PME-1 Modulates Protein Phosphatase 2A Activity to Promote the Malignant Phenotype of Endometrial Cancer Cells

Ewa Wandzioch, Michelle Pusey, Amy Werda, Sophie Bail, Aishwarya Bhaskar, Mariya Nestor, Jing-Jing Yang, and Lyndi M. Rice

Abstract

Protein phosphatase 2A (PP2A) negatively regulates tumorigenic signaling pathways, in part, by supporting the function of tumor suppressors like p53. The PP2A methylesterase PME-1 limits the activity of PP2A by demethylating its catalytic subunit. Here, we report the finding that PME-1 overexpression correlates with increased cell proliferation and invasive phenotypes in endometrial adenocarcinoma cells, where it helps maintain activated ERK and Akt by inhibiting PP2A. We obtained evidence that PME-1 could bind and regulate protein phosphatase 4 (PP4), a tumor-promoting protein, but not the related protein phosphatase 6 (PP6). When the PP2A, PP4, or PP6 catalytic subunits were overexpressed, inhibiting PME-1 was sufficient to limit cell proliferation. In clinical specimens of endometrial adenocarcinoma, PME-1 levels were increased and we found that PME-1 overexpression was sufficient to drive tumor growth in a xenograft model of the disease. Our findings identify PME-1 as a modifier of malignant development and suggest its candidacy as a diagnostic marker and as a therapeutic target in endometrial cancer. Cancer Res; 74(16); 1–11. ©2014 AACR.

Introduction

Protein phosphatase 2A (PP2A), a heterotrimeric serine/threonine phosphatase, composed of a scaffolding subunit (A), a catalytic subunit (C), and a B regulatory subunit, is implicated as a human tumor suppressor (reviewed in refs. 1–4). The core AC dimer recruits a B regulatory subunit giving the complex substrate specificity. There are 4 families of B regulatory subunits, composed of several genes that code for multiple isoforms (2, 3). Specific heterotrimers dephosphorylate and stabilize tumor suppressors, such as p53 (5, 6) and p107 (7); thus, PP2A activity is important in cell-cycle regulation and tumor suppression. PP2A has been shown to dephosphorylate positive regulators of cell signaling pathways such as ERK and Akt (8–11), inhibiting them and promoting senescence or apoptosis. Inhibition of PP2A promotes enhanced cell proliferation, impairment of cell differentiation, malignant cell transformation (reviewed in refs. 3, 4) and is thought to be a key step in cellular transformation (12).

The carboxy-terminal tail of the catalytic subunit of PP2A is highly posttranslationally modified, affecting PP2A activity through mechanisms such as altering B subunit recruitment and the physical blockade of the catalytic site (13, 14). The C-terminal tail of Ppp2ca/b, the two isoforms of its catalytic subunit, are conserved with the C-terminal tails of Ppp4c and Ppp6c, the catalytic subunits of protein phosphatases 4 and 6 (PP4, PP6), respectively, suggesting they may be regulated similarly to PP2A. The reversible methylation of the C-terminal leucine of the catalytic subunit of PP2A provides an interesting molecular mechanism of regulation. Methylation is catalyzed by leucine carboxyl methyltransferase (LCMT1; ref. 15) and has been shown to enhance both the catalytic activity of PP2A and the recruitment of specific B subunits to the PP2A complex (16, 17). The removal of the methyl group is catalyzed by protein phosphatase methylesterase 1 (PME-1; refs. 14, 18) and renders PP2A inactive (13, 19, 20). The conservation of catalytic subunits of PP4 and PP6 with that of PP2A suggests that PME-1 may also modulate PP4 and PP6 activities.

PME-1–mediated PP2A inhibition causes increased proliferation and activation of the ERK pathway and promotes malignant cell growth of human glioblastoma cells (21). PME-1 expression forces progression of low-grade astrocytic gliomas to malignant glioblastomas (22). PP2A targets RalA, Ras/Raf/MEK/ERK, and PI3K/AKT pathways (18, 23–25), and it has been shown that the simultaneous activation of ERK and PI3K/Akt pathways is highly oncogenic in breast cancer cells (26).

