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 serial analysis of gene expression 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 with 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 protein kinase B (AKT) signaling, and reduced tumorigenic potential of ovarian cancer cells in a nude mouse model. PELP1/MNAR overexpression in nontumorigenic immortalized surface epithelial cells (IOSE cells) promoted constitutive activation of c-Src and AKT signaling pathways and promoted anchorage-independent growth. Immunohistochemical 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. [Cancer Res 2008;68(12):4902–9]

Introduction

Among the gynecologic 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 (NR), 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 showed the presence of NRs, including ERα, ERβ, GR, PR, AR, ERRα, and ERRβ 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 seem 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 coreguators 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; ref. 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 nongenomic actions of the NRs (5, 23). PELP1/MNAR promotes cell proliferation by sensitizing cells to G_1>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, phosphatidylinositol-3-OH kinase (PI3K)-AKT, and epidermal growth factor receptor-signal transducers and activators of transcription 3 (STAT3; refs. 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.

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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 plays a significant role in the survival and progression of ovarian tumors. Using human tumor tissue arrays, we show 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 and maintained in RPMI 1640 supplemented with 20% serum. SKOV3 cells obtained from ATCC were maintained in McCoys 5A supplemented with 10% serum. BG1 cells were earlier described (28) and were maintained in RPMI 1640 supplemented with 10% FCS. Nontumorigenic 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.) containing 15% fetal bovine serum (Hyclone Laboratories Ltd.), 100 U/mL penicillin G, and 100 mg/mL streptomycin (Life Technologies, Inc.). Antibodies against vinculin were purchased from Sigma Co. Green fluorescent protein (GFP)-epitope antibody was purchased from Clontech. PELP1/MNAR antibody was purchased from Bethyl Laboratories. Antibodies against phospho-AKT, phospho-MAPK, Phospho-GSK3, phospho-nuclear factor-κB (NF-κB), and phospho-Src were purchased from Cell Signaling.

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) 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). With this method, we achieved 80% to 90% transfection efficiency as monitored by GFP expression. Briefly, 10^7 cells at 70% confluence were transfected with either 5 μg of GFP-PELP1/MNAR or PELP1/MNAR-ShRNA DNA or appropriate vectors. The cells were initially plated in a 6-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 μg 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) and control shRNA vector were purchased from SuperArray. 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 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 s.c. into the left flank of 6- to 7-week-old female nude mice ($n = 7$) and tumors were allowed to grow for 8 wk. Tumor volumes were measured with a caliper at weekly intervals. After 8 wk, the mice were sacrificed, and the tumors were removed and processed for immunohistochemistry (IHC). Before sacrifice, BrdUrd was injected via i.p. route into the mice.

Tissue microarrays. The tissue microarrays (TMA) used in this study were obtained from the University of Texas M. D. Anderson Cancer Center, and IHC was performed as described earlier (5, 30). These TMAs 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: 0, 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 up-regulated 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 National Cancer Institute's Cancer Genome Anatomy Project databases. The results from this search suggested that PELP1/MNAR expression indeed is up-regulated 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 with the normal ovary (Fig. 1*A*). 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 with 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 with normal ovarian tissues (Fig. 1*B*). We have then analyzed PELP1/MNAR expression by using Western blotting in three commonly used ovarian cancer cells (SKOV3, OVCAR3, and 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 NRs including ERα, ERβ, ERRα, ERRγ, and PR. However, expression of ERα 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 with IOSE cells (Fig. 1*C*). These results suggest that NR coregulator PELP1/MNAR expression is up-regulated 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, 31). The representative staining for each type of ovarian tumor is shown in Fig. 1*D*. In these studies using human ovarian cancer tissue array ($n = 123$), we observed a weak focal

