PEA3 Is Necessary for Optimal Epidermal Growth Factor Receptor–Stimulated Matrix Metalloproteinase Expression and Invasion of Ovarian Tumor Cells

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Abstract
Elevated expression of the epidermal growth factor (EGF) receptor (EGFR) is detected in human ovarian tumors and is associated with decreased recurrence-free and overall survival. EGFR activation affects tumor progression in part by promoting tumor invasion through the induction of prometastatic matrix metalloproteinases (MMP). PEA3, an ETS family transcription factor, is elevated in advanced and metastatic ovarian cancer and regulates MMPs in various cell types, therefore, we investigated whether PEA3 is required for the EGFR-dependent induction of MMP mRNA. MMP-9 and MMP-14 mRNA levels were selectively increased in response to EGFR activity in ovarian tumor cells. EGFR activation resulted in nuclear accumulation of PEA3 and direct binding of PEA3, but not the related proteinETS-1, to the endogenous MMP-9 and MMP-14 promoters. Furthermore, PEA3 overexpression was sufficient to induce MMP-9 and MMP-14 mRNA, tumor cell migration, and invasion, suggesting that PEA3 is an important contributor to the metastatic phenotype. Additionally, inhibition of PEA3 expression via short interfering RNA reduced the EGF induction of MMP-9 and MMP-14 gene expression by 92% and 50%, respectively, and impaired EGF-stimulated tumor cell invasion. These results suggest that PEA3 is regulated by EGFR and that the elevated PEA3 expression detected in human ovarian cancer may divert cells to a more invasive phenotype by regulating MMP-9 and MMP-14. (Mol Cancer Res 2007;5(5):413–21)

Introduction
Ovarian cancer is the fifth leading cause of cancer death among women in the U.S. (1). The disease is treatable if detected prior to metastasis; however, ~68% of women have distant metastases at diagnosis resulting in a 5-year survival rate of only 29% (1). Ovarian tumors are pathologically heterogeneous, and although the initiating events in ovarian tumor development are poorly understood, ovarian tumorigenesis is characterized by multiple genetic alterations and molecular signatures (2). The epidermal growth factor (EGF) receptor (EGFR) is overexpressed or mutated in a wide variety of human tumors including ovarian carcinomas (3-8). Cellular responses to EGFR activation include those that play a critical role in tumor growth, survival, and progression, whereas EGFR overexpression in tumors has been associated clinically with progression and metastasis (9-11). We are particularly interested in mechanisms by which EGFR mediates cancer metastasis, and our previous work has shown that EGFR activation promotes migration and invasion of ovarian cancer cells (12-15). EGFR activity stimulates the expression and activation of proinvasive molecules including several matrix metalloproteinases (MMP; refs. 12, 13, 16). We have shown previously that EGFR regulates MMP-9 protein, activity, and cell surface association in ovarian tumor cells and that this increased proteinase production stimulates cell invasion through an artificial basement membrane (12, 13, 17, 18). EGF also induces MMP-7 (19, 20) and MMP-14 (21, 22) in other cell types. Therefore, proteinase regulation by EGFR may lead to aggressive tumor cell behavior.

In this study, we investigate the role of the ETS family transcription factor PEA3 in promoting an invasive tumor cell phenotype. Furthermore, we show that PEA3 is a potential mediator of EGF-dependent MMP transcriptional regulation. PEA3 is not detected in normal ovarian tissue but is overexpressed in human ovarian tumors, and its expression in ovarian tumor cells has been correlated with poor overall survival (23-26). In one study, 92% of stage III and IV ovarian tumors (both primary lesions and metastases) expressed elevated PEA3 (24). This suggests that PEA3 plays a significant role in ovarian cancer progression. In a variety of cell types, PEA3 regulates proinvasive MMPs including MMP-1, MMP-7, MMP-9, MMP-13, and MMP-14 (19, 27-37). However, the importance and mechanisms of how PEA3 and PEA3 transcriptional activity contribute to ovarian cancer metastasis has yet to be elucidated. Based on the significant overlap of MMPs regulated by EGF (12, 13, 16) and PEA3 (28), and evidence that EGF can regulate PEA3 (19), we
wanted to ascertain (a) whether PEA3 represents a candidate downstream effector of EGFR signaling leading to MMP production and increased invasion in ovarian tumor cells and (b) whether PEA3 expression alone promotes an invasive ovarian cancer phenotype. We found that EGFR regulates PEA3 nuclear localization and binding to endogenous MMP-9 and MMP-14 promoters, and disruption of PEA3 by short interfering RNA (siRNA) interferes with EGFR-stimulated MMP expression and invasion. Furthermore, ectopic expression of PEA3 alone is sufficient to promote a migratory and invasive phenotype. These studies suggest that PEA3 is an important regulator of ovarian tumor cell behavior.