Although a few PP2A tumor-promoting mechanisms have been reported (9, 21), the role of PME-1 in cancer induction and progression remains to be elucidated. Since endometrial
cancer is the most common gynecologic cancer affecting women in the United States, we asked whether PME-1 plays a role in endometrial cancer progression. We show that increased PME-1 correlates to decreased PP2A activity and increased proliferation and metastatic phenotypes through the maintenance of increased ERK and Akt signaling. We demonstrate an interaction between PME-1 and PP4, suggesting that PME-1 is not specific for PP2A. Increased levels of PME-1 were detected in endometrial adenocarcinoma tumors, suggesting that PME-1 may be a diagnostic marker for patients with endometrial cancer. Currently, there are no available specific biomarkers for endometrial adenocarcinoma. Finally, increased PME-1 led to increased anchorage-independent growth and increased tumor burden in a xenograft model. Our results suggest a role for PME-1 in the promotion of cancer progression in endometrial cancer and may be a valid drug target for endometrial cancer treatment.

Materials and Methods

Detailed experimental procedures can be found online in Supplementary Materials.

Cell cultures and generation of stable cell lines

All cell lines were purchased from ATCC and were maintained according to ATCC recommendations. Analyses of ECC-1 cells (ATCC #CRL-2923) have determined ECC-1 cells purchased from ATCC to be genetically similar and redundant to the Ishikawa endometrial cancer cell line (27). All cell lines were authenticated in November of 2013 via STR Analysis (Genetica DNA Laboratories). Vectors were transfected into cells with Lipofectamine 2000 (Life Technologies) or using (Genetica DNA Laboratories). Vectors were transfected into cells with Lipofectamine 2000 (Life Technologies) or using lentivirus (System Biosciences, Inc). shRNA sequences are in the Supplementary Table S1. Lentivirus (5.0 × 10^6 ifu/mL) was added to cell cultures (Sigma Aldrich). Cells were selected with puromycin after 48 hours (Invivogen).

Foci formation assays

To measure cell proliferation, 1,000 cells were plated in a 6-well tissue culture dish in selection media and were grown for 10 to 14 days. Experiments were repeated at least three times. BrdUrd incorporation experiments were conducted as described in Supplementary Materials.

Analysis of endometrial cancer patient samples

Matched pairs harvested from 30 patients with type I endometrial adenocarcinoma purchased from Proteogenex (see Supplementary Table S2) were used to determine the mRNA and protein levels of PME-1 in tumor versus normal adjacent tissue. Further analysis was conducted via IHC techniques and immunofluorescence assays (see Supplementary Materials).

Phosphatase activity assay

Whole-cell lysates were used with the DuoSet IC PP2A Phosphatase Assay Kit (R&D Systems). The procedure was completed per kit instructions with 250 μg protein. The assay was repeated at least three times with similar results.

TaqMan RT-PCR analysis

Total RNA was used to generate cDNA for analysis of gene expression. TaqMan probes and PCR-mix were purchased from Life Technologies. Results are representative of three independent experiments in which genes of interest were normalized to the housekeeping genes, 18S or GAPDH.

ERK/Akt inhibition, protein extraction, and Western blot analysis

RL95-2 cells were incubated with either 50 μmol/L Akt inhibitor (LY294002, Cell Signaling Technologies) or 40 μmol/L ERK inhibitor (U0126, Cell Signaling Technologies) for 1 to 2 hours at 37°C. Laemml buffer (1.5 × 0.5 mol/L Tris, pH 6.8, 100% glycerol, 10% SDS, 100 mmol/L EDTA) was used to prepare cell lysates. All experiments were repeated several times with similar results.

Colony formation assays

To measure invasive growth phenotypes, the 3D On-Top Matrigel Assay was completed in 24-well dishes as described previously (28).

In vivo tumor formation

A total of 1 × 10^6 endometrial carcinoma cells (ECC-1) diluted in 100 μL 1× PBS expressing empty vector (Control) or overexpressing PME-1 (+PME-1) were injected subcutaneously into the flank of nude female mice (n = 7 per group). Tumor formation was measured weekly for 7 weeks with a caliper, and tumor volume was calculated according to the formula V = \( \frac{1}{2} xy^2 \), where \( y \) = tumor length and \( x \) = tumor width. At 8 weeks postinjection, mice were euthanized and tumors were resected for analysis. All animal work was approved by and conducted according to the guidelines of the Genesis Biotechnology Group Institutional Animal Care and Use Committee.

Statistical analysis

Statistical analysis was completed using GraphPad Prism version 5.02 for Windows, GraphPad Software (www.graphpad.com). The data were analyzed with the Mann–Whitney \( U \) test for significance (patient samples) or the Student standard \( t \) test; SEMs were calculated for all sample batches. ROC analysis plotting the sensitivity and specificity of PME-1 mRNA levels in endometrial cancer patient samples was calculated with 95% confidence interval (CI) to determine the validity of PME-1 as a biomarker for endometrial cancer using a likelihood ratio of 21. The cutoff was determined using the GraphPad Software. The determined \( P \) value (\( P < 0.0001 \)) and area under the curve (AUC, 0.9601) suggest that PME-1 could be a valuable predictor of endometrial cancer. Data from animal studies were analyzed using 2-way ANOVA. * \( P < 0.05 \); ** \( P < 0.01 \); *** \( P < 0.001 \).