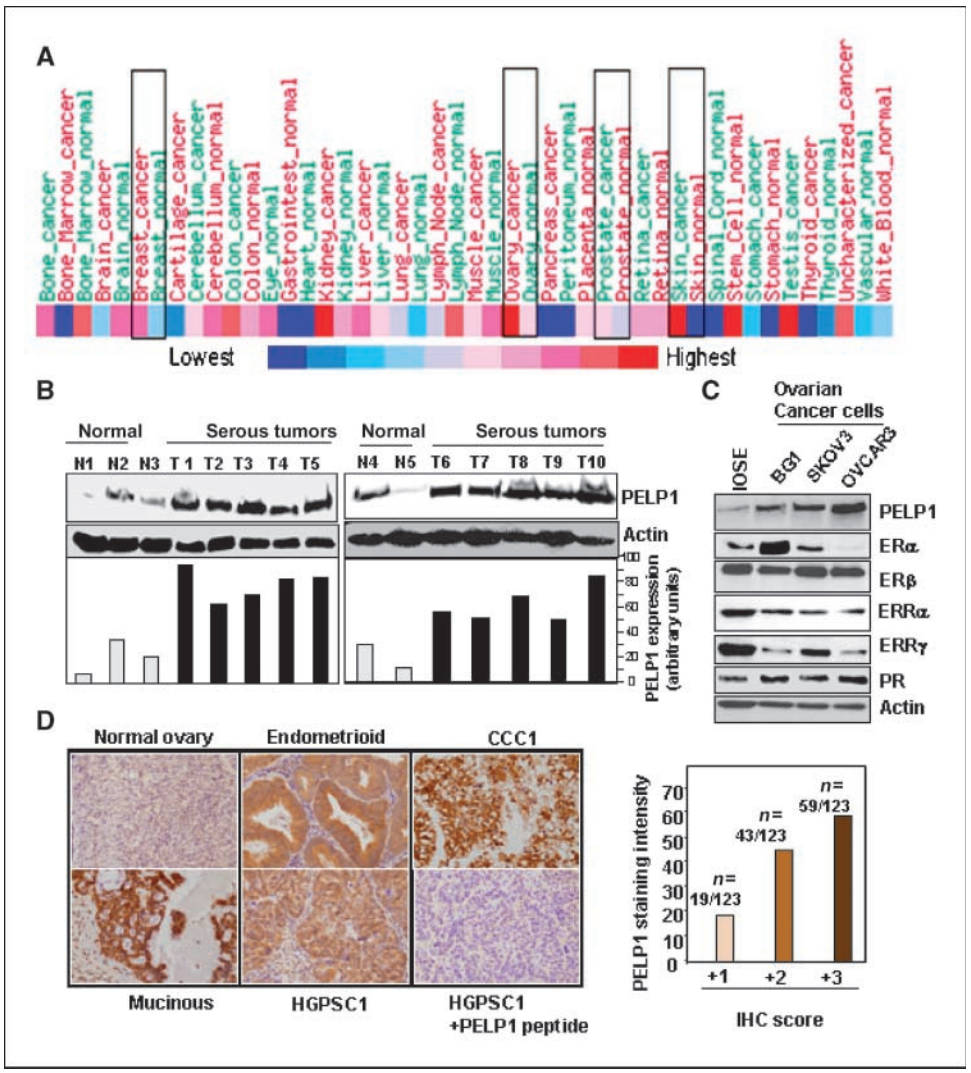

Figure 1. PELP1/MNAR expression is up-regulated in ovarian tumors. *A,* serial analysis of gene expression of PELP1/MNAR in various tumors and in ovarian cancer cells using publicly available data bases. *B,* total lysates from normal and high-grade serous ovarian tumor tissues was Western blotted with PELP1/MNAR-specific antibody. Actin was used as a loading control. Intensity of PELP1 bands was 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 NR 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; *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, ×200.

