PME-1 modulates protein phosphatase 2A activity to promote the malignant phenotype of endometrial cancer cells

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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 diagnostic marker and therapeutic target in endometrial cancer.
Precis

Findings identify PME-1 in endometrial cancer as a modifier of malignant development and a theranostic target.
Introduction

Protein phosphatase 2A (PP2A), a heterotrimeric serine/threonine phosphatase, comprised of a scaffolding subunit (A), a catalytic subunit (C) and a B regulatory subunit, is implicated as a human tumor suppressor (reviewed in (1-4)). The core AC dimer recruits a B regulatory subunit, giving the complex substrate specificity. There are four families of B regulatory subunits, comprised 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 (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 post-translationally 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) (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) (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 PME-1 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 (EC) is the most common gynecologic cancer affecting women in the United States, we asked if PME-1 plays a role in EC 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 EC patients.
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 EC and may be a valid drug target for EC treatment.

**Materials and Methods**

Detailed experimental procedures can be found online in Supplemental 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 lentivirus (System Biosciences, Inc). shRNA sequences are in the Supplemental Table S1. 5.0 x 10⁶ ifu/ml lentivirus was added to cell cultures (Sigma Aldrich). Cells were selected with puromycin after 48 hrs (Invivogen).

**Foci Formation Assays.** To measure cell proliferation, 1,000 cells were plated in a six well tissue culture dish in selection media and were grown for 10 to 14 days. Experiments were repeated at least three times. BrDU incorporation experiments were conducted as described in Supplemental Materials.
Analysis of EC patient samples. Matched pairs harvested from 30 patients with Type I endometrial adenocarcinoma purchased from Proteogenex (see 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 immunohistochemistry techniques and immunofluorescence assays (see Supplemental 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 house-keeping genes, 18S or GAPDH.

ERK/Akt inhibition, protein extraction, and western analysis. RL95-2 cells were incubated with either 50 μM Akt inhibitor (LY294002, Cell Signaling Technologies) or 40 μM ERK inhibitor (UO126, Cell Signaling Technologies) for 1-2 hours at 37°C. 1.5 x Laemelli buffer (0.5 M Tris pH6.8, 100% glycerol, 10% sodium dodecyl sulfate, 100 mM 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.** $1 \times 10^6$ endometrial carcinoma cells (ECC-1) diluted in $100 \mu l$ 1X PBS expressing empty vector (Control) or over-expressing PME-1 (+PME-1) were injected subcutaneously into the flank of nude female mice ($n = 7$ per group). Tumor formation was measured weekly for seven weeks with a caliper and tumor volume was calculated according to the formula $V = \frac{1}{2}yx^2$, where $y =$ tumor length and $x =$ tumor width. At 8 weeks post-injection, 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 IACUC.

**Statistical Analysis** was completed using GraphPad Prism version 5.02 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. The data was analyzed with the Mann-Whitney U test for significance (patient samples) or the student’s standard $t$ test; standard errors of the mean (SEM) 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 to determine the validity of PME-1 as a biomarker for EC 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) suggests that PME-1 could be a valuable predictor of
endometrial cancer. Data from animal studies were analyzed using two-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 ((20-21), data not shown), but the role(s) of PME-1 in EC initiation and/or progression has not been elucidated. EC is the most common gynecologic cancer in the US with no reliable non-invasive diagnostic available for early detection of EC. We examined the expression levels of PME-1 in EC cell lines by western analysis (Figure 1A) and found that the endometrioid adenocarcinoma cell lines, RL95-2, Ishikawa, and ECC-1, express more PME-1 than the immortalized endocervical cell line, End1. No immortalized endometrial cell lines exist due to misidentification (27), thus, we used the endocervical cell line since it is derived from a similar tissue.