Results

Increased PME-1 levels correlate with increased cancerous phenotypes and decreased PP2A activity

Increased PME-1 has previously been noted in glioblastomas (21) and increases cell proliferation in the cervical cancer cell line, HeLa (refs. 20, 21 and data not shown), but the role(s) of
PME-1 in endometrial cancer initiation and/or progression has not been elucidated. Endometrial cancer is the most common gynecologic cancer in the United States with no reliable noninvasive diagnostic available for early detection of endometrial cancer. We examined the expression levels of PME-1 in endometrial cancer cell lines by Western blot analysis (Fig. 1A).

**Figure 1.** PME-1 promotes cancer phenotypes through inhibition of PP2A activity in endometrial cancer (EC) cells. A, Western blot analysis of PME-1 levels in End1 (immortalized endocervical cell line) and the endometrial cancer cell lines, RL95-2, ECC-1, and Ishikawa cells. GAPDH serves as a loading control. B, Western blot analysis of PME-1 protein in RL95-2 cells expressing empty vector (Control), overexpressing PME-1 (+PME-1), or expressing two different shRNAs (sh #1, sh #2) against PPME1 mRNA (−PME-1); GAPDH serves as a loading control. C, a total of 1,000 cells per well were plated for each RL95-2 cell line and were grown for 10 days to measure cell proliferation. Data from seven independent studies are represented as percent of control. D, BrdUrd incorporation assays were completed to monitor changes in cell proliferation by altering PME-1 levels and were completed three times in triplicate. E, RL95-2 cells were transfected with nontargeting siRNA or siRNA against the 3′-UTR of PPME1. Cells were cotransfected with empty vector (Control) or inactive PME-1 S156A. Data are normalized to RL95-2 cells treated with control siRNA and the empty vector. See Materials and Methods for more detail. Foci experiments were completed as above. Data represent five independent experiments. F, a total of 1 × 10⁵ cells were grown in Matrigel and were stained with 1% crystal violet and counted after 14 days of growth to measure anchorage-independent growth. Data are presented as percent of control and represent four independent experiments. Analysis of PP2A activity in RL95-2 cells when PME-1 was depleted using shRNA (G) or PME-1 was overexpressed (H). Control samples were normalized to 100% PP2A activity and each experiment was conducted three times. Significance was calculated by the standard Student t test, where *, P < 0.05; **, P < 0.01; ***, P < 0.001.
and found that the endometrioid adenocarcinoma cell lines, RL95-2, Ishikawa, and ECC-1, express more PME-1 than the immortalized endocervical cell line, Emd1. No immortalized endometrial cell lines exist due to misidentification (27), thus, we used the endocervical cell line as it is derived from a similar tissue.

We next developed RL95-2 cell lines expressing empty vector (control), overexpressing PME-1 (+PME-1), or expressing shRNA (sh1, sh2) against PPME1 mRNA, which codes for PME-1 protein (−PME-1) and confirmed the appropriate level of PME-1 via real-time RT-PCR (data not shown) and Western blot analysis (Fig. 1B). Similar cell lines were constructed for ECC-1 cells as well as the KLE endometrial cancer cell line (data not shown). Multiple shRNAs and siRNAs were used for these experiments (see Supplementary Table S1), all with similar results. To determine the role of PME-1 in cell proliferation, we completed foci formation assays, which demonstrated a significant 50% increase in focus when PME-1 is overexpressed and a significant 75% decrease in focus when depleted for PME-1 compared with control cells (Fig. 1C). We noted a significant 2-fold increase in foci formed in KLE EC cells when PME-1 was overexpressed (data not shown). To confirm that altering PME-1 levels in endometrial cancer cells affects their rate of proliferation, we completed BrdUrd incorporation assays (Fig. 1D). We found a significant 40% increase in BrdUrd incorporation in +PME-1 cells compared with empty vector control and about 40% decrease proliferation in −PME-1 cells compared with a scrambled shRNA control. Taken together, these data suggest that PME-1 promotes cell proliferation.

