earlier (5).

Tumorigenesis assays. For the tumorigenesis studies, either OVCAR3 or OVCAR3-PELP1/MNAR-shRNA clone (5×10^7 cells) were implanted subcutaneously into the left flank of 6- to 7-week-old female nude mice ($n = 7$) and tumors were allowed to grow for 8 weeks. Tumor volumes were measured with a caliper at weekly intervals. After 8 weeks, the mice were sacrificed, and the tumors were removed and processed for immunohistochemistry (IHC). Prior to sacrifice BrdU was injected via intra-peritoneal route into the mice.

Tissue microarrays (TMAs). The TMAs used in this study were obtained from the UTMDACC and IHC was performed as described earlier (5;30). These TMA arrays are suitable for use in the investigation of differences in the prevalence of potential markers in various types of ovarian cancer: serous, mucinous, endometrioid, and clear cell. The preparation of negative controls was accomplished by replacing the primary antibody with control rabbit IgG or peptide-absorbed PELP1/MNAR antibody. The finding that no cells or $< 10\%$ of the cells were immunoreactive was considered to be a negative result, and the finding that $> 10\%$ of the cells were immunoreactive was considered a positive result. PELP1/MNAR staining and scoring was performed according to the established method (5;30), and the results were classified as follows: zero, no expression; 1, weak expression; 2, moderate expression; and 3, strong expression. The differences in the staining intensity between experimental groups was analyzed using one-tailed Student's *t* test analysis.

Results

PELP1/MNAR Expression is Upregulated in Ovarian Cancer Cells and Tumors. Emerging studies have shown that PELP1/MNAR expression is deregulated in breast, prostate and endometrial tumors (25). To examine if this deregulation occurs in other cancers, we initially examined the expression of PELP1/MNAR ESTs in the recently published NCI's Cancer

Genome Anatomy Project (CGAP) databases. The results from this search suggested that PELP1/MNAR expression indeed is upregulated in breast and prostate tumors confirming the published studies. Interestingly, this analysis also revealed increased presence of PELP1/MNAR expressed sequence tags (EST) in ovarian tumors compared to the normal ovary (Fig. 1A). To confirm that PELP1/MNAR expression indeed is deregulated in ovarian tumors, we have analyzed expression of PELP1/MNAR in high grade serous ovarian tumor tissues (n=10) and compared to the PELP1/MNAR expression in the normal ovary (n=5) using Western blotting. The results showed that PELP1/MNAR expression is 2 to 4 fold higher in serous tumors compared to normal ovarian tissues (Fig. 1B). We have then analyzed PELP1/MNAR expression by using Western blotting in three commonly used ovarian cancer cells (SKOV3, OVCAR3, BG1) and one immortalized normal ovarian surface epithelial cell line IOSE-80 (which represent a benign ovarian neoplasty). Western analysis also revealed that ovarian cells express several PELP1/MNAR interacting nuclear receptors including ER α , ER β , ERR α , ERR γ , PR. However, expression of ER α varied among cells with high in BG1, moderate in IOSE, SKOV3 and low/undetectable in OVCAR3 cells. Western blot results also showed increased expression of coregulator PELP1/MNAR in ovarian cancer cells compared to IOSE cells (Fig. 1C). These results suggest that NR coregulator PELP1/MNAR expression is upregulated in ovarian tumors and cancer cell lines.

PELP1/MNAR expression is deregulated in ovarian tumors. We used an ovarian cancer tissue array to investigate whether PELP1/MNAR deregulation occurs in different types of ovarian cancer. We measured the expression levels of PELP1/MNAR by IHC, and the PELP1/MNAR expression was scored as previously described (5;30;34). The representative staining for each type of ovarian tumor is shown in Fig. 1D. In these studies using human

ovarian cancer tissue array (n=123), we observed a weak focal staining (0 to 1+) in 19 (15.4%) and clearly detectable PELP1/MNAR staining (+2 to +3) in 104 (84.6%) samples. A total of 59 (56.7%) tumors showed 3+ staining while 45 (43.3%) showed 2+ staining compared to normal tissues which showed PELP1 staining of +1 (Fig. 1D). Further examination of several subtypes of ovarian tumors (including serous, endometrioid, clear cell carcinoma, and mucinous tumors) suggested deregulation of PELP1/MNAR in all subtypes of ovarian cancer and 60 % of the tumors have +3 staining (Fig. 1D). However, there is no major difference in PELP1 staining between different ovarian tumors types. All tumors with high staining (+3) also showed high cytoplasmic PELP1/MNAR compared to normal ovary. Collectively, these results suggest that PELP1/MNAR expression is altered during ovarian tumor progression and is upregulated in several subtypes of ovarian tumors.