**Results**

**EGF Induces PEA3 Nuclear Accumulation and MMP-9 Promoter Binding**

EGF induces MMP-9 production in many ovarian tumor cell lines (12, 13), and we found that EGFR activation by ligand (20 nmol/L EGF) or mutation (expression of EGFRvIII) also regulates MMP-9 in OVCA 433 cells (Fig. 1). EGF treatment for 24 h led to an increase in MMP-9 mRNA (Fig. 1A) and MMP-9 activity (Fig. 1B). Similarly, cells expressing the constitutively active EGFRvIII (A1 cells) showed elevated MMP-9 mRNA and gelatinase activity, which was nearly abolished when cells were grown in the presence of AG1478, a catalytic inhibitor of the EGFR kinase activity (Fig. 1A and B).

Because the ETS factor PEA3 is overexpressed in ovarian cancer and is known to regulate MMP-9 (24, 29, 30, 32, 38), we analyzed the PEA3 dependence of EGFR-stimulated MMP-9 gene expression through PEA3.

We first determined whether EGFR activation influences PEA3 nuclear accumulation. PEA3 was present in the nuclei of EGF-treated cells and cells expressing EGFRvIII (Fig. 2). PEA3 immunofluorescence was more diffuse in the untreated OVCA 433 cultures (Fig. 2). Next, chromatin immunoprecipitation (ChIP) was done to determine if EGF induced PEA3 binding to the endogenous MMP-9 promoter. PEA3 binding to the MMP-9 promoter increased after 1 and 6 h of EGF exposure (Fig. 3A). PEA3 also occupied the MMP-9 promoter in EGFRvIII (A1) cells, and the presence of PEA3 at this site was abolished in EGFRvIII (A1) cells maintained in AG1478 (Fig. 3A). PCR on input chromatin indicated that an equivalent amount of chromatin was used for each sample in the ChIP assays. As a negative control, immunoprecipitations were done with normal rabbit serum (Fig. 3A, lane 4). As an additional specificity control, PCR was done on ChIP samples using primers to a region upstream of the MMP-9 promoter that does not contain an ETS/PEA3 binding site (Fig. 3A). PCR product was only detected on input chromatin. These results were consistent with electrophoretic mobility shift assays that showed PEA3 bound to MMP-9 promoter elements in as little as 30 min after EGF treatment (data not shown). Expression of ER81 and ERM transcription factors that are highly related to PEA3 have not been investigated in ovarian cancer. We detect no (ERM) or little (ER81) EGF-dependent regulation of these genes in OVCA 433 cells (data not shown). However, ETS-1 has also been shown to be overexpressed in ovarian cancer, and its expression correlates with poor survival (39, 40). Therefore, we investigated whether EGF treatment induced binding of ETS-1 to the MMP-9 promoter. ETS-1 transiently associated with the MMP-9 promoter after 1 h of EGF treatment (Fig. 3B). However, by 6 h, the association of ETS-1 with the MMP-9 promoter was markedly decreased and little binding was detected in cells expressing the constitutively active EGFRvIII (Fig. 3B). These data suggest that EGF induces MMP-9 mRNA by stimulating PEA3 nuclear accumulation, DNA binding activity, and PEA3 association with the endogenous MMP-9 promoter.