We next treated RL95-2 cells with siRNA against the 3'-untranslated region (UTR) of PPME1 to decrease endogenous levels of PPME1 and overexpressed either empty vector or a catalytically inactive form of PME-1, S156A, in which the nucleophilic serine residue was mutated to an alanine (Fig. 1E). The decrease in focus due to decreased endogenous PME-1 was not rescued by overexpression of the S156A mutant; however, increased foci formation was rescued by the overexpression of wild-type PME-1 (data not shown), suggesting that active PME-1 is required for increased cell proliferation in RL95-2 cells. To determine whether PME-1 promotes anchorage-independent growth, we completed soft agar assays and counted the colonies formed. We determined that PME-1 overexpression led to a significant 50% increase in colony formation, whereas PME-1 inhibition led to a significant 50% decrease in colony formation (Fig. 1F). Thus, PME-1 promotes several cancer phenotypes.

The decrease in cell proliferation due to loss of PME-1 was not due to apoptosis, as demonstrated by a terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay, but was instead due to cells senescing, evidenced by substantial increase in the senescence protein marker, DcR2 (data not shown). This was accompanied by a 20- to 40-fold increase in PP2A activity (Fig. 1G) likely due to increased methylation of the catalytic subunit of PP2A (13, 19). Overexpression of PME-1 inhibited PP2A activity by about 90% (Fig. 1H). Thus, PME-1 inhibition leads to decreased cell proliferation and senescence via increased PP2A activity in endometrial cancer cell lines.

**Overexpression of PME-1 increases ERK/Akt activation**

Because increased PME-1 levels correlate with increased cellular proliferation and PP2A is known to negatively regulate the ERK and Akt pathways (24, 29, 30), we asked whether increased PME-1 led to the activation of these signaling pathways. Western blot analysis demonstrated that the ERK phosphorylation was altered with manipulation of PME-1 levels in RL95-2 cells (Fig. 2A) and in the KLE endometrial cancer cell line (data not shown); when PME-1 is overexpressed, there is an increase in phosphorylated ERK, whereas total ERK levels remain unchanged (Fig. 2A). Upon PME-1 depletion, there is decreased phospho-ERK compared with control cells. We noted decreased levels of phosphorylated ERK, as expected, upon treatment of cells with UO126, an upstream inhibitor of ERK phosphorylation.

Similarly, phosphorylation of Akt on threonine 308 (T308) and on serine 473 (S473) increased when PME-1 was overexpressed compared with control and decreased when PME-1 was depleted (Fig. 2B). Similar results were recently published by Jackson and Pallas (9), although they noted increased phosphorylation only at T308. We detected decreased phosphorylation of Akt, as expected, upon treatment of cells with

![Figure 2. Overexpression of PME-1 induces increased cell proliferation through activation of ERK and Akt signaling pathways. Western blot analysis of RL95-2 cells expressing control vector, vector with PME-1, or vector carrying shRNA against PME-1 examining levels of ERK phosphorylation compared to total ERK (A) and Akt phosphorylation at threonine 308 and serine 473 compared with total Akt levels (B). GAPDH serves as a loading control. Samples treated with the inhibitors of ERK (UO126) and Akt (LY294002) serve as controls.](Image)
the upstream inhibitor, LY294002. Therefore, PME-1 is a positive regulator of the ERK and Akt cancer signaling pathways in endometrial cancer cells.

**PME-1 is overexpressed in endometrial cancer patient tumors**

Uterine tissue samples from 30 patients diagnosed with type I endometrioid adenocarcinoma were purchased from Proteogenex (Supplementary Table S2). RNA and protein were extracted from each matched pair, tumor (T), and normal adjacent tissue (NAT, N). Note that the ratio of \( PPME1 \) mRNA expression is increased in tumor versus NAT in 24 of 29 patient samples (\( \sim 83\% \), Supplementary Table S2), suggesting that increased \( PPME1 \) mRNA is indicative of endometrial cancer. \( PPME1 \) expression was increased about 20-fold in tumor samples versus normal samples (Fig. 3A). ROC analysis (Fig. 3B) demonstrates that detection of \( PPME1 \) mRNA levels may be a diagnostic marker, as the area under the curve (AUC) score is >0.9, the sensitivity of the assay is 80.77%, and the specificity is 96.15%, suggesting that \( PPME1 \) levels may be predictive of endometrial cancer. Moreover, \( PPME1 \) mRNA expression with a cutoff of 8.14 (determined using Prism software) demonstrates a 95.5% positive predictive value (PPV) and an 83.3% negative predictive value (NPV).