PELP1/MNAR down regulation affects ovarian cell proliferation. To examine the function of endogenous PELP1/MNAR in ovarian cancer cells, we use the recently developed shRNA methodology (31;32) as a means of reverse genetics to assess the function PELP1/MNAR in ovarian cancer cells. Initially using transient transfection assay, we have screened four shRNAs and identified two shRNAs (PELP1/MNAR-shRNA1 and PELP1/MNAR-shRNA2) that caused greater than 80% reduction in the endogenous PELP1/MNAR levels. These two shRNAs target two distinct regions of PELP1/MNAR thus serve as confirmatory controls for non-specific actions of shRNA. We then established OVCAR3 stable cell lines that express PELP1/MNAR-shRNA1 and -shRNA2. As a control, OVCAR3 cells were transfected with shRNA vector. Western blot analysis of total lysates revealed that the PELP1/MNAR-shRNA clones showed down regulation of PELP1/MNAR expression to ~80 % of the level seen in the parental and the vector-transfected clones (Fig. 2A). To further analyze

the role of PELP1/MNAR on proliferation of OVCAR3 cells, we measured proliferation rate of these clones under low and high serum conditions. The results show that PELP1/MNAR shRNA cells had a decrease in proliferation compared to the parental cells and that the effect of PELP1/MNAR down regulation on cell proliferation is more pronounced in low serum conditions compared to high serum (Fig. 2B). Similarly, PELP1/MNAR shRNA clones exhibited decreased anchorage independence in soft agar colony assays (Fig. 2B, right panel). Western analysis showed that PELP1 down regulation did not affect the expression levels of nuclear receptors in these cells (Fig. 2C, left panel). However, PELP1/MNAR down regulation affected the expression of PELP1/MNAR regulated NR target genes such as PR and CyclinD1 (Fig. 2C, right panel). Reporter gene assays using ERE and Cyclin D1 luciferase reporters also confirmed that PELP1 deregulation affect the expression of these genes (Fig. 2D). Collectively these results suggest that PELP1/MNAR play a critical role in the proliferation of ovarian cancer cells and PELP1/MNAR mediated NR-genomic functions may play role in the proliferation.

PELP1/MNAR down regulation affects non-genomic signaling in ovarian cancer cells. Recent studies suggest that PELP1/MNAR in addition to its participation in the NR-genomic functions, also play a critical role on NR mediated non-genomic signaling. To examine the possibility that PELP1/MNAR has a role in the activation of non-genomic signaling pathways in ovarian cells, we measured the activation of non-genomic signaling pathways that are shown to be modulated by PELP1/MNAR signaling including Src, AKT and MAPK (5;21). Total cell lysates from OVCAR3 stable cells expressing either vector or PELP1/MNAR-shRNA were analyzed using Western blotting with phospho-specific antibodies. PELP1/MNAR-shRNA-expressing cells had significantly less Src, AKT and MAPK activation compared to shRNA vector transfected cells (Fig. 3A). However, PELP1 down regulation did not affect the status of

Phospho-NF-kappa-B p65. (Fig. 3A) and we have used this as a control for phospho antibody blots in PELP1 clones.. To confirm that the signaling changes observed are due to PELP1/MNAR down regulation in OVCAR3 cells, we generated two additional ovarian model cells: BG1 cells stably expressing PELP1/MNAR (BG1-PELP1/MNAR) and SKOV3 cells expressing PELP1/MNAR shRNA. Down regulation of PELP1/MNAR in SKOV3 cells utilizing two distinct shRNAs that target PELP1/MNAR also resulted in substantial reduction of the Src and AKT signaling pathways (Fig. 3A, *right panel*) with no reduction in the phospho-NF-Kappa-B signaling. Similarly, BG1-PELP1/MNAR cells (pooled clones 1 and 2) showed 3-fold increase in PELP1/MNAR expression compared to the vector-transfected cells (Fig. 3B, *left panel*). Western analysis of the total protein lysates showed that PELP1/MNAR over expression in BG1 cells promotes increased Src, AKT and MAPK signaling with no increase in phospho-NF-kappa-B signaling (Fig. 3B, *left panel*). PELP1 over expression in BG1 cells also proliferation potential of the cells (Fig. 3B, *right panel*). Collectively, the results from these three ovarian cancer model cells suggest that PELP1/MNAR signaling plays an essential role in Src, AKT, and MAPK signaling in ovarian cancer cells.