staining (0–1+) in 19 (15.4%) and clearly detectable PELP1/MNAR staining (+2–+3) in 104 (84.6%) samples. A total of 59 (56.7%) tumors showed 3+ staining, whereas 45 (43.3%) showed 2+ staining compared with normal tissues, which showed PELP1 staining of +1 (Fig. 1*D*). 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. 1*D*). 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 with normal ovary. Collectively, these results suggest that PELP1/MNAR expression is altered during ovarian tumor progression and is up-regulated 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 (32, 33) 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 >80% reduction in the endogenous PELP1/MNAR levels. These two shRNAs target two distinct regions of PELP1/MNAR thus serve as confirmatory controls for nonspecific actions of shRNA. We then established OVCAR3 stable cell lines that express PELP1/MNAR-shRNA1 and PELP1/MNAR-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. 2*A*). 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 with the parental cells and that the effect of PELP1/MNAR down-regulation on cell proliferation is more pronounced in low-serum conditions compared with high serum (Fig. 2*B*). Similarly, PELP1/MNAR shRNA clones exhibited decreased anchorage independence in soft agar colony assays (Fig. 2*B, right*). Western analysis showed that PELP1 down-regulation did not affect the expression levels of NRs in these cells (Fig. 2*C, left*). However, PELP1/MNAR down-regulation affected the expression of PELP1/MNAR regulated NR target genes such as PR and CyclinD1 (Fig. 2*C, right*). Reporter gene assays using ERE and

Cyclin D1 luciferase reporters also confirmed that PELP1 deregulation affect the expression of these genes (Fig. 2*D*). Collectively, these results suggest that PELP1/MNAR plays 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 nongenomic signaling in ovarian cancer cells. Recent studies suggest that PELP1/MNAR, in addition to its participation in the NR-genomic functions, also plays a critical role on NR-mediated nongenomic signaling. To examine the possibility that PELP1/MNAR has a role in the activation of nongenomic signaling pathways in ovarian cells, we measured the activation of nongenomic 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 with shRNA vector–transfected cells (Fig. 3*A*). However, PELP1 down-regulation did not affect the status of Phospho–NF-κB p65. (Fig. 3*A*), 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 using two distinct shRNAs that target PELP1/MNAR also resulted in substantial reduction of the Src and AKT signaling pathways (Fig. 3*A, right*) with no reduction in the phospho-NF-κB signaling. Similarly, BG1-PELP1/MNAR cells (pooled clones 1 and 2) showed 3-fold increase in PELP1/MNAR expression compared with the vector-transfected cells (Fig. 3*B, left*). Western analysis of the total protein lysates showed that PELP1/MNAR overexpression in BG1 cells promotes increased Src, AKT, and MAPK signaling with no increase in phospho-NF-κB signaling (Fig. 3*B, left*). PELP1 overexpression in BG1 cells also increased the proliferation potential of the cells (Fig. 3*B, right*). 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 promotes excessive nongenomic signaling and anchorage-independent growth. Most ovarian neoplasms arise from the ovarian surface epithelium (OSE; ref. 2). In earlier studies, we developed IOSE model cells from normal OSE by transfecting SV40 large T antigen (34). 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 overexpressed 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. 3*C*). This Amaxa's Nucleofector transfection typically resulted in transfection of >70% IOSE cells and generated IOSE model cells that overexpress 2- to 3-fold PELP1/MNAR compared with vector-transfected IOSE cells. The status of nongenomic 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 overexpression substantially increased Src, AKT, and MAPK signaling in IOSE model cells (Fig. 3*C*). Similarly,