We next tested the patient samples for PME-1 protein and compared it with the loading control, COX IV (Fig. 3C). Representative Western blots are displayed for Federation Internationale des Gynaecologistes et Obstetristes (FIGO) grades 1–3. Grade 1 samples (patients 06261, 06310, 06308, 06276) are shown in the top left and grade 2 samples (patients 06336, 06241, 06247) are shown in the top right. Grade 3 samples (patients 06268, 06294) are shown in the bottom. Most tumor samples (T) exhibit increased PME-1 protein when compared with normal adjacent tissue (N).

Patient samples were assessed to determine the stage and grade according to the FIGO guidelines and were analyzed with
hematoxylin and eosin (H&E) staining and/or α-PME-1 antibodies (Fig. 3D and E). Grade 1 tumors are well-differentiated cancers with clear cellular boundaries and normal cell morphology. Grade 2 tumors are moderately differentiated with abnormal cell morphology. Grade 3 tumors are poorly differentiated exhibiting loss of clearly defined boundaries and highly abnormal cell morphology (Fig. 3D). PME-1 protein levels were assessed as follows: 0 indicates negative cytoplasmic PME-1 staining or faint staining observed in <50% of the cells; 1+ indicates weakly positive cytoplasmic PME-1 staining in >50% of tumor cells or moderate/strong staining in <50% of tumor cells; and 2+ indicates strongly positive staining in >50% of tumor cells or moderate/strong staining in >50% of tumor cells. Representative images for PME-1 staining in >50% of the tumor cells. Representative images for PME-1 staining in >50% of the tumor cells. 

Table 1. PME-1 is increased in endometrial cancer and may correlate with grade

<table>
<thead>
<tr>
<th>FIGO grade</th>
<th>PME-1 stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1+</td>
<td>5</td>
</tr>
<tr>
<td>2+</td>
<td>4</td>
</tr>
</tbody>
</table>

NOTE: Patient tissues were sectioned and analyzed after H&E and immunostaining with anti-PME-1 antibody. PME-1 protein levels were assessed as follows: 0 indicates negative PME-1 cytoplasmic staining or faint staining observed in <50% of the cells; 1+ indicates weakly positive PME-1 cytoplasmic staining in >50% of tumor cells or moderate/strong staining in <50% of the cells; and 2+ indicates strongly positive staining in >50% of the tumor cells.

PME-1 and P-cadherin costaining may indicate cancer aggressivity

Our data suggested that PME-1 levels correlate with increased cancerous phenotypes (Fig. 1) and that PME-1 levels are increased in endometrial cancer versus normal tissue (Fig. 3). E-cadherin is a marker for epithelial cells and is commonly decreased in advanced tumors, whereas increased P-cadherin is indicative of more aggressive endometrial cancer (reviewed in ref. 31). We completed immunofluorescent studies (representative images are shown) to determine whether there was a correlation among PME-1 and E-cadherin or P-cadherin in grade 1 and 3 endometrial cancer patient samples. We found that in both grade 1 (patient 06313) and grade 3 (patient 06294), PME-1 and P-cadherin expression was increased compared with corresponding NAT samples (Fig. 4A). In grade 1 samples, more cells expressed PME-1 and P-cadherin, but in grade 3 samples, we noted single cells staining brightly for both proteins (arrows in merge). We then examined PME-1 and E-cadherin expression in grade 1 and 3 tumors. There is overlap in PME-1 and E-cadherin expression in the grade 1 sample (Fig. 4B, arrows in merge), but in grade 3 endometrial cancer, PME-1 is not co-expressed with E-cadherin, suggesting that the cells expressing PME-1 in grade 3 tumors are no longer epithelial but may be mesenchymal in nature.

PME-1 interacts with other protein phosphatases family members

Several reports suggest that PME-1 is specific for PP2A (13, 18, 32); however, we hypothesized that this is unlikely as the C-terminal tail of the catalytic subunit of PP2A (TPDYFL), the substrate for PME-1 demethylation, is similar to that of protein phosphatases 4 (VADYFL) and 6 (TPPYFL), or PP4 and PP6, respectively. The catalytic subunit of PP2A is coded for by two genes, PPP2CA and PPP2CB that produce two proteins, Ppp2ca and Ppp2cb, respectively, which are 97% identical. Their C-termini are 100% identical. Ppp4c and Ppp6c share 63% and 56% identity with Ppp2ca/b, respectively. To determine whether PME-1 specifically targets PP2A, we completed several co-immunoprecipitation studies in the endometrial cancer cell line, ECC-1. Empty vector (FLAG), wild-type PME-1 (FLAG-PME-1), or the inactive mutant of PME-1 (FLAG-PME-1 S156A) were transiently expressed in ECC-1 cells before immunoprecipitation on FLAG resin. Samples were analyzed by Western blot analysis and we probed for endogenous levels of Ppp2ca (Fig. 5A) and Ppp2cb (data not shown). We found that both wild-type and the inactive form of PME-1 (FLAG-PME-1 S156A) are capable of binding Ppp2ca (Fig. 5A), although the interaction of wild-type PME-1 with Ppp2ca is markedly weaker than the interaction of the inactive form of PME-1. This is likely due to a transient interaction between wild-type PME-1 and Ppp2ca, which does not occur with the inactive form of PME-1 as it cannot hydrolyze the methyl group from Ppp2ca. We also confirmed that wild-type PME-1 and the S156A mutant are capable of interacting with endogenous Ppp2cb (data not shown).