We next developed RL95-2 cell lines expressing empty vector (control), over-expressing PME-1 (+PME-1), or expressing shRNA (sh #1, sh #2) 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 analysis (Figure 1B). Similar cell lines were constructed for ECC-1 cells as well as the KLE EC cell line (data not shown). Multiple shRNAs and siRNAs were used for these experiments (see Supplemental 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 foci when PME-1 is over-expressed and a significant 75% decrease in foci when depleted for PME-1 compared to control cells (Figure 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 EC cells affects their rate of proliferation, we completed BrDU incorporation assays (Figure 1D). We found a significant ~40% increase in BrDU incorporation in +PME-1 cells compared to empty vector control and a ~40% decrease proliferation in -PME-1 cells compared 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'-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 (Figure 1E). The decrease in foci due to decreased endogenous PME-1 was not rescued by over-expression of the S156A mutant; however, increased foci formation was rescued by the over-expression 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 if PME-1 promotes anchorage-independent growth, we completed soft agar assays and counted the colonies formed. We determined that PME-1 over-expression led to a significant 50% increase in colony formation, while PME-1 inhibition led to a significant 50% decrease in colony formation (Figure 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 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-fold to 40-fold increase in PP2A activity (Figure 1G) likely due to increased methylation of the catalytic subunit of PP2A (13, 19). Over-expression of PME-1 inhibited PP2A activity by ~90% (Figure 1H). Thus, PME-1 inhibition leads to decreased cell proliferation and senescence via increased PP2A activity in EC cell lines.

**Over-expression of PME-1 increases ERK/Akt activation.** Since 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 if increased PME-1 led to the activation of these signaling pathways. Western analysis demonstrated that the ERK phosphorylation was altered with manipulation of PME-1 levels in RL95-2 cells (Figure 2A) and in the KLE EC cell line (data not shown); when PME-1 is over-expressed there is an increase in phosphorylated ERK, while total ERK levels remain unchanged (Figure 2A). Upon PME-1 depletion, there is decreased phospho-ERK compared to control cells. We noted decreased levels of phosphorylated ERK, as expected, upon treatment of cells with UO126, an up-stream inhibitor of ERK phosphorylation.

Similarly, phosphorylation of Akt on threonine 308 (T308) and on serine 473 (S473) increased when PME-1 was over-expressed compared to control and decreased when PME-1 was depleted (Figure 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 the up-stream inhibitor, LY294002. Therefore, PME-1 is a positive regulator of the ERK and Akt cancer signaling pathways in EC cells.

**PME-1 is overexpressed in EC patient tumors.** Uterine tissue samples from 30 patients diagnosed with type I endometrioid adenocarcinoma were purchased from Proteogenex (Supplemental 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 (~83%, Table S2), suggesting that increased $PPME1$ mRNA is indicative of EC. $PPME1$ expression was increased > 20-fold in tumor samples versus normal samples (Figure 3A). ROC analysis (Figure 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 EC. Moreover, $PPME1$ mRNA expression with a cut-off 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 to the loading control, COX IV (Figure 3C). Representative westerns are displayed for FIGO grades 1 – 3. Grade 1 samples (patients 06261, 06310, 06308, 06276) are shown in the top left panels and grade 2 samples (patients 06336, 06241, 06247) are shown in the top right panels. Grade 3 samples (patients 06268,
06294) are shown in the bottom panels. Most tumor samples (T) exhibit increased PME-1 protein when compared to normal adjacent tissue (N).

Patient samples were assessed to determine the stage and grade according to the International Federation of Gynecology and Obstetrics (FIGO) guidelines and were analyzed with H&E staining and/or α-PME-1 antibodies (Figure 3D, 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 (Figure 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; 2+ indicates strongly positive staining with moderate/strong cytoplasmic staining observed in > 50% of the tumor cells. Representative images for PME-1 grading are shown (Figure 3E). Of the 9 FIGO grade 1 samples examined, 55% were 1+ and 45% were 2+, whereas of the 7 FIGO grade 2 samples examined, 43% were 1+ and 57% were 2+. All FIGO grade 1 and grade 2 samples exhibited weak to strong staining for PME-1. Only three FIGO grade 3 samples were examined by IHC for PME-1 and no trend was determined (Table 1).