**Exogenous PEA3 Expression Mimics the Effects of EGFR Activation**

In order to determine whether PEA3 activity potentiated the invasive potential in ovarian cancer cells, OVCA 433 cells were stably transfected with human PEA3 cDNA or the corresponding empty vector (pCDNA3). Two independent clones overexpressing PEA3 (P2 and P6) or empty vector were selected for detailed analysis. DNA binding activity to an ETS/PEA3 site from the MMP-9 promoter was increased in cells that overexpressed PEA3 (data not shown), consequently, P2 and P6 cells had increased secretion of MMP-9 as determined by gelatinase activity in zymography analysis (Fig. 4A). MMP-9 production by P2 and P6 cells was similar to that of cells overexpressing MMP-9 (Fig. 4A) or vector control cells treated with EGF for 24 h (data not shown). Vector control cells exhibited an epithelial morphology identical to the parental OVCA 433 cells as observed by phase contrast microscopy.

![FIGURE 1](mcr.aacrjournals.org)  EGFR activation increases MMP-9 mRNA and gelatinase activity. A, OVCA 433 cells were either serum-starved for 24 h or serum-starved and then treated with 20 nmol/L of EGF for an additional 24 h. EGFRvIII (A1) cells were untreated or grown in the presence of the EGFR inhibitor (5 μmol/L AG1478 for 7 d. To assure stability, AG1478 was replenished every 2 d. Reverse transcription-PCR was done for MMP-9 expression (top) and 18s RNA was used as a control (bottom). B, OVCA 433 cells were either serum-starved for 24 h or serum-starved and then treated with 20 nmol/L of EGF for 24 h. A1 cells were untreated or grown in the presence of 2 μmol/L of AG1478 for 7 d. Conditioned medium was collected from the different treatments and zymography analysis was done.
Next, the influence of PEA3 overexpression on migration and invasion was investigated. EGF increased vector control migration by 19.6-fold (Fig. 5A), whereas expression of PEA3 increased migration through cell culture inserts by 8.3- and 1.9-fold over untreated vector control cells in the P2 and P6 cell clones \((P < 0.05\) and \(P = 0.05\), respectively; Fig. 5A). Invasive potential was measured using cell culture inserts coated with artificial basement membrane (Matrigel). Vector control cells exhibited a 12.8-fold increase in invasion with the addition of EGF \((P < 0.01\); Fig. 5B). Invasion of P2 and P6 cells was similar to EGF stimulation with an 11.3- and 8.3-fold increase above untreated vector control cells, respectively \((P < 0.01\); Fig. 5B). Therefore, PEA3 overexpression is, in part, sufficient to promote gelatinase activity, migration, and \textit{in vitro} invasion.

**PEA3 Expression Induces Endogenous MMP-9 and MMP-14 Expression**

PEA3 has been reported to regulate several MMPs that are implicated in cancer cell invasion including MMP-1, MMP-7, MMP-9, MMP-13, and MMP-14 (19, 27, 29-36, 41, 42). To further examine the role of PEA3 in the regulation of MMP gene expression in ovarian cancer, quantitative PCR (Q-PCR) was done on parental OVCA 433 cells and cells ectopically expressing PEA3. Expression of MMP-1, MMP-7, and MMP-13 was unchanged in cells overexpressing PEA3, and EGF treatment did not affect the expression of these genes in OVCA 433 cells (data not shown). MMP-9 mRNA was increased by 6.4- and 7.5-fold in the P2 and P6 cells compared with vector cells \((P < 0.05\); Fig. 6A), and PEA3 overexpression increased MMP-14 mRNA production by 15.4- and 8.7-fold over vector cells \((P < 0.05\); Fig. 6A). Because PEA3 overexpression increased MMP-14 mRNA, we sought to determine if EGFR activation induced MMP-14 through PEA3. Increased binding of PEA3 to the endogenous MMP-14 promoter was detected by ChIP analysis in EGF-treated OVCA 433 cells and EGFRvIII-expressing cells (Fig. 6B). Again, we examined the ability of EGFR to regulate another ETS family member, ETS-1, in MMP-14 activation. ChIP analysis was done on OVCA 433 cells treated with EGF for 0, 1, and 6 h and EGFRvIII cells with and without AG1478 treatment. Under all conditions, there was
only a low level of ETS-1 association with the MMP-14 promoter that did not change with treatment (Fig. 6C). PCR on input chromatin indicated that equal chromatin was used in the immunoprecipitations. No PCR product was produced from immunoprecipitations in the absence of anti-PEA3 (immunoprecipitations done with normal rabbit serum) or the anti–ETS-1 antibodies (Fig. 6B and C).