We next asked whether PME-1 is able to associate with the conserved PP4 and PP6 catalytic subunits. The same constructs were expressed as above and we probed for endogenous levels of Ppp4c and Ppp6c to confirm association with PME-1. PME-1 does in fact bind the catalytic subunit of PP4, but we could not detect an interaction with PP6 (Fig. 5B). The inactive mutant of PME-1 bound more strongly to Ppp4c compared with wild-type PME-1, suggesting that PME-1 may demethylate PP4.

We next determined whether PME-1 has a preference for binding PP2A or PP4. For this study, we co-expressed empty FLAG vector or FLAG-tagged Ppp2ca or Ppp4c, with empty V5 vector or V5-tagged wild-type PME-1 or inactive PME-1 S156A in HEK293T cells. Figure 5C demonstrates that PME-1 and PME-1 S156A were expressed equally across all studies (see input) and Ppp2ca and Ppp4c were expressed to equal levels. Likewise, when the catalytic subunits were immunoprecipitated (see FLAG elutions), there was equal Ppp2ca and Ppp4c pulled down. Weak interactions were detected with wild-type PME-1 (V5-PME-1) with either Ppp2ca or Ppp4c.
stronger association between V5-PME-1 S156A and Ppp2ca than with Ppp4c, suggesting that while PME-1 can associate with Ppp4c, it has a higher affinity for Ppp2ca.

These data suggest that PME-1 can target both PP2A and PP4. While PP2A has tumor suppressor activity (4, 33, 34), PP4 is implicated in tumor-promoting pathways and is overexpressed in certain cancers (35, 36). Thus, we were concerned that inhibition of PME-1 could promote PP4-dependent activity and counteract the reactivation of PP2A. Although our in vitro data suggest that PME-1 inhibition decreases cell proliferation and reduces cancer phenotypes (Figs. 1 and 2), we asked whether PME-1 inhibition had deleterious effects when PP4 is overexpressed. We transiently transfected empty vector or overexpressed PPP2CA, PPP4C, or PPP6C in ECC-1 cells stably expressing scrambled (black bars) or PME-1 shRNA (white bars) and completed foci-forming assays to determine the effects of PME-1 inhibition when various phosphatases were overexpressed (Fig. 5D). We found that overexpression of PPP2CA decreased foci formation by about 65% (+PPP2CA, black bar), suggesting that increased PP2A alone decreases cell proliferation. Depletion of PME-1 in this background leads to a further significant decrease in foci (+PPP2CA, white bar). Interestingly, overexpression of PPP4C led to about 35% increase in cell proliferation (+PPP4C, black bar) when compared with control cells (Empty, black bar), substantiating other reports that PP4 has a tumor-promoting role. Overexpression of PPP6C led to a significant 25% decrease in foci formation (+PPP6C, black bar), suggesting that PP6 may also have antitumor effects. Importantly, when PME-1 was depleted in all cases, cell proliferation was significantly decreased, even when PPP4C and PPP6C were overexpressed (+PPP4C and +PPP6C, white bars). These data suggest that inhibition of PME-1 is sufficient to decrease cell proliferation regardless of its role in PP4 and/or PP6 regulation.