Deregulation of PELP1/MNAR expression in IOSE cells promote excessive non-genomic signaling and anchorage independent growth. Most ovarian neoplasms arise from the ovarian surface epithelium (OSE) (2). In earlier studies, we developed immortalized OSE model cells (IOSE) from normal OSE by transfecting simian virus 40 large T antigen (33). The SV40T/t antigen inactivates both the p53 and pRb pathways and extends the life span of these cells to 10 passages while maintaining many of the properties of normal ovarian epithelium. Several studies suggested that these are good model cells to study potential oncogene functions in ovarian cancer (29). To examine the putative function of PELP1/MNAR deregulation in

ovarian tumors, we have used IOSE cells. Using Amaxa's Nucleofector transfection kit, we over expressed PELP1/MNAR in the IOSE model cells. To enable the monitoring of the transfected cells, we used GFP epitope-tagged PELP1/MNAR expression vector in these assays and GFP-vector was used as a control. The expression of PELP1/MNAR in transfected IOSE cells was analyzed by using Western analysis (Fig. 3C). This Amaxa's Nucleofector transfection typically resulted in transfection of >70% IOSE cells and generated IOSE model cells that over express 2-3 fold PELP1/MNAR compared to vector transfected IOSE cells. The status of non-genomic signaling in GFP-vector and GFP-PELP1/MNAR expressing cells was then analyzed by using Western blot analysis using phospho-specific antibodies. The results showed that PELP1/MNAR over expression substantially increased Src, AKT and MAPK signaling in IOSE model cells (Fig. 3C). Similarly, PELP1/MNAR over expressing IOSE cells exhibited increased proliferation potential and showed increased anchorage independence in soft agar colony assays (Fig. 3D).

PELP1 modulates ovarian cancer cell proliferation via PI3K-AKT pathway. Earlier studies have shown that PELP1 interacts with Src leading to activation of PI3K–AKT pathway. Since PELP1 shRNA clones exhibited decreased activation of Src and AKT kinases, we examined, whether PELP1 down regulation contributes to decreased PI3K activity. Results of PI3- kinase assay showed that PELP1 shRNA clones indeed have low PI3K activity (Fig. 4A). To examine whether functional PELP1-PI3K-Src complexes exists in ovarian cancer cells, we have performed immunoprecipitation of OVCAR3 cell lysates using antibodies that recognize endogenous Src and PELP1. The results showed that PELP1 interacts with p85 subunit of PI3K kinase and PELP1 form complexes with Src, and p85 subunit of PI3K (Fig. 4B). We also confirmed PELP1 interactions with p85 subunit of PI3K in SKOV3 cells (Fig. 4B, right panel). Since PELP1 interactions with Src kinase leads to activation of PI3K pathway, we hypothesized

that over expression of activated form of PI3K will rescue the defects in PELP1 shRNA cells. To test this hypothesis, we have performed rescue experiments in PELP1 shRNA clones using activated p110 catalytic subunit of PI3K. We have transfected the OVCAR3-PELP1shRNA cells with vector alone or vector that express activated and membrane targeted PI3K catalytic subunit (myristolated p100*) using Nucleofector transfection methodology that facilitated 80-90% transfection efficiency. The results from this experiment showed that over expression of Myr-p110 α can restore the proliferation defect seen in PELP1shRNA cells (Fig. 4C, right panel). Further, Myr-p110* over expression also restored the defect seen in the cyclin D1 levels in PELP1 shRNA clones (Fig. 4C, left panel). To further examine how defects in AKT activation leads to decreased proliferation in PELP1 shRNA clones, we examined the status of down stream effectors of AKT in PELP1 shRNA clones. We have observed a dramatic decrease in the phosphorylation of GSK3 β , a down stream target of AKT in the PELP1 shRNA clones. To further delineate the mechanism, we performed restoration experiments using activated form of AKT (Myr-AKT). Interestingly, over expression of Myr-AKT restored the defect seen in the GSK3 β phosphorylation, restored cyclin D1 levels and rescued the proliferation defect in PELP1 shRNA clones (Fig. 4D). Collectively these restoration experiments provide evidence that blockage of PI3K-AKT-GSK3 β -cyclin D1 pathway by PELP1 down regulation contributed the defect in the cells proliferation seen in the PELP1 shRNA clones.

PELP1/MNAR down regulation decreases tumorigenic potential of OVCAR3 cells *in vivo*. We then used a nude mouse xenograft model to examine whether PELP1/MNAR is required for tumorigenic potential of ovarian cancer cells *in vivo*. OVCAR3 cells stably expressing vector (n=7) or PELP1/MNAR-shRNA (n=7) were injected subcutaneously into mice and tumorigenic potential was monitored for 8 weeks (Fig. 5A). Under those conditions,

OVCAR3 vector-transfected cells formed tumors and tumor grew linearly with time. However OVCAR3-PELP1/MNAR-shRNA injected sites showed tumors with substantial reduction in growth compared to control (Fig. 5B, C). These results suggested that PELP1/MNAR expression is essential for optimal growth of ovarian tumor cells *in vivo*. IHC examination of the tumors revealed that PELP1/MNAR-shRNA tumors retained the low expression of PELP and exhibited activation of Src and AKT to a lesser degree than the activation seen in OVCAR3 vector-transfected tumors (Fig. 5D). Further BrdU staining of the tumor sections revealed increased proliferation in the OVCAR3 vector transfected xenograft tumors compared to PELP1/MNAR shRNA tumors. Also, the PELP1/MNAR shRNA tumors showed increased apoptosis as revealed by annexin staining (Fig. 5D). These results suggest that PELP1/MNAR plays an essential role in the growth of ovarian tumor cells *in vivo*.