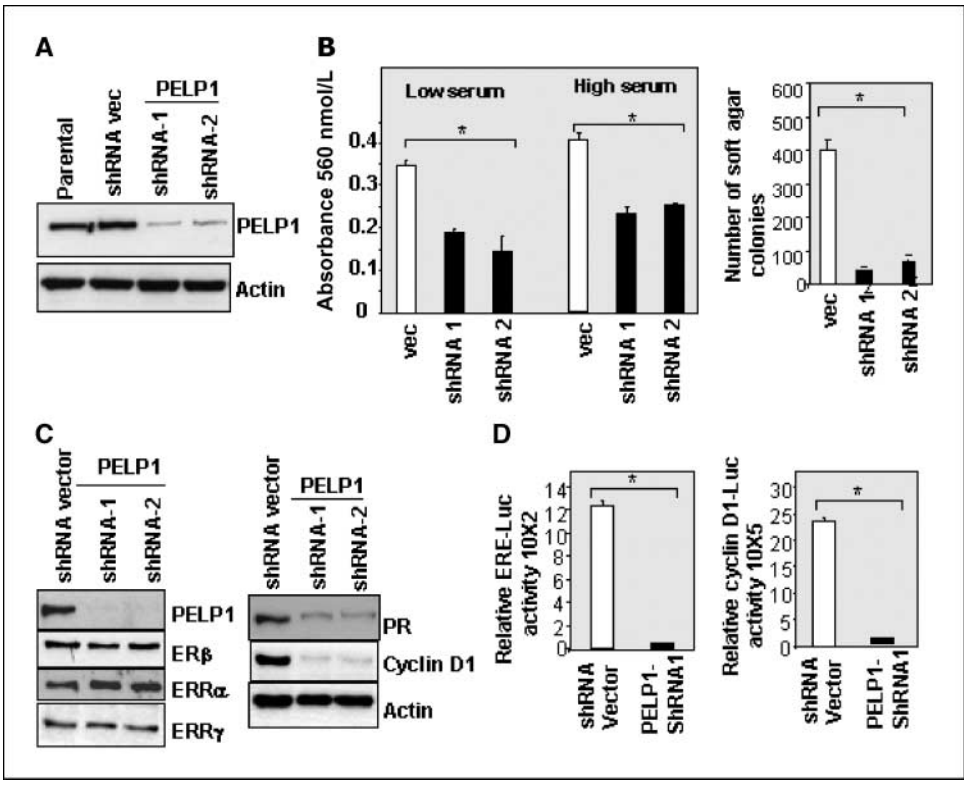

Figure 2. PELP1/MNAR down-regulation reduces ovarian cancer proliferation. OVCAR3 cells, OVCAR3-vector, and OVCAR3 cells stably expressing PELP1/MNAR shRNA vectors (1 and 2) were lysed in radioimmunoprecipitation assay buffer, and PELP1/MNAR expression in these clones was analyzed by using Western blotting. *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*). Anchorage independence of PELP1/MNAR shRNA clones was analyzed by soft agar colony formation assay (*right*). Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$, Student's *t* test. *C,* total lysates from OVCAR3-vector and OVCAR3 PELP1/MNAR shRNA-1 cells were analyzed for the status of various NRs 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.

