Glycerol-3-phosphate Acyltransferase 1 Promotes Tumor Cell Migration and Poor Survival in Ovarian Carcinoma

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Abstract

Glycerophosphodiesterase EDI3 (GPCPD1; GDE5; GDPD6) has been suggested to promote cell migration, adhesion, and spreading, but its mechanisms of action remain uncertain. In this study, we targeted the glycerol-3-phosphate acyltransferase GPAM along with choline kinase-α (CHKA), the enzymes that catabolize the products of EDI3 to determine which downstream pathway is relevant for migration. Our results clearly showed that GPAM influenced cell migration via the signaling lipid lysophosphatidic acid (LPA), linking it with GPAM to cell migration. Analysis of GPAM expression in different cancer types revealed a significant association between high GPAM expression and reduced overall survival in ovarian cancer. Silencing GPAM in ovarian cancer cells decreased cell migration and reduced the growth of tumor xenografts. In contrast to these observations, manipulating CHKA did not influence cell migration in the same set of cell lines. Overall, our findings show how GPAM influences intracellular LPA levels to promote cell migration and tumor growth. Cancer Res; 77(17): 1–13. ©2017 AACR.

Introduction

Altered metabolism is increasingly linked to cellular transformation, tumor progression, and poor outcome in several cancer types (1–3). Although most studies have focused on changes in glucose and glutamine metabolism (2), abnormal choline metabolism is also relevant, as elevated levels of phosphorycholine and total choline have been consistently reported in different tumors compared with normal tissue (4). Some of the key proteins and processes involved in choline metabolism that are altered in cancer have already been elucidated (4), most recently the glycerophosphocholine phosphodiesterase (GPC-PD), EDI3 (GDE5; GDPD6; GPCPD1), which hydrolyzes the glycerophosphodiester GPC to choline and glycerol-3-phosphate (G3P; refs. 4–6). Both products are key intermediates in metabolic pathways, including choline, lipid, glucose, and carbohydrate metabolism, suggesting that EDI3 is a crucial link between de novo phospholipid synthesis and energy metabolism (Fig. 1A; ref. 7). Indeed, downregulating EDI3 altered choline metabolite levels in several cell lines, decreased the signaling lipids, phosphatidic acid (PA), and lysophosphatic acid (LPA), as well as the major membrane phospholipid, phosphatidylcholine (6).

EDI3 was initially identified in a screen for potential markers of metastasis in endometrial cancer, where high EDI3 expression in primary tumors was associated with shorter metastasis-free survival (MFS; ref. 6). To elucidate EDI3’s contribution to metastasis and cancer, phenotypic studies conducted after altering EDI3 expression demonstrated a role in cellular migration, attachment, and spreading, processes altered in cancer (6, 8), but the exact mechanism linking EDI3 to these processes remained unknown. Our initial work showed an association between EDI3 expression, PKCα and integrin β1 (6, 8). However, most of the key signaling proteins known to regulate migration and adhesion remained unchanged after manipulating EDI3 levels in vitro (6), suggesting that EDI3-induced metabolic changes may be responsible.

The enzymatic products of EDI3, choline and G3P, are rapidly metabolized via different pathways to products that serve as substrates for other enzymes (Fig. 1A). Both products are linked to diverse cellular functions, including the production of structural and signaling phospholipids, triglyceride synthesis, the G3P shuttle, glycolysis, and the methylation cycle via choline’s oxidiation product, betaine. Therefore, to systematically address which of the downstream metabolic pathways is relevant for migration,
we focused on two well-characterized enzymes that directly metabolize choline and G3P, namely choline kinase-alpha (CHKA) and G3P acyltransferase 1 (GPAT1; GPAM; Fig. 1A), respectively. CHKA has been previously linked to migration (9–12); however, a role for GPAM in this process has never been described.

Four GPATs have been identified to date, which are primarily characterized for their roles in the de novo synthesis of intracellular glycerophospholipids and triglycerides (TAG; ref. 13). GPATs esterify long-chain fatty acyl coenzyme-A to G3P to produce LPA, which is further metabolized to PA, signaling lipids implicated in many diverse cellular processes (14–16), including cell migration. GPAM is the most studied member of the family, especially regarding its role in triglyceride metabolism (17); however, very little is known about its role in cancer or cancer-related phenotypes.