Because EGF stimulated PEA3 binding to the endogenous MMP-9 and MMP-14 promoters, we investigated whether PEA3 expression is required for EGF-induced MMP-9 and MMP-14 expression in OVCA 433 cells using a siRNA approach. We tested three different siRNA oligonucleotides for their ability to knockdown PEA3 mRNA expression (siRNA no. 1, siRNA no. 2, and siRNA no. 3). Both siRNA no. 1 and siRNA no. 3 significantly knocked down PEA3 expression at 72 and 96 h post-transfection, respectively (Fig. 7A). Both of these siRNA oligonucleotides specific for PEA3 had similar effects on target gene expression and invasion. OVCA 433 cells were untransfected, mock-transfected, or transfected with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) siRNA, a negative control (nonspecific siRNA provided by Ambion), siRNA no. 1, or siRNA no. 3. Cells were then serum-deprived for 24 h and then treated with EGF for 24 h. Q-PCR analyses revealed that EGF induced MMP-9 mRNA expression by 5.4-fold ($P < 0.05$; Fig. 7B) and transfection of siRNA against PEA3 reduced the expression of MMP-9 in the untreated as well as the EGF-treated OVCA 433 cells ($P < 0.01$; Fig. 7B), suggesting that PEA3 may be involved in both the regulation of basal levels of MMP-9 and EGF-induced MMP-9 expression. EGF treatment induced MMP-14 expression by 3-fold ($P < 0.05$), and this expression was reduced to 1.9-fold in EGF-treated cells transfected with PEA3 siRNA ($P < 0.05$). These results indicate that PEA3 is an EGF-regulated transcription factor important for MMP-9 and MMP-14 expression in ovarian cancer cells.

Finally, because MMPs play key roles in tumor invasion, we sought to determine if the disruption of PEA3 alters EGF-induced invasion. Matrigel invasion assays were done on cells transfected with negative control siRNA or siRNA no. 3. Untreated control OVCA 433 cells exhibited little invasion in the absence of stimulus, whereas EGF treatment increased cell invasion by at least 2-fold (Fig. 7C and D). SiRNA no. 3 did not alter invasion in the absence of EGF stimulation ($P = 0.7$; Fig. 7D). EGF stimulated invasion by 2.3-fold in negative control siRNA cells ($P = 0.01$) but only by 1.5-fold in siRNA no. 3–transfected cells, resulting in a significant reduction in invasion ($P = 0.03$; Fig. 7D). PEA3 expression is an important element of this EGFR-driven invasion.

**Discussion**

Tumor metastasis results from numerous changes in gene expression and cell behavior. Identifying transcription factors that regulate cell- or tumor type–specific metastatic programs may aid in the development of novel treatments and the prevention of tumor spread. In this study, we show for the first time that EGFR regulates PEA3 activity resulting in MMP-9 and MMP-14 gene transcription in ovarian cancer cells. EGFR activation by ligand or mutation increased PEA3 nuclear accumulation (Fig. 2) and stimulated PEA3 occupancy on the endogenous MMP promoters (Figs. 3 and 6). Furthermore, EGF induced MMP-9 and MMP-14 gene expression through a PEA3-dependent mechanism based on siRNA analysis. EGFR seems to regulate PEA3 through multiple mechanisms. PEA3 DNA binding activity was increased within 1 h of EGF treatment (Fig. 3A), suggesting that EGFR activates an existing latent pool of PEA3 protein. Additionally, EGF treatment for 24 h results in a 3-fold increase in PEA3 mRNA as detected by Q-PCR (data not shown), which may promote a more durable effect on PEA3 activity by increasing the total levels of available PEA3 in cells.