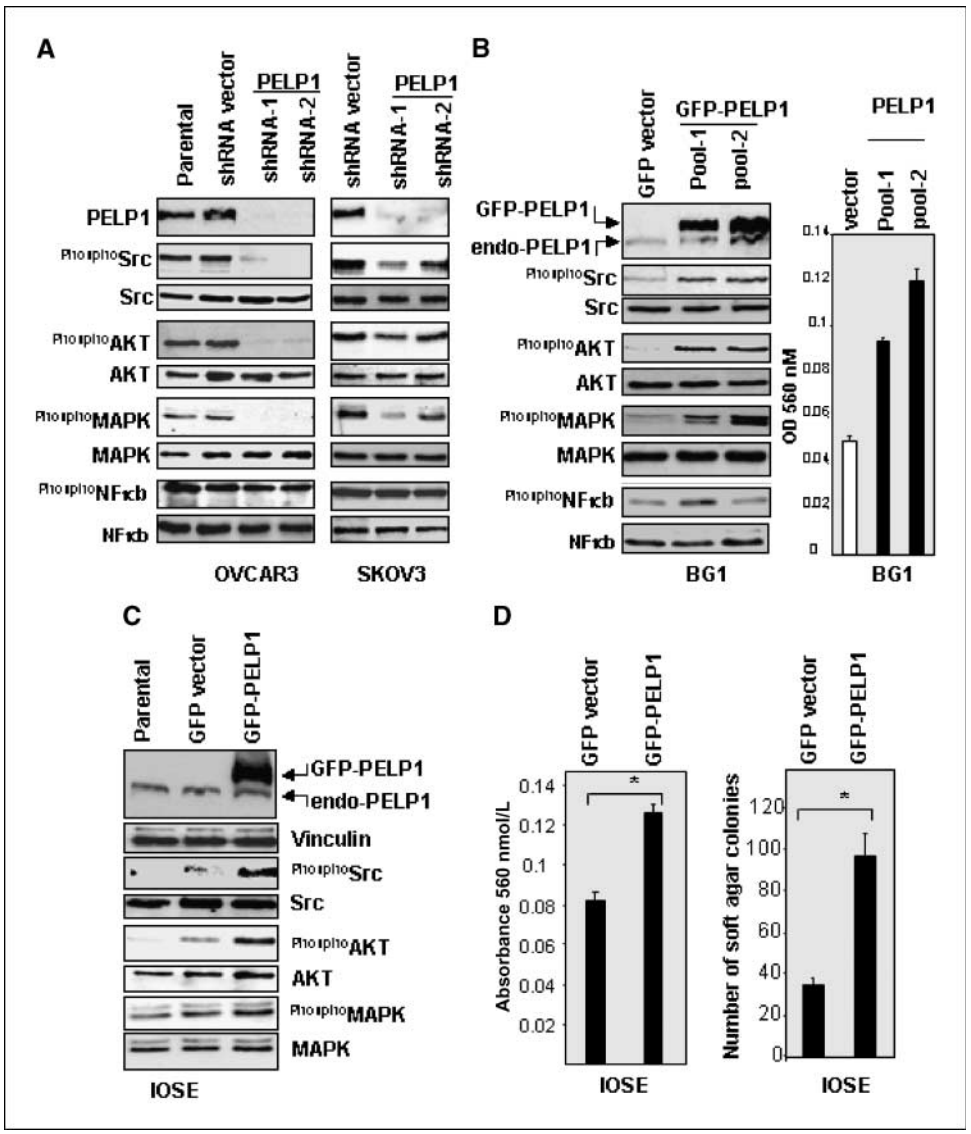

Figure 3. PELP1/MNAR signaling contributes to constitutive activation of nongenomic signaling pathways. *A,* OVCAR3 cells (*left*) or SKOV3 cells (*right*) expressing vector-specific or PELP1/MNAR-specific shRNA were cultured in 10% serum-containing medium, and the activation of signaling pathways was 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 h, 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*). 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 h of transfection, cells were serum starved for a further 48 h, and activation of signaling pathways in IOSE cells overexpressing PELP1/MNAR was analyzed by Western analysis using phospho-specific antibodies. *D,* cell proliferation (*left*) and anchorage independence (*right*) of PELP1/MNAR-overexpressing IOSE cells were analyzed by soft agar colony formation assay. Columns, mean of three independent experiments; bars, SE. *, $P < 0.05$, Student's *t* test.

PELP1/MNAR-overexpressing IOSE cells exhibited increased proliferation potential and showed increased anchorage independence in soft agar colony assays (Fig. 3*D*).

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. Because PELP1 shRNA clones exhibited decreased activation of Src and AKT kinases, we examined whether PELP1 down-regulation contributes to decreased PI3K activity. Results of PI3K assay showed that PELP1 shRNA clones indeed have low PI3K activity (Fig. 4*A*). To examine whether functional PELP1-PI3K-Src complexes exits 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. 4*B*). We also confirmed PELP1 interactions with p85 subunit of PI3K in SKOV3 cells (Fig. 4*B, right*). Because PELP1 interactions with Src kinase leads to activation of PI3K pathway, we hypothesized that overexpression 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% to 90% transfection efficiency. The results from this experiment showed that overexpression of Myr-p110α can restore the proliferation defect seen in PELP1shRNA cells (Fig. 4*C, right*). Furthermore, Myr-p110* overexpression also restored the defect seen in the cyclin D1 levels in PELP1 shRNA clones (Fig. 4*C, left*). To further examine how defects in AKT activation leads to decreased proliferation in PELP1 shRNA clones, we examined the status of downstream effectors of AKT in PELP1 shRNA clones. We have observed a dramatic decrease in the phosphorylation of GSK3β, a downstream 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, overexpression of Myr-AKT restored the defect seen in the GSK3β phosphorylation, restored cyclin D1 levels, and rescued the proliferation defect in PELP1 shRNA clones (Fig. 4*D*). Collectively, these restoration experiments provide evidence that blockage of