**PME-1 and P-cadherin co-staining may indicate cancer aggressivity.**

Our data suggested that PME-1 levels correlate with increased cancerous phenotypes (Figure 1) and that PME-1 levels are increased in EC versus normal
tissue (Figure 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 EC (reviewed in (31)). We completed immunofluorescence studies (representative images are shown) to determine if there was a correlation among PME-1 and E-cadherin or P-cadherin in grade 1 and grade 3 EC patient samples. We found that both grade 1 (patient 06313) and grade 3 (patient 06294), PME-1 and P-cadherin expression was increased compared to corresponding NAT samples (Figure 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 grade 3 tumors. There is overlap in PME-1 and E-cadherin expression in the grade 1 sample (Figure 4B, arrows in merge), but in grade 3 EC, 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 (PPP) family members.** Several reports suggest that PME-1 is specific for PP2A (13, 18, 32); however, we hypothesized that this is unlikely since 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 (TTPYFL), 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 if PME-1 specifically targets PP2A, we completed several co-
immunoprecipitation studies in the EC 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 prior to immunoprecipitation
on FLAG resin. Samples were analyzed by western 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), though 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 since 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 if 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 to wild type PME-1, suggesting that PME-1
may demethylate PP4.
We next determined if 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. There is a 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 suggests 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 over-expressed 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. Though our in vitro data suggests that PME-1 inhibition decreases cell proliferation and reduces cancer phenotypes (Figures 1, 2), we asked if PME-1 inhibition had deleterious effects when PP4 is overexpressed. We transiently transfected empty vector or over-expressed 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 over-expression of PPP2CA decreased
foci formation by ~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, over-expression of PPP4C led to a ~35% increase in cell proliferation (+PPP4C, black bar) when compared to control cells (Empty, black bar), substantiating other reports that PP4 has a tumor-promoting role. Over-expression of PPP6C led to a significant 25% decrease in foci formation (+PPP6C, black bar), suggesting that PP6 may also have anti-tumor effects. Importantly, when PME-1 was depleted in all cases, cell proliferation was significantly decreased, even when PPP4C and PPP6C were over-expressed (+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.

**PME-1 promotes tumor formation in an in vivo model.** Since our data suggests that PME-1 promotes more aggressive EC, we asked if the over-expression of PME-1 in EC 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 EC cells with high levels of endogenous PME-1 (Figure 1A) that have been used previously for similar studies (38). ECC-1 cells expressing either the empty vector (Control) or over-expressing PME-1 (+PME-1) were subcutaneously injected into the flank of seven female nude mice per group and tumor size was measured weekly. Prior to injection, we completed western analysis to confirm the proper expression of PME-1 (Figure 6A). We found that mice injected with +PME-1 cells formed
tumors with a significantly larger tumor compared to control mice by 7 weeks post injection (Figure 6B). qRT-PCR and western 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.

**Discussion**

PME-1 regulates PP2A activity (13-14, 21) and we have shown that increased PME-1 correlates with increased cancer phenotypes in EC cell lines. Exogenous expression of PME-1 led to increased cell proliferation (Figure 1C, D) via increased activity of ERK ((21), Figure 2A) and Akt pathways ((9), Figure 2B), which promote EMT when constitutively activated (26). Recent work has indicated up-regulation 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 EC likely contributes to this phenomenon.

PP2A dephosphorylates both MEK1/2 and ERK1/2 (42), inhibiting the MAP kinase pathway and its downstream targets (reviewed in (43)). While the specific B subunits regulating MEK1/2 phosphorylation status have not been identified, B/56β- and B/56γ-dependent PP2A are known to dephosphorylate ERK (10). We demonstrated that over-expression of PME-1 correlates with an increase in the activated and phosphorylated ERK (Figure 2A), thereby stabilizing the pathway. Previous work suggests PME-1 regulates the
association of B/55α subunits with the PP2A core dimer (16-17) and that reduced methylation of PP2A results in decreased B/55α-PP2A formation (44-45). Our data suggests that PME-1 may affect the activity of B/56-dependent PP2A in the regulation of ERK or that B/55α-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 (43)), promoting cell proliferation.