Discussion

Ovarian cancer is the fifth leading cause of cancer death in women and is the most lethal gynecological malignancy. The lethality of ovarian carcinoma primarily stems from the inability to detect the disease at an early, organ-confined stage. The molecular basis of this disease is not completely understood (3). In this study, we identified that nuclear receptor PELP1/MNAR play a role in ovarian cancer progression and its expression is deregulated in ovarian tumors. Using normal and commonly used ovarian cancer cells and shRNA methodology, we have provided evidence that PELP1/MNAR deregulation contributes to excessive activation of non-genomic signaling pathways.

Although the progression from early- to advanced-stage ovarian cancer is a critical determinant of survival, little is known about the molecules that contribute to the progression and

metastasis of ovarian tumors. The role of NR coregulators as proto-oncogenes is an emerging area in the field of cancer research and thus represents a potential area for therapeutic targeting (16;35-37). A common theme is the occurrence of marked alterations in the levels and functions of coregulators during the progression of cancer (35;36). Recent data on several coregulator proteins support the concept of coregulators as master genes (16). For example, the coregulator AIB1 is over expressed or amplified in breast and ovarian tumors and over expression of AIB1 in ovarian carcinomas correlates with poor survival (17;38). Deregulation of another NR coregulator MTA1 in Tg mouse models is shown to induce mammary tumors (15;16;39) and also to promote the formation B cell lymphomas (40). Using shRNA methodology, we provided evidence that the NR coregulator PELP1/MNAR is another coregulator that may play a critical role in ovarian cancer progression. Wide expression of nuclear receptors and decreased expression of PR, Cyclin D1 in OVCAR3-PELP1/MNAR-shRNA clones along with decreased activity of ERE, Cyclin D1 reporter genes, suggest that PELP1/MNAR mediated NR-genomic actions may play an important role in ovarian cancer cell proliferation.

Earlier studies suggested that ovarian cancer cells have deregulated AKT signaling (41). A recent study revealed activation of PI3K and constitutive AKT phosphorylation in ovarian carcinoma (42). Studies utilizing the OVCAR3 cell line revealed that AKT plays an important role in ovarian cancer progression and that PI3K induces AKT activation in ovarian cancer cells (43). Interestingly, over expression of the NR coactivator AIB1 in a transgenic murine model, has been shown to promote a high incidence of tumors via activation of the PI3K-AKT pathway (44). In the current study, analysis of cellular signaling pathways using phospho-specific antibodies in PELP1/MNAR-overexpressing model cells revealed constitutive activation of c-Src kinase and AKT. We have also found that PELP1 form functional complexes with Src and p85

subunit of PI3K in ovarian cancer cells. The ability of PELP1/MNAR to modulate the c-Src-PI3K pathways and its potential deregulation in ovarian cancer cells suggest that the c-Src-PI3K pathway may represent one potential mechanism by which PELP1/MNAR promotes tumorigenesis in ovarian cancer cells.

PI 3-kinase has been increasingly recognized as one of the important signaling molecules required for G₁-S cell cycle progression and PI3K/Akt pathway was previously shown to regulate cell cycle progress through inducing cyclin D1 expression (46). Earlier studies have also shown that GSK3 β phosphorylation by AKT to down regulate GSK3 β activity leading to up regulation of cyclin D1 levels via stabilization (47). Knock down of PELP1 expression significantly affected cellular proliferation and reduced activation of AKT with a decrease in the GSK3 β phosphorylation. Overexpression of activated forms of PI3K catalytic subunit or activated form of AKT kinase rescued the defects in the proliferation seen in PELP1 shRNA clones. In our studies, we also found that over expression of active AKT in PELP1 shRNA clones also restores phosphorylation of GSK3 β with a concomitant increase in the cyclin D1 levels. Collectively our results suggest that PELP1 deregulation promotes ovarian cancer cell proliferation via PI3K-AKT-GSK3 β -cyclin D1 pathway.