In the current work, we analyzed the contribution of CHKA and GPAM to migration and metabolism in relation to EDI3. We observed no influence of CHKA on migration in the studied cell types, and EDI3 knockdown/overexpression results were compared with cells transfected with scrambled siRNA (si-NEG).

Figure 1.
Knockdown of GPAM and EDI3, but not CHKA inhibits cell migration. A, Overview of EDI3 metabolic pathway identifying potential links to cell function. EDI3 cleaves GPC to choline (Cho) and G3P, making it relevant in choline, glycerophospholipid, and triglyceride metabolism. EDI3 may play a role in glycolysis and gluconeogenesis due to the conversion of G3P to dihydroxyacetone phosphate (DHAP), and in signaling pathways activated by the lipids, LPA, PA, and diacylglycerol (DAG). The study concept was to compare the effect of EDI3, GPAM (GPAT1), and CHKA knockdown/overexpression on cell migration and metabolite levels. B, Successful silencing of EDI3, GPAM, and CHKA in MCF7 cells compared with cells transfected with scrambled siRNA (si-NEG). Representative images of MCF7 (C) and HeLa (D) cells scratch assay after EDI3, GPAM, and CHKA knockdown with accompanying quantification indicating percentage of wound closure compared with control (si-NEG). All data are mean ± SD of five and three independent experiments for MCF7 and HeLa cells, respectively (**, P < 0.01; ***, P < 0.001). Images were taken using a 10× objective. CDP-Cho, cytidine 5′-diphosphocholine; CHKA, choline kinase alpha; CT, phosphocholine cytidyltransferase; DAGK, DAG kinase; DAGAT, DAG acyltransferase; FM, full media control; GPAT, G3P acyltransferase; LPAAT, LPA acyltransferase; PAP, PA phosphatase; PCho, phosphocholine; PCT, diacylglycerol choline phosphotransferase; PLA, phospholipase A; PLB, phospholipase B; PLC, phospholipase C; PtdCho, phosphatidylcholine; TAG, triacylglycerol.
Materials and Methods

Cell lines and cell culture

MC7, MDA-MB-231, and HeLa cells were purchased in 2011, OVCAR-3 in 2012, and EFO-27 in 2016 from the ATCC. HEK293, A2780, and OVCAR3 cells were maintained in RPMI1640 (PAN-Biotech) supplemented with 10% heat-inactivated FBS (PAN-Biotech). ES-2 cells were bought from the ATCC in 2012, and ES-2-shNEG and ES-2-shGPAM cells were maintained in ES-2 media supplemented with 1 μg/mL puromycin (Thermo Fisher Scientific). Cells were maintained in a humidified incubator at 37°C with 5% CO₂. Experiments were conducted using cells passaged twice after thawing from liquid nitrogen storage and cultured for approximately 12 passages. Mycoplasma assays were performed prior to storage in liquid nitrogen and around passage five for cultured cells using Venor GeM Classic Mycoplasma Detection Kit (Minerva Biolabs). All cells were sent to the DSMZ for authentication using STR DNA typing according to ANSI/ATCC ASN-0002-2011 guidelines.

Expression alteration and analysis

Expressions of ED13, GPAM, and CHKA were transiently down-regulated using siRNA (Supplementary Table S1A; ref. 6). CHKA and GPAM were overexpressed in HEK293 cells with vectors from AMSBio. For in vivo experiments, ES-2 cells were infected with lentiviral particles containing either non-targeting or two shRNA sequences targeting GPAM cloned into the pLenti-U6-shRNA-Rsv (GFP-Puro) vector following the manufacturer’s protocol (AMSBio; Supplementary Table S1B). Total RNA was isolated using the RNaseasy Mini Extraction Kit (Qiagen), quantified using the NanoDrop N-2000 spectrophotometer (NanoDrop), and expression measured with qRT-PCR using Quantifast SYBR Green RT-PCR Kit and primer assays (Qiagen; Supplementary Table S2). Relative quantification was calculated using the 2⁻ΔΔCt method. Affymetrix gene array analysis to compare overlapping genes after silencing GPAM and ED13 is described in detail in the Supplementary Information. Whole-cell lysates for Western blotting were prepared as described previously (8), and protein expression was detected with the following primary antibodies: anti-ED13 (custom-made AMSBio); GPAM (Santa Cruz Biotechnology and Abcam); CHKA (Abcam and Sigma); calnexin and PCNA (Cell Signaling Technology); and β-actin (Sigma-Aldrich). Images were taken on a VILBER Fusion Fx7 imager (VILBER Lourmat), and bands were quantified using ImageJ (NIH, Bethesda, MD).