EGFR regulates multiple ETS family transcription factors; however, there seems to be selectivity in which ETS proteins are activated and which MMPs are targets in different model systems. MMP-9 and MMP-14 were selectively regulated by PEA3 in the ovarian tumor cells. In contrast, EGF induces
MMP-7 via PEA3 in colon and esophageal cancer cell lines (19, 36) and even though ChIP identified EGF-dependent PEA3 binding to the MMP-7 promoter in OVCA 433 cells, there was no corresponding increase in mRNA by Q-PCR (data not shown). There were striking differences between EGF-induced PEA3 and ETS-1 activity in the ovarian tumor cells. In contrast to PEA3, ETS-1 only transiently associates with the MMP-9 promoter after 1 h of EGF treatment (Fig. 3) and basal level binding of ETS-1 to the MMP-14 promoter is insensitive to EGF activation (Fig. 6). These observations imply that PEA3 is the more relevant ETS family member for the observed EGF-dependent responses in these studies. PEA3 also regulates MMPs through cooperation with other transcription factors (29, 30, 34, 41, 43). Perhaps subtle differences in the transcription factor network between different cells types may direct EGF signaling to distinct cohorts of MMPs.

PEA3 is elevated in ovarian carcinomas (24, 25, 38), suggesting that PEA3 is an important determinant in the development or progression of ovarian cancer. In OVCA 433 cells, PEA3 overexpression alone is sufficient to stimulate morphologic and functional changes similar to those observed following EGF activation including cell dissociation, increased cell motility, and invasion (Figs. 4 and 5). Ectopic PEA3 overexpression also greatly increased MMP-9 and MMP-14 expression. PEA3-dependent regulation of these MMPs may have significant consequences for metastatic progression in ovarian cancer. MMP-9 expression in either ovarian epithelial tumor cells or the adjacent malignant stroma was indicative of poor prognosis (44-46). MMP-14 is expressed in borderline and malignant, but not benign ovarian tumors, suggesting that MMP-14 is associated with more advanced disease (47). Our findings of PEA3-dependent regulation of MMP-9 and MMP-14 in ovarian tumor cells may reflect an important biological role for PEA3 overexpression in ovarian cancer based on the observations that epithelial expression of MMP-9 or MMP-14 correlate with decreased patient survival (44-46). Understanding oncogenic signaling cascades that culminate in the expression of metastatic genes will be critical to understanding the etiology of ovarian cancer, and in identifying new potential therapeutic targets.

Materials and Methods

Materials
EGF was purchased from Biomedical Technologies, Inc. Matrigel was purchased from Becton Dickinson. Protease inhibitors were obtained from Sigma. Anti-PEA3 polyclonal antibody was obtained from Santa Cruz Biotechnology, and FITC-conjugated anti-rabbit secondary antibody was purchased from Chemicon. AG1478 was purchased from LC Laboratories. Cell culture reagents were also obtained from Sigma.