PELP1/MNAR appears to function as a scaffolding protein by coupling NRs with several proteins implicated in oncogenesis. PELP1/MNAR modulates the ER's interaction with c-Src, stimulating c-Src enzymatic activity, leading to the activation of the mitogen activated protein kinase (MAPK) pathway (21). A recent study reported direct correlation between PELP1/MNAR expression levels and E2-induced activation of PI3K and AKT kinases and provides evidence that PELP1/MNAR exists as a complex with ER α , cSrc, and p85, the regulatory subunit of PI3 kinase (45). With the enormous potential of PELP1/MNAR as a modulator of NRs and

protoncogenes such as PI3K, Src, and STAT3, the deregulation of PELP1/MNAR expression could provide cancer cells with a advantage in survival, growth, and metastasis (25). We found that down regulation of PELP1/MNAR decreased cell proliferation, increased annexin staining and decreased ovarian tumor growth in a nude mouse model. As non-genomic functions of NRs are implicated in different cellular processes, including cell survival and apoptosis, PELP1/MNAR-mediated non-genomic actions may play a role in ovarian cancer cell survival.

PELP1/MNAR expression and localization are deregulated in breast (20;49) and endometrial tumors (30). Elevated PELP1/MNAR expression is also reported in high-grade prostate tumors (50). We used human ovarian cancer tissue arrays and found that PELP1/MNAR is over expressed 2 to 3 fold in 60% of ovarian tumors. PELP1/MNAR is deregulated in several ovarian tumor subtypes, including serous tumors, endometrioid tumors, clear cell carcinomas, and mucinous tumors. A substantial amount of PELP1/MNAR is localized in the cytoplasm in ovarian tumors. PELP1/MNAR cytoplasmic localization in ovarian tumors, increased activation of the Src, MAPK, and AKT pathways in PELP1/MNAR-overexpressing IOSE and BG1 model cells and decreased activation of these signaling pathways in PELP1/MNAR down regulated OVCAR3 and SKOV3 model cells suggests that PELP1/MNAR-mediated non-genomic signaling contributes to some extent to the tumorigenic potential of PELP1/MNAR. Future studies using a large panel of ovarian tumors are needed to evaluate the prognosis and/or diagnosis value of PELP1 expression status in ovarian cancer.

In summary, the results of our study provide the first evidence for the contribution of the NR coregulator PELP1/MNAR to the tumorigenic potential in ovarian cancer cells. Our findings also suggest that alterations in the levels or localization of PELP1/MNAR could occur during ovarian cancer progression and such alterations may provide survival advantage by excessively

activating NR mediated genomic and non-genomic signaling. Collectively, these findings suggest a possibility that the PELP1-Src-AKT axis could be used as a potential diagnostic and/or therapeutic target in ovarian cancer.

Reference List

- (1) Perez RP, Godwin AK, Hamilton TC, Ozols RF. Ovarian cancer biology. *Semin Oncol* 1991;18:186-204.
- (2) Auersperg N, Wong AS, Choi KC, Kang SK, Leung PC. Ovarian surface epithelium: biology, endocrinology, and pathology. *Endocr Rev* 2001;22:255-88.
- (3) Cannistra SA. Cancer of the ovary. *N Engl J Med* 1993;329:1550-9.
- (4) Collingwood TN, Urnov FD, Wolffe AP. Nuclear receptors: coactivators, corepressors and chromatin remodeling in the control of transcription. *J Mol Endocrinol* 1999;23:255-75.
- (5) Vadlamudi RK, Manavathi B, Balasenthil S, Nair SS, Yang Z, Sahin AA, et al. Functional implications of altered subcellular localization of PELP1 in breast cancer cells. *Cancer Res* 2005;65:7724-32.
- (6) Losel R, Wehling M. Nongenomic actions of steroid hormones. *Nat Rev Mol Cell Biol* 2003;4:46-56.
- (7) Bjornstrom L, Sjoberg M. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. *Mol Endocrinol* 2005;19:833-42.
- (8) Woenckhaus J, Franke FE, Hackethal A, Von GR, Munstedt K. Glucocorticosteroid receptors in ovarian carcinomas. *Oncol Rep* 2006;15:1137-40.
- (9) Wang PH, Chang C. Androgens and ovarian cancers. *Eur J Gynaecol Oncol* 2004;25:157-63.
- (10) Treeck O, Pfeiler G, Mitter D, Lattrich C, Piendl G, Ortmann O. Estrogen receptor {beta}1 exerts antitumoral effects on SK-OV-3 ovarian cancer cells. *J Endocrinol* 2007;193:421-33.
- (11) Fujimoto J, Alam SM, Jahan I, Sato E, Sakaguchi H, Tamaya T. Clinical implication of estrogen-related receptor (ERR) expression in ovarian cancers. *J Steroid Biochem Mol Biol* 2007;104:301-4.
- (12) Cunat S, Hoffmann P, Pujol P. Estrogens and epithelial ovarian cancer. *Gynecol Oncol* 2004;94:25-32.
- (13) McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev* 1999;20:321-44.
- (14) Lonard DM, O'Malley BW. The expanding cosmos of nuclear receptor coactivators. *Cell* 2006;125:411-4.