Cell migration

Migration was investigated using the scratch and transwell assays as described previously (6). The Scratch Assay Analyzer operator for the ImageJ (NIH) plugin, Microscope Image Analysis Toolbox (MiToBo) was used to quantify the open area of the scratch.

Xenograft mouse model

Untreated (control) ES-2 cells (2 × 10⁶) or cells stably transfected with nontargeting control (shNEG), siRNA oligos for transient (siGPAM1), and shRNA lentiviral plasmids for stable GPAM knockdown (shGPAM1 and shGPAM2) were injected subcutaneously into 6-week-old, female CD1 NU/NJ mice (Charles River Laboratories) in 0.1 mL × 1 PBS. Five mice were injected per condition and monitored daily and weighed three times weekly. Tumor size (greatest longitudinal diameter, L and greatest traverse diameter, W) was measured with a caliper three times weekly and volume calculated using the modified elliptical formula (length × width²)/2 (18). All animal studies were done in accordance with the Principles of Laboratory Care following the recommendations of the Society of Laboratory Animal Science (84-02.04.2013.A379 - Gesellschaft für Versuchstierkunde, GV-SOLAS).

Analysis of TAG, G3P, choline-containing compounds, and LPA

Nuclear magnetic resonance (NMR) at 14.1 T (Bruker) was used to measure intracellular G3P, GPC, choline, phosphocholine, glycerophosphoethanolamine (GPE), phosphatidylcholine and TAG, and mass spectrometry and the liquid chromatography system, UFLC XR Prominence (Shimadzu) coupled to a QTRAP 5500 LC/MS-MS system (Sciex) was used to measure 1-oleoyl LPA (O-LPA = 18:1 LPA) and 1-palmitoyl-LPA (P-LPA = 16:0 LPA) as described in the Supplementary Methods. The mass spectrometry settings and the fragment m/z data are listed in Supplementary Table S3.

GPAM expression in human cancers

GPAM expression was assessed using Affymetrix GeneChip Human Genome U133B and Plus 2.0 microarray data (225420_at and 225424_at). Publicly available microarray gene expression datasets with information on MFS, disease-free survival (DFS), or OS, including data from breast (n = 635), ovarian (n = 524), colon (n = 838), and non–small cell lung cancer (n = 1,117) patients were accessed via the Gene Expression Omnibus (GEO) data repository (http://www.ncbi.nlm.nih.gov/geo/; accessed June 2015). Frozen robust multiarray analysis (19) was used for normalization, except for GSE3141, where only MASS-normalized data were available. GEO accession numbers for analyzed datasets and clinicopathologic characteristics for all patients are listed in Supplementary Table S4A–S4D.

Statistical analysis

Analyses were performed using GraphPad Prism, version 6, SAS/STAT, version 9.4, and the statistical programming language R version 3.2.1. Unless stated, data are presented as mean ± SD; a Student paired t test and ANOVA were used to determine
Results
Silencing of GPAM, but not CHKA, reduces cell migration
To determine which metabolic pathway downstream of EDI3 is relevant for migration, EDI3, GPAM, and CHKA were downregulated in two tumor cell lines, MCF7 and HeLa, and the effect on migration directly compared. Knockdown efficiency was comparable for all genes in MCF7 (88%–72% 24 hours posttransfection; Fig. 1B) and HeLa cells (85%–48 hours posttransfection; Supplementary Fig. S1A) cells without any obvious off-target effects. The scratch assay confirmed our previous findings that EDI3 is important in migration (6). Here, we observe a 58% delay in wound closure in MCF7 cells transfected with EDI3 siRNA 48 hours after the wound was made (Fig. 1C), as well as a 40% delay in migration after 24 hours in HeLa cells (Fig. 1D).