Cell Culture and Generation of Stable Cell Lines
The epithelial ovarian carcinoma cell line OVCA 433 (and all of the stable cell lines generated from the OVCA 433 cells) were grown in Eagle’s MEM supplemented with 10% fetal bovine serum, 0.2 mmol/L of l-glutamine, 1 mmol/L of sodium pyruvate, 50 units/mL of penicillin, and 50 μg/mL of streptomycin. OVCA 433 cells were a kind gift from Robert Bast, Jr. (M.D. Anderson Cancer Center, Houston, TX). OVCA 433 cells stably expressing the constitutively active EGFRvIII (vIII A1 cells) were described previously (14, 15). Where indicated, OVCA 433 cells were grown to 30% confluency, serum-starved in medium containing bovine serum albumin for 24 h, and treated with 20 nmol/L of EGF for the indicated time points. EGFRvIII (A1) cells alone or treated with 5 μmol/L of the EGFR tyrosine kinase inhibitor AG1478 for 7 days to fully reverse the EGFRvIII migratory fibroblastic phenotype were also used in various assays (15). A human PEA3 cDNA image clone was obtained from American Type Culture Collection. PEA3 was subcloned into EcoRV and HindIII sites in pCDNA3. OVCA 433 cells were transfected with 10 μg of PEA3/pCDNA according to the manufacturer’s instructions using Superfect (Qiagen) and stable cell lines expressing PEA3 were selected using 2 mg/mL of G418 and maintained in 0.75 mg/mL of G418. Fifteen PEA3-expressing cell lines were generated, and two were chosen for further analysis. Stable cell lines were selected based on their DNA binding in an electrophoretic mobility shift assay and gelatinase activity in a zymography assay.

FIGURE 5. EGF and PEA3 expression induces migration and invasion. 

A. Vector control, P2, and P6 cells were plated on cell culture inserts with 8 micron pores and allowed to migrate in the presence of serum-free medium for 48 h. Vector cells were also allowed to migrate towards EGF for 48 h. After 48 h, migratory cells were stained with crystal violet, the membranes were photographed, and cells were counted. Columns, fold change over the untreated control cells (*, P < 0.01; **, P < 0.05).

B. To measure invasion, vector (in the presence and absence of 20 nmol/L of EGF), P2, and P6 cells were plated on porous cell culture inserts coated with Matrigel. Cells were allowed to invade for 48 h, after which, they were stained with crystal violet, photographed, and the number of cells were counted. Columns, fold change over the untreated vector control cells (*, P < 0.05).
Microscopy

Phase contrast microscopy and immunofluorescence microscopy were done using an Olympus BH-2 inverted microscope or an Olympus 1X70 fluorescence microscope, respectively. Images were collected using an Olympus America camera and Magnafire 2.1 software. Briefly, cells were plated on chamber slides and fixed for 10 min at room temperature in ice-cold acetone, washed in PBS, permeabilized with PBS/0.2% Triton X-100 for 5 min, and blocked in 0.2% (w/v) bovine serum albumin in PBS with 0.1% Tween 20 for 10 min. Cells were then incubated with a 1:200 dilution of anti-PEA3 primary polyclonal antibody (H120, Santa Cruz Biotechnology) in PBS/0.1% Tween 20 at 4°C overnight, washed thrice for 5 min with PBS/0.1% Tween 20, followed by incubation with 1:500 of FITC-conjugated anti-rabbit secondary antibody (Chemicon) in PBS with Tween 20 at room temperature for 1 h. Slides were again washed thrice for 5 min with PBS/0.1% Tween 20 prior to mounting coverslips on slides using Vectashield mounting media with 4',6-diamidino-2-phenylindole (Vector Laboratories) to visualize nuclei.

Zymogram Analysis

For all zymography analyses, cells were serum-deprived for 24 h and zymography was done on conditioned media as described previously (12, 13). Briefly, after 24 h of serum deprivation, samples of conditioned media were collected and whole cell lysates were made by lysing cells in 0.1% Triton X-100. The lysate was vortexed, centrifuged for 10 min at 12,000 × g at 4°C and supernatant was collected. The protein concentration was determined from the whole cell lysates and 5 to 15 μg of protein-equivalent medium was used for zymogram analysis. A nonreducing sample buffer was added to each sample. Samples were analyzed for proteinase activity by resolving on 7.5% SDS-polyacrylamide gels containing 0.1% gelatin. Following electrophoresis, gels were washed with 2.5% Triton X-100 for 30 min at room temperature and then incubated with substrate buffer (50 mmol/L Tris, 0.2 mol/L NaCl, 5 mmol/L CaCl2, and 0.02% Brij; pH 7.6) for 12 h at 37°C. Proteinase activity was visualized as zones of clearance in a Phastgel Blue R (Sigma)—stained gel. Results shown are representative of a minimum of three independent experiments.