PI3K-AKT-GSK3β-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 s.c. into mice and tumorigenic potential was monitored for 8 weeks (Fig. 5*A*). 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 with control (Fig. 5*B* and *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 PELP1 and exhibited activation of Src and AKT to a lesser degree than the activation seen in OVCAR3 vector–transfected tumors (Fig. 5*D*). Further BrdUrd staining of the tumor sections revealed increased proliferation in the OVCAR3 vector–transfected xenograft tumors compared with PELP1/MNAR shRNA tumors. Also, the PELP1/MNAR shRNA tumors showed increased apoptosis as revealed by annexin staining (Fig. 5*D*). 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 gynecologic 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 NR PELP1/MNAR plays 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 nongenomic 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 overexpressed or amplified in breast and ovarian tumors and overexpression 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 NRs 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

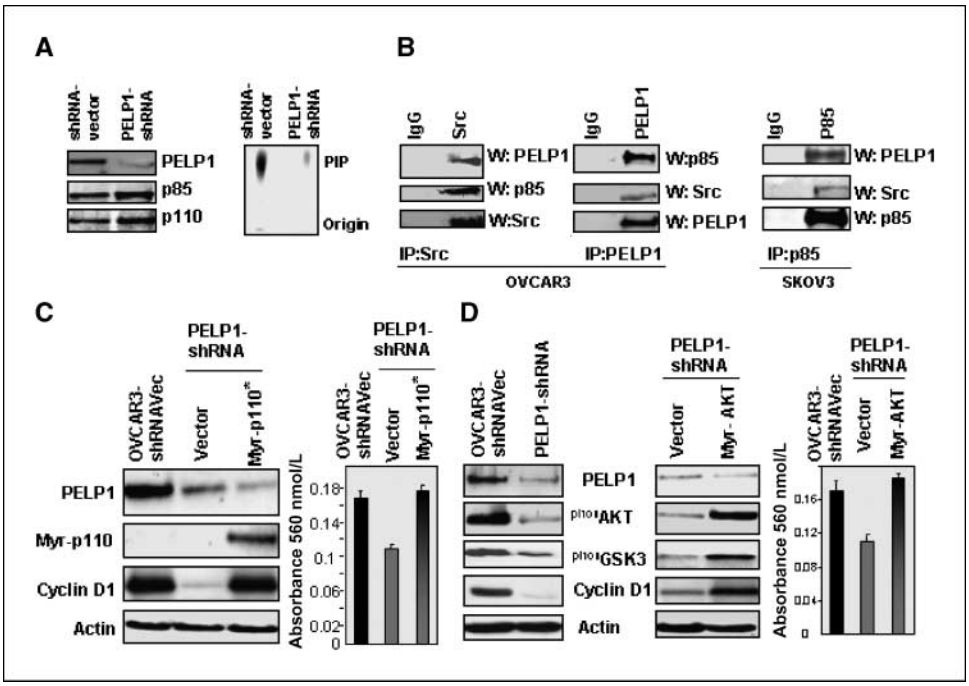

Figure 4. PELP1/MNAR modulates ovarian cancer cell proliferation via PI3K-AKT pathway. *A,* OVCAR3 and OVCAR3 PELP1-shRNA clones were cultured in 10% serum, and the total lysates were subjected to immunoprecipitation (*IP*) using anti-p85 antibody followed by *in vitro* PI3K assay (*right*). Expression of PELP1, p85, p110 in the PELP1 and PELP1 siRNA lysates used in the assay was analyzed by Western analysis (*left*). *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 p110α using Amaxa's Nucleofection kit. Half of the transfected cells were used to measure proliferation using MTT assay (*right*), and the remaining cells were used for Western analysis (*left*). *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*), and the remaining cells were used for Western analysis of total protein lysates using phospho antibodies (*left*). Total protein lysates from OVCAR3 cells expressing shRNA vector and PELP1 shRNA were used as a control.