Figure 4. PME-1–positive cells undergo transition to E-cadherin–negative and P-cadherin–positive mesenchymal cells. Immunostainings of tissue sections from endometrial cancer patient matched pairs comparing grade 1 endometrial cancer (patient 06313) and grade 3 endometrial cancer (patient 06294) using a ×40 objective. A, tissues were immunostained with antibodies specific for PME-1 or P-cadherin, a marker of aggressive endometrial cancer, and were imaged with fluorescent microscopy. B, tumor samples were immunostained with antibodies specific for PME-1 and E-cadherin. 4′,6-Diamidino-2-phenylindole (DAPI) is a nuclear stain; merged images and white arrows demonstrate the co-expression of PME-1 and P-cadherin (A) or PME-1 and E-cadherin (B).
PME-1 promotes tumor formation in an in vivo model

Since our data suggest that PME-1 promotes more aggressive endometrial cancer, we asked whether the overexpression of PME-1 in endometrial cancer cells promoted the formation of tumors in a xenograft model. Instead of RL95-2 cells, which require a high number of cells to induce tumor formation (37), we used ECC-1 cells, which are aggressive endometrial cancer cells with high levels of endogenous PME-1 (Fig. 1A) that have been used previously for similar studies (38). ECC-1 cells expressing either the empty vector (Control) or overexpressing PME-1 (+PME-1) were subcutaneously injected into the flank of 7 female nude mice per group and tumor size was measured weekly. Before injection, we completed Western blot analysis to confirm the proper expression of PME-1 (Fig. 6A). We found that mice injected with +PME-1 cells formed tumors with a significantly larger tumor than control mice by 7 weeks post-injection (Fig. 6B). Quantitative RT-PCR and Western blot analysis of tumor tissue harvested at the end of the study confirmed that PME-1 levels were still increased in +PME-1 cells versus control cells (data not shown). These in vivo data correlate well with our in vitro data and further substantiate our hypothesis that PME-1 promotes cancer progression.
Overexpression of PME-1 causes increased tumor growth in a xenograft model. A total of \(1 \times 10^6\) ECC-1 cells expressing empty vector (control, \(n = 7\)) or overexpressing PME-1 (+PME-1, \(n = 7\)) in PBS were injected subcutaneously into the flank of nude female mice. A, Western blot analysis of ECC-1 cells before injection. B, tumors were measured (length and width) weekly with a caliper to approximate volume over 7 weeks. Averages are shown with SEM. Data were analyzed by 2-way ANOVA for significance, where *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\).

**Figure 6.** Overexpression of PME-1 causes increased tumor growth in a xenograft model.

**Discussion**

PME-1 regulates PP2A activity (13, 14, 21) and we have shown that increased PME-1 correlates with increased cancer phenotypes in endometrial cancer cell lines. Exogenous expression of PME-1 led to increased cell proliferation (Fig. 1C and D) via increased activity of ERK (Fig. 2A; ref. 21) and Akt pathways (Fig. 2B; ref. 9), which promote EMT when constitutively activated (26). Recent work has indicated upregulation of the Akt and ERK signaling pathways in endometrial cancers through several mechanisms (39–41), including the accumulation of mutations (40, 41). Increased PME-1 activity in endometrial cancer likely contributes to this phenomenon.

PP2A dephosphorylates both MEK1/2 and ERK1/2 (42), inhibiting the MAPK pathway and its downstream targets (reviewed in ref. 43). While the specific B subunits regulating MEK1/2 phosphorylation status have not been identified, B/56\(\beta\)- and B/56\(\gamma\)-dependent PP2A are known to dephosphorylate ERK (10). We demonstrated that overexpression of PME-1 correlates with an increase in the activated and phosphorylated ERK (Fig. 2A), thereby stabilizing the pathway. Previous work suggests PME-1 regulates the association of B/55\(\alpha\) subunits with the PP2A core dimer (16, 17) and that reduced methylation of PP2A results in decreased B/55\(\alpha\)-dependent PP2A (44, 45). Our data suggest that PME-1 may affect the activity of B/56-dependent PP2A in the regulation of ERK or that B/55\(\alpha\)-PP2A may also regulate ERK dephosphorylation. Sustained activation of the ERK pathway leads to increased activity of transcription factors, such as oncogenic c-Myc and Elk1 transcription factors (reviewed in ref. 43), promoting cell proliferation.

PP2A has a well-defined role in other signaling pathways. Several isoforms of PP2A are capable of dephosphorylating Akt. Kuo and colleagues identified B/55\(\alpha\)-dependent PP2A as a regulator of Akt signaling via dephosphorylation of T308 in lymphoid and NIH3T3 cell lines (46). More recently, Rodgers and colleagues showed the B/56\(\beta\) subunit is activated by Cdc2-like kinase 2 (Cik2), targeting the B/56\(\beta\)-PP2A holoenzyme to Akt, dephosphorylating Akt at T308 and S473 (11). We found that overexpression of PME-1 led to a dramatic increase in phosphorylation of Akt on T308 and S473 (Fig. 2B) promoting cell proliferation (Fig. 1C and D) supporting the recent findings (9). Activation of Akt correlates with decreased E-cadherin expression and upregulation of EMT-promoting genes, such as Twist, Snail, and Slug (47–49). We note similar trends in decreased E-cadherin and increased vimentin and noggin expression upon PME-1 overexpression (Pusey and Rice, unpublished data).