mRNA Detection

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. cDNA was generated from 2 μg of sample RNA using High Capacity cDNA Archive kit according to the instructions of the manufacturer (Applied Biosciences). Briefly, 25 μL reactions were made for each sample containing a 2× Master Mix (10× reverse transcriptase buffer, 25× deoxynucleotide triphosphates, 10× random primers, MultiScribe reverse transcriptase, and water). cDNA reactions were incubated in a thermocycler at 25°C for 10 min and then at 37°C.
for 2 h. The following primers were used for reverse transcription-PCR: MMP-9 forward, 5′-GAGACCGGTGAGCTGGATAG-3′; MMP-9 reverse, 5′-TACACCGGAGTGAAGTGAG-3′; 18s forward, 5′-AAACGGCTACCACATCCAAG-3′; 18s reverse, 5′-CCTCCAATGGATCCTCGTTA-3′; PEA3 forward, 5′-CAGTCACTTCTCTCTCTAGTC-3′; and PEA3 reverse, 5′-CCTTCTGCTTATACCCAGCAC-3′. The MMP-9 and PEA3 PCR program used was: 94°C for 4 min, 94°C for 30 s, 63°C for 30 s and 72°C for 30 s for 29 cycles and then 72°C for 4 min (MMP-9 generated a 236-bp product and PEA3 had a 302-bp product). The 18s PCR program used was: 94°C for 4 min, 94°C for 30 s, 55°C for 30 s and 72°C for 30 s for 19 cycles and then 72°C for 4 min (100 bp product). PCR products were analyzed on a 2% agarose gel.

For Q-PCR, total RNA was extracted and cDNA was prepared as described above. SYBR Master Mix solution was obtained from Applied Biosciences, and commercially available primer/probe Quantitect Primer Assays for GAPDH, MMP-9, and MMP-14 were obtained from Qiagen. cDNA was then assayed in a 20 μL total volume primer probe pair/Master Mix solution (2× SYBR green Master Mix, 10× Quantitect Primer assays, 2 μL of cDNA, and water). All reactions were done in duplicate and all experiments were done at least thrice using a 7900HT sequence detection system (Applied Biosciences). ΔΔct calculations were used to normalize signal versus GAPDH controls.

ChIP Assays

ChIP assays were done as described previously (48). Briefly, OVCA 433 cells were grown to 30% density on 15 cm plates, serum-starved in medium containing bovine serum albumin for 24 h, and treated with 20 nmol/L of EGF for 1 or 6 h.