Currently, there is very little information on the role of the GPAT enzymes in migration (20), and only a small number of studies (9–12) reporting a decrease in migration after silencing CHKA in cells. This latter observation was not supported in the current study where silencing CHKA in both MCF7 (Fig. 1C) and HeLa cells (Fig. 1D) had no effect. Instead, downregulating GPAM resulted in an analogous decrease in migration as observed after EDI3 knockdown (Fig. 1C and D), suggesting a role for EDI3 and GPAM, but not CHKA, in this process. Importantly, this is the first study to implicate GPAM in migration. Silencing the three genes had no effect on cell number at the time when migration was measured (48 hours for MCF7 and 24 hours for HeLa cells; Supplementary Fig. S1B and S1C), indicating that the reduced motility observed with EDI3 and GPAM was not due to decreased viability.

To clarify the contribution of CHKA to migration, we transfected MCF7 cells with three siRNA oligos targeting different exons of the CHKA gene. Compared with cells transfected with nontargeting siRNA (si-NEG), CHKA expression significantly decreased at both RNA and protein levels (Fig. 2A), with no obvious changes to cell confluency or PCNA expression as a measure of proliferation (Supplementary Fig. S2A and S2B). The selected siRNA oligos were specific for the alpha isoform as no change in choline kinase-β (CHKB) expression was observed (Supplementary Fig. S2C). To confirm the inhibition of CHKA activity, high-resolution 1H-NMR was used to quantify choline and choline-containing metabolites. Representative spectra indicate that compared with control cells, silencing CHKA caused the expected decrease in phosphocholine (76.7 ± 3.5%; Fig. 2B), with a concurrent increase in choline (168.3 ± 20.0%) and GPC (189.7 ± 16.6%), and an overall increase in the GPC/phosphocholine ratio, as reported previously (21). In support of our initial results (Fig. 1C), silencing CHKA had no influence on wound closure in MCF7 cells (Fig. 2C). Furthermore, overexpressing CHKA in HEK293 cells also had no effect on migration (Fig. 2D). Altogether, these data suggest that despite the comparable changes in the GPC/phosphocholine ratio after CHKA (Fig. 2B) and EDI3 (6) knockdown, the influence of EDI3 on migration is not mediated via this pathway in the investigated cell lines.

To validate our initial observation that GPAM influences migration, three siRNA oligos targeting different exons were used to silence GPAM in MCF7 cells. Successful knockdown was confirmed at both RNA and protein levels (Fig. 3A), with no obvious change in cell confluency, an indicator of viability (Supplementary Fig. S3A). Furthermore, GPAM silencing did not influence the expression of other GPAT members, suggesting that no compensatory mechanisms were involved, and that the selected oligos were specific for GPAM (Supplementary Fig. S3B). Migration was significantly inhibited with all oligos (Fig. 3B), thus confirming a role for GPAM in this process. A similar effect was also observed in HeLa cells (Supplementary Fig. S4A and S4B), where we also excluded the participation of other GPATs after GPAM knockdown (Supplementary Fig. S4C). Silencing GPAM in the highly migratory MDA-MB-231 cells (Supplementary Fig. S5A) also significantly inhibited migration, measured using the transwell assay (Supplementary Fig. S5B). Finally, transient overexpression of GPAM in HEK293 cells (Fig. 3C) increased migration by 35% compared with vector-transfected cells. Overall, the results imply that alterations in the pathways downstream of the EDI3-released product, G3P, and not choline and CHKA, are relevant in explaining EDI3’s role in migration.

To address whether the effect of EDI3 and GPAM on migration was mediated via the same pathway or was independent of each other, MCF7 cells were cotransfected with siRNA against both genes. Transfecting twice the amount of siRNA produced no off-target effects and resulted in a comparable decrease in the expression of both genes (Fig. 4A). Interestingly, compared with the single knockdown, combined knockdown of GPAM and EDI3 elicited no additional effect on migration (Fig. 4B), suggesting that the contribution of both proteins to migration occurred via the same metabolic pathway.