PME-1 regulation of PP2A activity varies among different cell types and conditions. Previous work demonstrated that PME-1 regulates ERK signaling, but not Akt signaling, in human gliomas (21), whereas recent findings suggest that PME-1 overexpression stabilizes Akt phosphorylation at T308 independent of S473 in HEK-TERC cells (9) expressing shRNA against B/56\(\gamma\). We found that in endometrial cancer cells, overexpression of PME-1 induces phosphorylation of ERK and Akt, indicating inhibition of PP2A holoenzymes containing B/55\(\alpha\), among other B subunits. These data suggest that PME-1 acts as a global inhibitor of PP2A and may not target specific heterotrimers.

Because of the high identity among catalytic subunits of PP2A, PP4, and PP6, we asked whether PME-1 can interact with other protein phosphatases. We identified a novel role for PME-1 in the regulation of PP4. Our data suggest a strong affinity for PME-1 to associate with PP2A and a lesser affinity for PP4, whereas PME-1 does not associate with PP6 (Fig. 5B and C). Interestingly, PP2A has tumor suppressor roles (4, 34, 50) whereas PP4 has tumor-promoting roles (35, 36), suggesting that PME-1 may counterbalance its own activity toward PP2A by also inhibiting PP4 or that PME-1 inhibits PP2A while activating PP4 to promote cell proliferation. These hypotheses require more investigation; however, we have shown that inhibition of PME-1 in the context of PP2A, PP4, or PP6 overexpression decreases cell proliferation (Fig. 5D), substantiating our findings that inhibition of PME-1 in endometrial cancer cells is beneficial to decrease cancer phenotypes, regardless of PP4 and PP6 expression levels.

PME-1 is increased in tumor samples versus NAT in patient samples (Fig. 3 and Table 1; Supplementary Table S2), indicating PME-1 as a potential diagnostic marker for patients with type I endometrial cancer. On the basis of the analysis of 30 patient samples, it is difficult to determine whether there is a correlation with PME-1 levels and cancer grade; however, we noted increased PME-1 mRNA and protein levels in endometrial cancer tumors compared with NAT (Fig. 2A and C) and increased PME-1 immunopositivity in 18 of 19 samples (~95%) tested by IHC, suggesting that PME-1 strongly correlates with disease (Fig. 3D). A slight increase in PME-1 2++ staining was observed in grade 2 compared with grade 1 patient samples (57% vs. 44%); however, the acquisition of more samples is necessary to confirm these findings. Loss of PME-1 staining in 1 of 3 FIGO grade 3 samples may be due to dedifferentiation and loss of histologic and immunohistochemical characteristics; however, 2 of 3 grade 3 samples were positive for PME-1 staining. Importantly, we correlated an increase in PME-1 and F-cadherin costaining and a concomitant loss of PME-1 and E-cadherin costaining in grade 3 samples, suggesting that PME-1

---

**Figure 6.** Overexpression of PME-1 causes increased tumor growth in a xenograft model.
may play a role in endometrial cancer aggressivity. While more patient samples are required to fully investigate a role for PME-1 in promoting cancer progression, we demonstrated that inhibition of PME-1 decreases cancer phenotypes (Fig. 1C–F), increases PP2A activity (Fig. IG), decreases Akt/ERK signaling (Fig. 2), and is therefore an attractive diagnostic marker and potential target for cancer drug therapy for type I endometrioid adenocarcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: E. Wandzioch, M. Pusey, S. Bail, L.M. Rice
Development of methodology: E. Wandzioch, M. Pusey, S. Bail, L.M. Rice
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Wandzioch, M. Pusey, A. Werda, S. Bail, A. Bhaskar
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Wandzioch, M. Pusey, A. Werda, S. Bail, J.-J. Yang, L.M. Rice

References


PME-1 Modulates Protein Phosphatase 2A Activity to Promote the Malignant Phenotype of Endometrial Cancer Cells

Ewa Wandzioch, Michelle Pusey, Amy Werda, et al.

Cancer Res  Published OnlineFirst June 13, 2014.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-13-3130

Supplementary Material  Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2014/06/16/0008-5472.CAN-13-3130.DC1
http://cancerres.aacrjournals.org/content/suppl/2014/06/16/0008-5472.CAN-13-3130.DC2

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.