- (15) Rosenfeld MG, Lunyak VV, Glass CK. Sensors and signals: a coactivator/ corepressor/ epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev* 2006;20:1405-28.
- (16) O'Malley BW. Coregulators: from whence came these "master genes". *Mol Endocrinol*. *Mol Endocrinol* 2007;21:1009-13.
- (17) Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, et al. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* 1997;277:965-8.
- (18) Quezada S, Avellaira C, Johnson MC, Gabler F, Fuentes A, Vega M. Evaluation of steroid receptors, coregulators, and molecules associated with uterine receptivity in secretory endometria from untreated women with polycystic ovary syndrome. *Fertil Steril* 2006;85:1017-26.
- (19) Hussein-Fikret S, Fuller PJ. Expression of nuclear receptor coregulators in ovarian stromal and epithelial tumours. *Mol Cell Endocrinol* 2005;229:149-60.
- (20) Vadlamudi RK, Wang RA, Mazumdar A, Kim Y, Shin J, Sahin A, et al. Molecular cloning and characterization of PELP1, a novel human coregulator of estrogen receptor alpha. *J Biol Chem* 2001;276:38272-9.
- (21) Wong CW, McNally C, Nickbarg E, Komm BS, Cheskis BJ. Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with Src/Erk phosphorylation cascade. *Proc Natl Acad Sci U S A* 2002;99:14783-8.
- (22) Nair SS, Mishra SK, Yang Z, Balasenthil S, Kumar R, Vadlamudi RK. Potential role of a novel transcriptional coactivator PELP1 in histone H1 displacement in cancer cells. *Cancer Res* 2004;64:6416-23.
- (23) Barletta F, Wong CW, McNally C, Komm BS, Katzenellenbogen B, Cheskis BJ. Characterization of the interactions of estrogen receptor and MNAR in the activation of cSrc. *Mol Endocrinol* 2004;18:1096-108.
- (24) Balasenthil S, Vadlamudi RK. Functional interactions between the estrogen receptor coactivator PELP1/MNAR and retinoblastoma protein. *J Biol Chem* 2003;278:22119-27.
- (25) Vadlamudi RK, Kumar R. Functional and biological properties of the nuclear receptor coregulator PELP1/MNAR. *Nucl Recept Signal* 2007;5:e004.
- (26) Manavathi B, Nair SS, Wang RA, Kumar R, Vadlamudi RK. 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* 2005;65:5571-7.
- (27) Rajhans R, Nair S, Holden AH, Kumar R, Tekmal RR VR. Oncogenic potential of the nuclear receptor coregulator proline-, glutamic acid-, leucine-rich protein 1 / Modulator of the nongenomic actions of the estrogen receptor. *Cancer Research* 2007;67:5505-12.

- (28) Salvo VA, Boue SM, Fonseca JP, Elliott S, Corbitt C, Collins-Burow BM, et al. Antiestrogenic glyceollins suppress human breast and ovarian carcinoma tumorigenesis. *Clin Cancer Res* 2006;12:7159-64.
- (29) Choi KC, Auersperg N, Leung PC. Expression and antiproliferative effect of a second form of gonadotropin-releasing hormone in normal and neoplastic ovarian surface epithelial cells. *J Clin Endocrinol Metab* 2001;86:5075-8.
- (30) Vadlamudi RK, Balasenthil S, Broaddus RR, Gustafsson JA, Kumar R. Dereglulation 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* 2004;89:6130-8.
- (31) Hammond SM, Caudy AA, Hannon GJ. Post-transcriptional gene silencing by double-stranded RNA. *Nat Rev Genet* 2001;2:110-9.
- (32) Paul CP, Good PD, Winer I, Engelke DR. Effective expression of small interfering RNA in human cells. *Nat Biotechnol* 2002;20:505-8.
- (33) Maines-Bandiera SL, Kruk PA, Auersperg N. Simian virus 40-transformed human ovarian surface epithelial cells escape normal growth controls but retain morphogenetic responses to extracellular matrix. *Am J Obstet Gynecol* 1992;167:729-35.
- (34) Vadlamudi RK, Balasenthil S, Sahin AA, Kies M, Weber RS, Kumar R, et al. Novel estrogen receptor coactivator PELP1/MNAR gene and ERbeta expression in salivary duct adenocarcinoma: potential therapeutic targets. *Hum Pathol* 2005;36:670-5.
- (35) Barnes CJ, Vadlamudi RK, Kumar R. Novel estrogen receptor coregulators and signaling molecules in human diseases. *Cell Mol Life Sci* 2004;61:281-91.
- (36) Hall JM, McDonnell DP. Coregulators in nuclear estrogen receptor action: from concept to therapeutic targeting. *Mol Interv* 2005;5:343-57.
- (37) O'Malley BW. Molecular biology. Little molecules with big goals. *Science* 2006;313:1749-50.
- (38) Li AJ, Lerner DL, Gapuzan ME, Karlan BY. AIB1 polymorphisms predict aggressive ovarian cancer phenotype. *Cancer Epidemiol Biomarkers Prev* 2005;14:2919-22.
- (39) Bagheri-Yarmand R, Talukder AH, Wang RA, Vadlamudi RK, Kumar R. Metastasis-associated protein 1 deregulation causes inappropriate mammary gland development and tumorigenesis. *Development* 2004;131:3469-79.
- (40) Bagheri-Yarmand R, Balasenthil S, Gururaj AE, Talukder AH, Wang YH, Lee JH, et al. Metastasis-associated protein 1 transgenic mice: a new model of spontaneous B-cell lymphomas. *Cancer Res* 2007;67:7062-7.