LPA decreases after both GPAM and EDI3 knockdown
To obtain an overview of the metabolic changes after knockdown, 1H-NMR and 31P-NMR were performed in MCF7 cells 72 hours after transfection with siRNA targeting EDI3, GPAM, and CHKA. As expected, loss of EDI3 significantly increased GPC and decreased both choline and G3P levels (Fig. 5A, white bars; data represented as a percentage of control with absolute concentrations provided in Supplementary Table S3). GPE also increased after EDI3 knockdown, suggesting that it too is an endogenous substrate of EDI3, and confirms previous findings using recombinant mouse Edi3 (Gde5; ref. 5). As already illustrated (Fig. 2B), silencing CHKA resulted in increased choline and GPC levels, and decreased phosphocholine compared with control (Fig. 5A, dotted bars). These changes translated to an increased GPC/phosphocholine ratio similar to that observed after EDI3 knockdown and therefore cannot explain the disparate effect of both proteins on migration in the current study.

Silencing GPAM led to a significant increase in its substrate, intracellular G3P (Fig. 5A, striped bars), whereas downregulating EDI3 decreased G3P. The opposing effect of both genes on G3P...
suggests that it is not a direct indicator of the migration capacity of a cell. Our previous work showed that knocking down EDI3 reduced quantities of several lipid moieties, including phosphatidylcholine, as well as the signaling lipids, LPA and PA (6). LPA is formed from the direct acylation of G3P by GPAM and is associated with cell proliferation and migration (16). However, the role of LPA in these processes has been explained by the binding of extracellularly produced LPA to one of seven LPA receptors found on the cell surface. No study to date has investigated intracellular LPA in migration. Consequently, LC/MS-MS
was used to measure intracellular 16:0 and 18:1 LPA after knockdown of EDI3, GPAM, and CHKA in MCF7 cells. Silencing CHKA had no effect on either LPA species (Fig. 5B and C, respectively).

Conversely, knocking down GPAM and EDI3 led to a comparable reduction of both 16:0 and 18:1 LPA levels (Fig. 5B and C). Furthermore, overexpressing GPAM in HEK293 cells, which

Figure 3.
GPAM expression is associated with cell migration. A, Downregulation of GPAM RNA and protein expression in MCF7 cells using three siRNA oligos compared with control (si-NEG). FM, full media control. B, Representative images of scratch assay at 0 and 48 hours (top). Initial scratch was made 72 hours post knockdown, with quantification showing the wound closure after 48 hours (bottom). C, Top, representative images of GPAM expression in HEK293 cells 48 hours after transient transfection with pCMV6 empty vector (−) or pCMV6-GPAM (+ GPAM). Middle, images of cells that migrated to the underside of the transwell; bottom, quantification of migrated cells 24 hours after plating, presented as the migration index. Data are mean ± SD of three to five independent experiments (**, P < 0.01; ***, P < 0.001). Images were taken using a 10× objective.
increased migration, resulted in higher 16:0 and 18:1 LPA levels compared with control (Fig. 5B and C).

To study whether intracellular LPA is important for cell migration and to circumvent the binding of extracellularly added LPA binding to one of its receptors on the plasma membrane, LPA was directly introduced into cells via transfection using the cationic transfection reagent Lipofectamine 2000 as a vehicle. A fluorescently labeled, albeit nonphysiologic LPA species (TopFluor Lyso PA) was used to demonstrate that LPA enters the cells when combined with the transfection reagent compared with LPA-only treatment (Supplementary Fig. S6). Consequently, transfecting cells with Lipofectamine, LPA significantly increased migration compared with cells treated with either LPA or Lipofectamine alone (Fig. 5D and E). Furthermore, LC/MS-MS measurements confirmed that the faster migrating cells did indeed have higher levels of intracellular LPA (Fig. 5F). Altogether, these results provide strong evidence that intracellular LPA is an important factor in cellular migration.

**Similar gene categories influenced by EDI3 and GPAM**

Because of the comparable effect of GPAM and EDI3 on migration and LPA levels, we next assessed whether there are common gene expression alterations after silencing both genes in MCF7 cells. Two oligos (GPAM-1 and -2) were used to down-regulate GPAM; therefore, we first calculated the differential gene expression ($P_{adj} < 0.01$, FC > 1.5) for each oligo compared with si-NEG, obtaining 501 and 409 differentially expressed genes (731 and 585 probe sets) for GPAT-1 and -2, respectively.
Second, we determined the number of deregulated genes and probe sets common to both GPAM-1 and GPAM-2, identifying an overlap of 240 genes (341 probe sets; Supplementary Table S6). Subsequently, overlapping genes for GPAM-1 and GPAM-2 siRNA oligos were compared with genes differentially expressed after EDI3 silencing (Supplementary Table S7; ref. 8), and the overlapping up- and downregulated genes and probe sets are illustrated using Venn diagrams.