PP2A has a well-defined role in other signaling pathways. Several isoforms of PP2A are capable of dephosphorylating Akt. Kuo et al. identified B/55α-dependent PP2A as a regulator of Akt signaling via dephosphorylation of T308 in lymphoid and NIH3T3 cell lines (46). More recently, Rodgers et al. showed the B/56β subunit is activated by Cdc2-like kinase 2 (Clk2), targeting the B/56β-PP2A holoenzyme to Akt, dephosphorylating Akt at T308 and S473 (11). We found that over-expression of PME-1 led to a dramatic increase in phosphorylation of Akt on T308 and S473 (Figure 2B) promoting cell proliferation (Figure 1C, D) supporting the recent findings (9). Activation of Akt correlates with decreased E-cadherin expression and up-regulation 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 over-expression (M. Pusey and L. 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 over-expression stabilizes Akt phosphorylation at T308 independent of S473 in HEK-TERT cells (9) expressing shRNA against B/56γ. We found that in EC cells, over-expression of PME-1 induces phosphorylation of ERK and Akt, indicating inhibition of PP2A holoenzymes containing B/55α, among other B subunits. These data suggest that PME-1 acts as a global inhibitor of PP2A and may not target specific heterotrimers.

Due to the high identity among catalytic subunits of PP2A, PP4, and PP6, we asked if 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, while PME-1 does not associate with PP6 (Figure 5B, C). Interestingly, PP2A has tumor suppressor roles (4, 34, 50) while PP4 has tumor-promoting roles (35-36), suggesting that PME-1 may counterbalance its own activity towards 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 over-expression decreases cell proliferation (Figure 5D), substantiating our findings that inhibition of PME-1 in EC 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 (Figure 3, Table 1, Table S2), indicating PME-1 as a potential diagnostic marker for patients with type I EC. Based on the analysis of 30 patient samples, it is difficult to determine if there is a correlation with PME-1 levels and cancer grade;
however, we noted increased PME-1 mRNA and protein levels in EC tumors compared to NAT (Figure 2A, C) and increased PME-1 immunopositivity in 18 of 19 samples (~95%) tested by IHC, suggesting that PME-1 strongly correlates with disease (Figure 3D). A slight increase in PME-1 2+ staining was observed in grade 2 compared to grade 1 patient samples (57% versus 44%); however, the acquisition of more samples is necessary to confirm these findings. Loss of PME-1 staining in 1 out of 3 FIGO grade 3 samples may be due to dedifferentiation and loss of histological and immunohistochemical characteristics; however, two of three grade 3 samples were positive for PME-1 staining. Importantly, we correlated an increase in PME-1 and P-cadherin co-staining and a concomitant loss of PME-1 and E-cadherin co-staining in grade 3 samples, suggesting that PME-1 may play a role in EC 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 (Figure 1C – F), increases PP2A activity (Figure 1G), decreases Akt/ERK signaling (Figure 2), and is therefore an attractive diagnostic marker and potential target for cancer drug therapy for type I endometrioid adenocarcinoma.

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Table 1. PME-1 is increased in endometrial cancer and may correlate with grade. 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; 2+ indicates strongly positive staining in > 50% of the tumor cells.

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Figure Legends

Figure 1. PME-1 promotes cancer phenotypes through inhibition of PP2A activity in EC cells. **A**, Western analysis of PME-1 levels in End1 (immortalized endocervical cell line) and the EC cell lines, RL95-2, ECC-1, and Ishikawa cells. GAPDH serves as a loading control. **B**, Western analysis of PME-1 protein in RL95-2 cells expressing empty vector (Control), over-expressing 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**, 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**, BrDU 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 non-targeting siRNA or siRNA against the 3’-UTR of PPME1. Cells were co-transfected with empty vector (Control) or inactive PME-1 S156A. Data is 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 5 independent experiments. **F**, 1 x 10^5 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 is presented as percent of control and represents four independent experiments. Analysis of PP2A activity in RL95-2 cells when **G**, PME-1 was depleted using shRNA, or **H**, PME-1 was over-expressed. Control samples were normalized to 100% PP2A activity and each
experiment was conducted three times. Significance was calculated by the Standard student’s t test; where *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 2. Over-expression of PME-1 induces increased cell proliferation through activation of ERK and Akt signaling pathways. Western analysis of RL95-2 cells expressing control vector, vector with PME-1 or vector carrying shRNA against PME-1 examining levels of A, ERK phosphorylation compared to total ERK and B, Akt phosphorylation at threonine 308 and serine 473 compared to total Akt levels. GAPDH serves as a loading control. Samples treated with the inhibitors of ERK (UO126) and Akt (LY294002) serve as controls.