- (41) Nakayama K, Nakayama N, Jinawath N, Salani R, Kurman RJ, Shih I, et al. Amplicon profiles in ovarian serous carcinomas. *Int J Cancer* 2007;120:2613-7.
- (42) Woenckhaus J, Steger K, Sturm K, Munstedt K, Franke FE, Fenic I. Prognostic value of PIK3CA and phosphorylated AKT expression in ovarian cancer. *Virchows Arch* 2007;450:387-95.
- (43) Meng Q, Xia C, Fang J, Rojanasakul Y, Jiang BH. Role of PI3K and AKT specific isoforms in ovarian cancer cell migration, invasion and proliferation through the p70S6K1 pathway. *Cell Signal* 2006;18:2262-71.
- (44) Torres-Arzayus MI, Font de MJ, Yuan J, Vazquez F, Bronson R, Rue M, et al. High tumor incidence and activation of the PI3K/AKT pathway in transgenic mice define AIB1 as an oncogene. *Cancer Cell* 2004;6:263-74.
- (45) Greger JG, Fursov N, Cooch N, McLarney S, Freedman LP, Edwards DP, et al. Phosphorylation of MNAR promotes estrogen activation of phosphatidylinositol 3-kinase. *Mol Cell Biol* 2007;27:1904-13.
- (46) Takuwa N, Fukui Y, and Takuwa Y. Cyclin D1 Expression Mediated by Phosphatidylinositol 3-Kinase through mTOR-p70^{S6K}-Independent Signaling in Growth Factor-Stimulated NIH 3T3 Fibroblasts. *Mol Cell Biol* 1999;19:1346-58.
- (47) Liang J, Slingerland JM. Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. *Cell Cycle* 2003;2:339-45
- (48) Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes dev* 1998;12:3499-511
- (49) Vadlamudi RK, Bagheri-Yarmand R, Yang Z, Balasenthil S, Nguyen D, Sahin AA, et al. Dynein light chain 1, a p21-activated kinase 1-interacting substrate, promotes cancerous phenotypes. *Cancer Cell* 2004;5:575-85.
- (50) Nair S, Seetharaman B, Mueller JM KS, Schule R, Kumar R, Vadlamudi RK. PELP1/MNAR modulates LIM-only coactivator FHL2 transactivation functions. *Mol Endocrinol*. 2007;21:613-24.

Figure Legends

Figure 1. PELP1/MNAR expression is upregulated in ovarian tumors. *A*, Serial analysis of gene expression (SAGE) of PELP1/MNAR in various tumors and in ovarian cancer cells using publicly available data bases. *B*, Total lysates from normal and high grade serous ovarian tumor tissues were Western blotted with PELP1/MNAR-specific antibody. Actin was used as a loading control. Intensity of PELP1 bands were quantitated by Sigma Scan program, normalized to actin levels and shown as graph with arbitrary units. *C*, Total lysates from normal and ovarian cancer cells were Western blotted with PELP1/MNAR and various nuclear receptor antibodies. *D*, Summary of immunoreactive staining of PELP1/MNAR in the human ovarian tumor array. A representative sample of PELP1/MNAR staining in different ovarian tumor subtypes is shown and a normal ovary is used as a control. PELP1/MNAR antiserum that was preadsorbed with target peptide was used as a negative control. Tumor array stained with PELP1/MNAR antiserum was scored according to IHC intensity in a range from 0 to 3. 1, low expression; 2, moderate expression; and 3, high expression. Summary of the tumors IHC scores analyzed in the ovarian tumor array (n = 123). *P < 0.05, Student's t test . Magnification, x200.