(Supplementary Table S6).
The overlap ratios for the up- and downregulated genes were 26.2 and 10.1 (P < 0.001), respectively, indicating that the number of genes influenced by both EDI3 and GPAM is higher than randomly expected (Supplementary Fig. S7A). The top up- and downregulated genes, ranked according to adjusted P values are listed in Supplementary Table S8. Among the most significantly altered are genes associated with cytoskeletal reorganization processes, including migration, adhesion, vesicle trafficking, and exocytosis, such as LAMB1, an extracellular matrix glycoprotein, and the phospholipid-binding protein Annexin A1 (ANXA1). How these changes contribute to the role of EDI3 and GPAM in migration needs further investigation.

GPAM expression in human cancers

Increased cell migration in vitro is often extrapolated to represent the increased propensity of a tumor to metastasize in vivo, as well as worse disease outcome. Therefore, to understand whether GPAM expression is associated with the latter, we analyzed publicly available Affymetrix gene expression array datasets of different cancers, including ovarian, breast, colon, and lung (Supplementary Table S4A–S4D). The results of the univariate survival analysis for all cohorts are summarized in Supplementary Table S9. The association of GPAM expression with worse outcome is illustrated in a Kaplan–Meier plot for GSE9891 (Fig. 6B; Supplementary Fig. S8B). Further, when GPAM expression was included together with age, stage, grade, and residual disease after surgical resection in a multivariate model, higher GPAM levels were found to be significantly associated with worse outcome (225420_at, HR = 1.45, P = 0.004; 225424_at, HR = 1.48, P = 0.002; Fig. 6B; Supplementary Fig. S8C). All findings were confirmed using different cutoffs for GPAM dichotomization and with survival time censored at 5 or 10 years (Supplementary Fig. S8D).

Figure 6.
GPAM expression is associated with worse outcome in human ovarian carcinoma. A, Higher GPAM expression was significantly associated with shorter OS in a meta-analysis of five publicly available ovarian cancer datasets (P = 0.0003). Results were visualized with forest plots, where HRs of all single studies and the pooled estimates along with their 95% confidence intervals are plotted. Significance of the overall effect was assessed by the P value of the random effects model. B, Left, Kaplan–Meier plot illustrating the association of GPAM expression (probe set: 225240_at), dichotomized to high (gray line) and low (black line) GPAM level according to the expression of the 75th percentile, with shorter OS in the dataset originally published by Tothill and colleagues (GSE9891; ref. 22). The x-axis provides OS in years and the corresponding numbers of patients at risk. Right, GPAM expression is significantly associated with OS in a multivariate Cox regression model with inclusion of clinicopathologic variables age, stage, grade, and residual disease in GSE9891.

( Supplementary Fig. S7A and S7B). The overlap ratios for the up- and downregulated genes were 26.2 and 10.1 (P < 0.001), respectively, indicating that the number of genes influenced by both EDI3 and GPAM is higher than randomly expected (Supplementary Fig. S7A). The top up- and downregulated genes, ranked according to adjusted P values are listed in Supplementary Table S8. Among the most significantly altered are genes associated with cytoskeletal reorganization processes, including migration, adhesion, vesicle trafficking, and exocytosis, such as LAMB1, an extracellular matrix glycoprotein, and the phospholipid-binding protein Annexin A1 (ANXA1). How these changes contribute to the role of EDI3 and GPAM in migration needs further investigation.

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In breast cancer, higher GPAM expression was associated with longer MFS or DFS in two cohorts for at least one probe set (GSE9195: HR = 0.36, P = 0.044; GSE42568: HR = 0.67–0.73, P = 0.016–0.019), supporting previous findings (23). Conversely, when the analysis was performed stratified according to ER and HER2 status, GPAM was found to be associated with shorter MFS or DFS in HER2-positive patient subsets in two cohorts (GSE19615: HR = 2.62, P = 0.040; GSE21653: HR = 1.46, P = 0.040; Supplementary Table S9). Altogether, the results suggest that the association of GPAM with MFS may be subtype and context dependent in human breast cancer. The disparate association between GPAM expression and outcome observed in ovarian and breast cancer prompted further analysis in other cancer types, more specifically colorectal and non–small cell lung cancer (Supplementary Table S9). Contradicting results were also observed for colorectal cancer, where GPAM expression was found to be associated with both longer and shorter DFS. Finally, no association was observed for GPAM expression and OS in non–small cell lung cancer.