Figure 3. PME-1 Expression is increased in EC patient samples compared to normal adjacent tissue. A, Analysis of 30 Type I EC patient samples by qRT-PCR examining the levels of PME-1 mRNA in tumor versus normal adjacent tissue (NAT). Means are marked by black bars, error bars represent standard error of the mean (SEM), p value is calculated with the non-parametric Mann-Whitney test. B, ROC analysis of data in figure 3A to determine the efficacy of PME-1 expression to be predictive of endometrial cancer. C, Western analysis of representative endometrial adenocarcinoma patient samples determining PME-1 protein levels in FIGO grade 1 (top left panels), FIGO grade 2 (top right panels), and FIGO grade 3 tumor samples (bottom panel). COX IV serves as a loading control. D, H&E staining of FIGO grades 1, 2, and 3 type I EC. Tissue samples stained with anti-PME-1 antibody are shown to illustrate PME-1 staining grading scale. 0 indicates a negative stain, in which no cytoplasmic staining is observed or faint staining is observed in less than 50% of cells; 1+ indicates a weakly
positive sample with weak cytoplasmic staining detected in more than 50% of the cells or moderate/strong staining detected in less than 50% of cells; 2+ indicates a strongly positive sample, in which moderate/strong staining is observed in more than 50% of cells.

**Figure 4. PME-1 positive cells undergo transition to E-cadherin-negative and P-cadherin-positive mesenchymal cells.** Immunostainings of tissue sections from EC patient matched pairs comparing grade 1 EC (patient 06313) and grade 3 EC (patient 06294) using a 40X objective. **A,** Tissues were immunostained with antibodies specific for PME-1 or P-cadherin, a marker of aggressive EC and were imaged with fluorescent microscopy. **B,** Tumor samples were immunostained with antibodies specific for PME-1 and E-cadherin. 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**).

**Figure 5. PME-1 interacts with the catalytic subunits of PP2A and PP4.** **A,** **B,** ECC-1 cells over-expressing FLAG empty vector, FLAG-PME-1, or FLAG-PME-1 S156A were used to prepare whole cell extracts that were used to immunoprecipitate (IP) with FLAG resin to pull-down PME-1. Western analysis was completed for inputs (10%) and IP (100%) and blots were probed for endogenous Ppp2ca (**A**) or Ppp4c and Ppp6c (**B**). **C,** HEK293T cells were co-transfected with V5-tagged empty vector, V5-PME-1 or V5-PME-1 S156A and FLAG-tagged Pppp2ca or Ppp4c. Immunoprecipitation was completed with FLAG-resin and western analysis was completed to confirm that all plasmids were expressed (input) and to identify which catalytic subunits PME-1 is capable
of binding.  

D, ECC-1 cells stably expressing scrambled shRNA (black bars) or PPME1 shRNA (white bars) were transfected with empty vector or vectors over-expressing the catalytic subunit for PP2A (PPP2CA), PP4 (PPP4C) or PP6 (PPP6C) and were plated at 3,000 cells per well. Foci were grown for 10 days prior to crystal violet staining and quantification and data represent percent foci formation compared to cells expressing scrambled shRNA and empty vector. The experiment was repeated 6 times. Significance was calculated by the Standard student’s \( t \) test; where \(* p < 0.05, ** p < 0.01, *** p < 0.001.\)

**Figure 6. Over-expression of PME-1 causes increased tumor growth in a xenograft model.** \( 1 \times 10^6 \) ECC-1 cells expressing empty vector (control, \( n = 7 \)) or over-expressing PME-1 (+PME-1, \( n = 7 \)) in PBS were injected subcutaneously into the flank of nude female mice. A, Western analysis of ECC-1 cells prior to injection. B, Tumors were measured (length and width) weekly with a caliper to approximate volume over 7 weeks. Averages are shown with standard error of the mean. Data was analyzed by two-way ANOVA for significance, where \(* p < 0.05, ** p < 0.01, *** p < 0.001.\)
Figure 2

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Figure 5

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\[ \alpha\text{-FLAG} \]

D

Graph showing percent foci formation (%) with shSCR and shPPME1 conditions.
Figure 6

A

Control  +PME-1

PME-1

GAPDH

B

Tumor Volume (mm³)

Time (Weeks)

Control

+ PME-1

** * * * * * * * *
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.