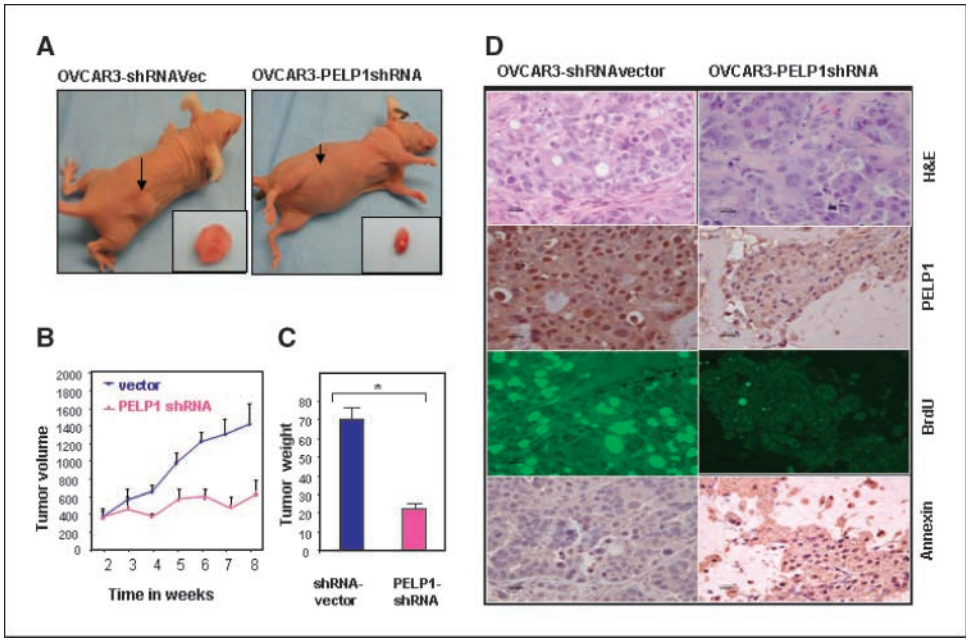

Figure 5. PELP1/MNAR signaling plays a critical role in proliferation of ovarian cancer cells *in vivo*. *A,* nude mice were injected s.c. with OVCAR3 cells ($n = 7$) or OVCAR3-PELP1/MNAR shRNA ($n = 7$), and tumor growth was measured at weekly intervals. A representative picture of a PELP1/MNAR-induced tumor in a nude mouse 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 H&E staining and PELP1/MNAR antibodies, respectively. Cell proliferation and apoptosis was analyzed by BrdUrd and annexin staining, respectively.

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 using 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, overexpression 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.

PI3K has been increasingly recognized as one of the important signaling molecules required for G_1-S cell cycle progression, and PI3K/Akt pathway was previously shown to regulate cell cycle progress through inducing cyclin D1 expression (45). Earlier studies have also shown that GSK3β phosphorylation by AKT to down-regulate GSK3β activity leading to up-regulation of cyclin D1 levels via stabilization (46, 47). Knockdown of PELP1 expression significantly affected cellular proliferation and reduced activation of AKT with a decrease in the GSK3β phosphorylation. Overxpression 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 overexpression of active AKT in PELP1 shRNA clones also restores phosphorylation of GSK3β with a concomitant increase in the cyclin D1 levels. Collectively, our results suggest that PELP1 deregulation promotes ovarian cancer cell proliferation via PI3K-AKT-GSK3β-cyclin D1 pathway.

PELP1/MNAR seems to function as a scaffolding protein by coupling NRs with several proteins implicated in oncogenesis. PELP1/MNAR modulates the interaction of ERs with c-Src, stimulating c-Src enzymatic activity, leading to the activation of the 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 exits as a complex with ERα, cSrc, and p85, the regulatory subunit of PI3K (48). With the enormous potential of PELP1/MNAR as a modulator of NRs and proto-oncogenes 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 nongenomic functions of NRs are implicated in different cellular processes, including cell survival and apoptosis, PELP1/MNAR-mediated nongenomic 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 overexpressed 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 nongenomic 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 nongenomic 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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