Silencing GPAM decreases migration and tumor growth in ovarian cancer cells

With the observation that high GPAM expression is associated with worse survival in ovarian cancer, GPAM expression was assessed in different ovarian cancer cell lines, with the highest expression measured in the rapidly migrating ES-2 cells (Fig. 7A; Supplementary Fig. S9A). Silencing GPAM and EDI3 (Fig. 7B) in these cells decreased cell migration (Fig. 7C), results that were confirmed with a second oligo for each gene (Supplementary Fig. S9B and S9C). No effect was observed after knocking down CHKA, supporting our observation in other cell lines (Figs. 1 and 2). To determine whether GPAM has any role in tumor growth in vivo, xenograft tumors were produced after subcutaneous injection of ES-2 cells in mice. Transient knockdown of GPAM, where expression remained significantly downregulated 10 days posttransfection (7 days postinjection; Fig. 7D), resulted in reduced tumor volume compared with control (Fig. 7E; Supplementary Fig. S9D). The recovery of GPAM expression (Fig. 7D), also confirmed in the tumors (Supplementary Fig. S9D, bottom), may explain the increased tumor growth at later time points. For long-term, consistent downregulation of GPAM in cell migration, the current study focused on pathways immediately downstream of EDI3 family member in cancer, as most of the available data focus on GPATs in triglyceride synthesis and obesity-related pathologies (13).

Silencing EDI3 significantly decreased intracellular G3P levels, indicating that GPC cleavage by EDI3 is an important source of G3P, the backbone of all cellular glycerophospholipids. Downregulating GPAM significantly increased G3P levels. The opposing effect indicates that G3P is not the common metabolic denominator linking both enzymes to migration. Silencing EDI3 was previously shown to decrease levels of the signaling lipids, LPA and PA (6). As LPA is the direct metabolic product of GPAM, and several studies have described a role for LPA in migration (34–36), we compared the effect of EDI3, GPAM, and CHKA on intracellular LPA levels. Upon silencing, both EDI3 and GPAM, but not CHKA caused a comparable decrease in intracellular LPA levels.

Thus far, no studies have implicated intracellular LPA with cell migration, instead focusing on extracellular LPA binding any one of seven G-protein–coupled LPA receptors at the cell surface (37, 38). To investigate intracellular LPA in migration, LPA was directly introduced into cells using a cationic transfection reagent (Lipofectamine 2000). The preferential uptake

Discussion

Aberrant cellular metabolism is an acknowledged feature of cancer, with implications in disease development and progression (1–3). Consequently, identifying the enzymes involved provides insight into the regulation of metabolite levels and also offers potential therapeutic targets. We recently identified the glycerophosphodiesterase EDI3 as a key enzyme in choline and phospholipid metabolism (6), which is also relevant in cell migration, adhesion, and spreading (6, 8). However, the link between EDI3’s enzymatic activity and altered phenotypes is not understood (6). Therefore, to further characterize EDI3 in migration, the current study focused on pathways immediately downstream of EDI3 metabolic products and the enzymes involved, namely CHKA and GPAM. Our results do not support a role for CHKA in cell migration. Rather, this work is the first to demonstrate a role for GPAM outside of glycero phospholipid and triglyceride metabolism, showing its importance in cell migration via changes to intracellular LPA. In addition, for the first time, an association is shown between GPAM expression and survival in ovarian cancer.