Figure 2. PELP1/MNAR down regulation reduces ovarian cancer proliferation. OVCAR3 cells, OVCAR3-vector and OVCAR3 cells stably expressing PELP1/MNAR shRNA Vectors (1 and 2) were lysed in RIPA buffer and PELP1/MNAR expression in these clones were analyzed by using Western blotting. *B*, OVCAR3 and OVCAR3-PELP1/MNAR shRNA clones were cultured either in low (2%) or high 20% serum containing medium and the cell proliferation was measured by MTT assay (Left panel). Anchorage independence of PELP1/MNAR shRNA clones were analyzed by soft agar colony formation assay (right panel). Columns, mean of 3 independent

experiments; bars, SEM. *, $P < 0.05$, Student's *t* test. *C*, Total lysates from OVCAR3-vector and OVCAR3 PELP1/MNAR shRNA-1 cells were analyzed for the status of various nuclear receptors and their target genes by Western blotting. *D*, OVCAR3-vector and OVCAR3 PELP1/MNAR shRNA-1 cells were transiently transfected with either ERE-Luciferase or Cyclin D1 luciferase reporter vectors. After 48 h, cells were lysed and reporter gene activity was measured.

Figure 3. PELP1/MNAR signaling contributes to constitutive activation of non-genomic signaling pathways. *A*, OVCAR3 cells (left panel) or SKOV3 cells (right panel) expressing vector- or PELP1/MNAR-specific shRNA were cultured in 10% serum containing medium and the activation of signaling pathways was analyzed by using Western analysis of total protein lysates with phospho-specific antibodies. *B*, BG1 cells were transfected with GFP or GFP-PELP1/MNAR expression vector and stable cells (pooled clones) were selected. Cells were serum starved for 48 hours, expression of endogenous- as well as GFP-PELP1/MNAR protein and activation of signaling pathways were analyzed by using Western analysis with phospho-specific antibodies (left panel). For proliferation assays, BG1 and BG1-PELP1/MNAR clones were cultured in 10% serum containing medium and the cell proliferation was measured by MTT assay *C*, IOSE cells were transfected with the GFP or the GFP-PELP1/MNAR expression vector using Amaxa's Nucleofection kit. After 48 hours of transfection, cells were serum starved for a further 48 hours, and activation of signaling pathways in IOSE cells-overexpressing PELP1/MNAR was analyzed by Western analysis using phospho-specific antibodies. *D*, Cell proliferation (left panel) Anchorage independence (right panel) of PELP1/MNAR

overexpressing IOSE cells were analyzed by soft agar colony formation assay. Columns, mean of 3 independent experiments; bars, SEM. *, $P < 0.05$, Student's t test.

Figure 4. PELP1/MNAR modulates ovarian cancer cell proliferation via PI3K-AKT pathway.

A, OVCAR3 and OVCAR3 PELP1-shRNA clones were cultured in 10% serum, and the total lysates were subjected to immunoprecipitation using anti p85 antibody followed by *in vitro* PI3K assay (right panel). Expression of PELP1, p85, p110 in the PELP1 and PELP1 siRNA lysates used in the assay was analyzed by Western analysis (left panel). **B**, Total protein lysates from OVCAR3 or SKOV3 cells grown in 10% serum were immunoprecipitated with Src or PELP1 or p85 and Western blotted with PELP1, Src and p85 antibodies. **C**, OVCAR3 PELP1shRNA clones were transfected with activated p110 α using Amaxa's Nucleofection kit. Half of the transfected cells were used to measure proliferation using MTT assay (right panel) and the remaining cells were used for western analysis (left panel) **D**, OVCAR3 PELP1shRNA clones were transfected with activated AKT (Myr-AKT) using Amaxa's Nucleofection kit. Half of the transfected cells were used to measure proliferation using MTT assay (right panel) and the remaining cells were used for western analysis of total protein lysates using phospho antibodies.(left panel). Total protein lysates from OVCAR3 cells expressing shRNA vector and PELP1 shRNA were used as a control.

Figure 5. PELP1/MNAR signaling plays a critical role in proliferation of ovarian cancer cells *in vivo*.

A, Nude mice were injected subcutaneously with OVCAR3 cells (n=7) or OVCAR3-PELP1/MNAR shRNA (n=7) and tumor growth was measured at weekly intervals. A representative picture of a PELP1/MNAR-induced tumor in a nude mice is shown. **B**, Tumor growth was measured at weekly intervals and tumor volume is shown in the graph **C**, Tumors

were excised after the mice were euthanized. The average tumor weight is shown in the graph.

D, Morphologic characteristics of PELP1/MNAR-induced tumors and the expression of PELP1/MNAR evaluated with hematoxylin and eosin staining and PELP1/MNAR antibodies, respectively. Cell proliferation and apoptosis was analyzed by BrdU and annexin staining respectively.