![PEA3 knockdown decreased MMP-9 and MMP-14 expression in EGF-treated OVCA 433 cells.](image-url)
EGFRvIII(A1) cells, alone or treated with 5 μmol/L of AG1478 for 7 days, were also collected. Cells were formaldehyde cross-linked followed by the addition of 0.125 mol/L of glycine. Collected cells were centrifuged (1,500 × g for 5 min). Cell pellets were washed with PBS and lysed (10 mmol/L Tris (pH 8.0), 10 mmol/L NaCl, 0.2% NP40, 50 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, and 1 μmol/L pepstatin). Nuclei were recovered by centrifugation (1,500 × g for 5 min at 4°C) and then lysed [50 mmol/L Tris (pH 8.1), 10 mmol/L EDTA, 1% SDS, and 50 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, and 1 μmol/L pepstatin]. Chromatin was sonicated and 500 μg of nuclear protein lysates were precleared and immunoprecipitated with normal rabbit serum or anti-PEA3 (H120, Santa Cruz Biotechnology) prebound to Protein A agarose. Immunoprecipitates were washed in buffer 1 [20 mmol/L Tris (pH 8.1), 2% EDTA, 50 mmol/L NaCl, 1% Triton X-100, and 0.1% SDS], buffer 2 [20 mmol/L Tris (pH 8.1), 2% EDTA, 50 mmol/L NaCl, 1% Triton X-100, 0.01% SDS], buffer 3 [10 mmol/L Tris (pH 8.1), 2% EDTA, 0.25 mol/L LiCl 1% NP40, and 1% deoxycholic acid], TE, and then eluted with 100 mmol/L of NaHCO₃ containing 1% SDS. Cross-links were reversed by overnight incubation with 0.3 mg/mL of protease K at 45°C. DNA was isolated and resuspended in water. PCR was done on MPP-9 and MPP-14 promoter elements and genomic region upstream of the MPP-9 promoter for specificity using the following Sigma Genosys primers: MPP-9F, 5'-TCCGAGCTCACCATGATGGT-3'; MPP-9R, 5'-CTTTTGACTCAGCTTCCTC-3'; MPP-14F, 5'-GAGGAATCGGCACCAGTCAAT-3'; MPP-14R, 5'-GTTCTCGTGAAGGCTCAGCTT-3'; and UpstreamF, 5'-GTGGGGCTCTGC TCATACAT-3', and UpstreamR, 5'-ATC TTCAAGGGATGAGGAGG-3'. The MMP-9 PCR program used was: 94°C for 4 min, 94°C for 30 s, 55°C for 30 s and 72°C for 30 s for 29 cycles and then 72°C for 4 min (336 bp product). The MMP-14 and upstream control PCR program used was: 94°C for 4 min, 94°C for 30 s, 63°C for 30 s and 72°C for 30 s for 35 cycles and then 72°C for 4 min (440 bp product for the MMP-14 promoter and 151 bp for the upstream control).

**Migration and Invasion Assays**

Cells (1.25 × 10⁵) were allowed to migrate for 48 h through 8 micron pore cell culture inserts (Becton Dickinson Labware). Nonmigratory cells were removed. Migratory cells were stained with 0.2% crystal violet in 10% ethanol. Invasion was measured using 8 micron pore BD BioCoat Matrigel Invasion Chambers (BD Biosciences) according to the manufacturer’s instructions. Cells (2.5 × 10⁵) were added to chambers and incubated for 48 h at 37°C. Matrigel and noninvasive cells were removed and chambers were stained as described above. To quantitate invasive cells, three independent fields of migratory or invasive cells per well were photographed under phase contrast microscopy. The number of cells per field were counted and averaged. Each migration or invasion assay was done at least thrice.

**SiRNA Analysis**

SiRNA against PEA3 was purchased from Ambion. Three different siRNA oligonucleotides were tested and the following two were used in the described knockdown experiments: ETV4 ID 115237 and ETV4 ID 106636. All experiments reported used ETV4 ID 115237, but similar siRNA results were obtained with ETV4 ID 106636. Ambion Silencer GAPDH siRNA controls (which includes GAPDH siRNA and a negative control siRNA) were used as siRNA controls. At 50% confluency, OVCA 433 cells were mock-transfected on 10 cm plates or transfected with 50 nmol/L of PEA3 siRNA, 50 nmol/L of GAPDH siRNA, or 50 nmol/L of negative control siRNA using Superfect (Qiagen). Forty-eight hours after transfection, cells were serum-starved for 24 h and then treated with 20 nmol/L of EGF for 24 h prior to RNA collection and Q-PCR analysis. SiRNA-treated cells were compared (normalized) to negative control siRNA (control si) for Q-PCR and invasion analyses. For invasion assays, 24 h after transfection, 2 × 10⁵ cells were placed in invasion chambers as described. Cells were allowed to invade for 48 h at 37°C. To quantitate invasive cells in Photoshop, histograms were used to generate mean color intensities. Histograms were generated for two images per plate. The mean color intensity was averaged for each plate. Experiments were done in duplicate in three independent experiments.

**Statistical Analysis**

Each experiment was done at least thrice. Error bars represent ± SE. Student’s t tests were done to determine statistical significance for the following assays: migration, invasion, and Q-PCR.

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**References**


Molecular Cancer Research

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