Altered choline metabolism is reported in several cancer types (10–29). These changes are primarily manifested by increased total choline and phosphocholine levels in cancerous tissue and more aggressive cell lines, due to increased choline uptake, and elevated expression and activity of CHKA, leading to increased phosphatidylcholine production (4). EDI3 is also relevant in this complex network of enzymatic reactions regulating choline metabolism as silencing EDI3 altered GPC, choline, and phosphatidylcholine levels in different cells (6). In the current study, comparable downregulation of CHKA and EDI3 produced similar changes to the GPC/phosphocholine ratio. However, only EDI3 affected cell migration, indicating that this ratio is not predictive of migratory capacity. Interestingly, our findings differ from those of previous studies where silencing CHKA decreased motility and invasion (9–12). In two studies (9, 10), the authors reported reduced proliferation, but no direct effect on apoptosis, or on MAPK and PI3K/Akt signaling pathways, as published by others after CHKA inhibition in other cell lines (30–33). Furthermore, a recent study describing a role for CHKA in migration observed no direct effect on proliferation (12). Altogether, the role of CHKA in processes such as migration and proliferation requires further evaluation and may be cell type and context dependent.

Our results strongly support a role for GPAM in cell migration. Silencing GPAM in different cell lines with multiple siRNA oligos significantly decreased migration, whereas overexpression of GPAM accelerated the process. Combined knockdown of both EDI3 and GPAM elicited no additive inhibitory effect on migration, suggesting that both genes influence this cellular process via the same pathway. This study is the first to demonstrate a role for GPAM in cell migration, and one of the first to implicate a GPAT family member in cancer, as most of the available data focus on the role of GPATs in triglyceride synthesis and obesity-related pathologies (13).
Silencing GPAM in ovarian cancer cells decreases migration and tumor growth. 

**A**, GPAM mRNA expression in a panel of ovarian cancer cell lines. **B**, RNA and protein expression of GPAM, EDI3, and CHKA in ES-2 cells 72 hours after siRNA knockdown compared with control (si-NEG). FM, full media control. **C**, Representative images of scratch assay with ES-2 cells transfected with siRNA oligos targeting GPAM, EDI3, and CHKA with accompanying quantification compared with si-NEG. **D**, GPAM RNA expression in ES-2 cells showing knockdown efficiency up to 10 days after transfection with GPAM siRNA. Inset, GPAM protein level 72 hours posttransfection with GPAM siRNA. **E**, Transiently silencing GPAM in ES-2 cells decreases subcutaneous tumor volume in CD1 NU/NU mice. **F**, GPAM RNA and protein expression in ES-2 cells after stable knockdown with two GPAM shRNA constructs compared with nontargeting control (shNEG). **G**, Effect of stable downregulation of GPAM on volume of ES-2 xenograft tumors produced in CD1 NU/NU mice. All *in vitro* data are mean ± SE of three to five independent experiments (*, P < 0.05; **, P < 0.01; ***, P < 0.001). Tumor volume measurements (E and G) represent the average tumor volume from 5 mice per treatment condition. Images were taken using a 10× objective.
of LPA with Lipofectamine was confirmed, and increased migration was directly associated with elevated intracellular LPA levels. This is the first study to associate intracellular LPA with migration. Although LPA may be relevant for EDI3- and GPAM-mediated cell migration, other downstream signaling lipids, such as PA, may contribute. LPA and PA are important precursors in phospholipid metabolism, and both influence membrane curvature (39, 40), which may affect processes, such as exocytosis and cytoskeletal organization. Therefore, further work is required to understand the influence of EDI3, GPAM, and other related enzymes (Fig. 1A) on cellular lipid and metabolic content, and how these changes are linked to observed phenotypic changes.

The current findings that GPAM influences cell migration and intracellular LPA led us to investigate whether GPAM has any link to disease outcome. More specifically, the association between GPAM expression and survival was examined in over 3,000 patients using publicly available datasets of ovarian, breast, colon, and lung cancer. Here, we report that high GPAM expression is significantly associated with shorter survival in ovarian cancer, independent of age, stage, grade, and residual disease after surgical resection. Importantly, this is the first study to link expression of a GPAT enzyme to worse prognosis in any cancer type. In the other three cancer types investigated, the association was more ambiguous where high GPAM expression was associated with either better and poor prognosis, or no association at all, suggesting tumor type and subtype specificity.

In support of the significant association between GPAM expression and survival in ovarian cancer, downregulating GPAM in ovarian cancer cells significantly delayed growth of tumor xenografts in mice. Previous studies have shown that LPA levels are elevated in the ascites of ovarian cancer patients, most probably due to increased LPA production by cancer cells and the surrounding mesothelium (36, 37, 41–43). The potential contribution of GPAM to this process is not known, thus justifying further investigation into its role in this disease.

**References**
