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Inhibition of Gastric Cancer Invasion and Metastasis by \textit{PLA2G2A}, a Novel $\beta$-Catenin/TCF Target Gene

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Abstract

Elevated expression of the \textit{PLA2G2A} phospholipase in gastric cancer (GC) is associated with improved patient survival. To elucidate function and regulation of \textit{PLA2G2A} in GC, we analyzed a panel of GC cell lines. \textit{PLA2G2A} was specifically expressed in lines with constitutive Wnt activity, implicating $\beta$-catenin–dependent Wnt signaling as a major upstream regulator of \textit{PLA2G2A} expression. The invasive ability of \textit{PLA2G2A}-expressing AGS cells was enhanced by \textit{PLA2G2A} silencing, whereas cellular migration in non–\textit{PLA2G2A}-expressing N87 cells was inhibited by enforced \textit{PLA2G2A} expression, indicating that \textit{PLA2G2A} is both necessary and sufficient to function as an inhibitor of GC invasion \textit{in vitro}. We provide evidence that antiinvasive effect of \textit{PLA2G2A} occurs, at least in part, through its ability to inhibit the S100A4 metastasis mediator gene. Consistent with its invasion inhibitor role, \textit{PLA2G2A} expression was elevated in primary gastric, colon, and prostrate early-stage tumors, but was decreased in metastatic and late-stage tumors. There was a strong association between \textit{PLA2G2A} promoter methylation status and \textit{PLA2G2A} expression, suggesting that the loss of \textit{PLA2G2A} expression in late-stage cancers may be due to epigenetic silencing. Supporting this, among the non–\textit{PLA2G2A}-expressing lines, pharmacologic inhibition of epigenetic silencing reactivated \textit{PLA2G2A} in Wnt-active lines, but in non–Wnt-active lines, a combination of Wnt hyperactivation and inhibition of epigenetic silencing were both required for \textit{PLA2G2A} reactivation. Our results highlight the complexity of \textit{PLA2G2A} regulation and provide functional evidence for \textit{PLA2G2A} as an important regulator of invasion and metastasis in GC.

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Introduction

Gastric cancer (GC) is the second highest cause of global cancer mortality, accounting for $700,000$ deaths annually (1). Although curable if detected early, most GC patients are diagnosed with late-stage disease, wherein current therapeutic strategies are still far from optimal (2). Surgery and combination chemotherapy have been shown to confer only modest survival benefits in advanced GC, resulting in an overall 5-year survival rate of $<24\%$ (3, 4). Thus, despite a steady decline in global incidence, GC still constitutes a disease of outstanding morbidity and mortality. A molecular understanding of the genetic factors involved in GC progression and advanced stage disease may contribute toward identifying novel GC biomarkers and highlight potential avenues for targeted therapies.

One promising strategy for identifying genes involved in GC progression is to focus on genes associated with important clinical variables, such as patient survival. In GC, patients with tumors expressing high levels of \textit{PLA2G2A}, a secreted phospholipase, have been shown to exhibit significantly improved survival compared with patients with low \textit{PLA2G2A}–expressing tumors (5). However, beyond this prognostic association, little is actually known about how \textit{PLA2G2A} might contribute to GC disease and progression. Currently, the major known functions of \textit{PLA2G2A} are largely related to inflammatory responses, antimicrobial defense, and phospholipid degradation in the gastrointestinal track (6). Furthermore, although \textit{PLA2G2A} has been proposed as a potential tumor suppressor, evidence supporting this model is conflicting (5, 7–10). Clarifying the functional relationship between \textit{PLA2G2A} and GC will thus require (a) characterizing the cellular pathways regulating \textit{PLA2G2A}, (b) testing if \textit{PLA2G2A} functionally contributes or is merely associated with improved patient survival, and (c) identifying \textit{PLA2G2A} downstream target genes that might mediate its prosurvival effect.

We recently reported an association between \textit{PLA2G2A} expression and components of the Wnt signaling pathway, including \textit{CTNNB1} ($\beta$-catenin) and the Wnt target gene \textit{Eph}R2 in a consensus gene coexpression meta-network of GC (11). In this current study, we sought to extend these studies to elucidate the mechanistic basis of \textit{PLA2G2A} prosurvival activity by examining \textit{PLA2G2A} activity and regulation in a panel of GC cell lines. Using a variety of experimental approaches, we show that \textit{PLA2G2A} is a direct target of Wnt/$\beta$-catenin signaling in GC cells, which functions to negatively regulate GC invasion and metastasis. This inhibition of invasion is mechanistically achieved through the negative regulation of downstream metastasis genes, such as \textit{S100A4} and \textit{NEDD9}. Our results support the notion that in addition to being a prognostic biomarker, \textit{PLA2G2A} plays an intimate functional role in inhibiting GC progression. One implication of our findings is that because \textit{PLA2G2A} is often underexpressed in late-stage and metastatic tumors, it is plausible that the reintroduction of \textit{PLA2G2A} into aggressive tumors, by either gene therapy, administration of \textit{PLA2G2A} protein, or intriguingly via epigenetic reactivation, might constitute a novel therapy for late-stage GC.
Materials and Methods

Cell culture. GC cell lines AGS, Kato III, SNU1, SNU5, SNU16, N87, and Hs746T were obtained from American Type Culture Collection and cultured as recommended. Fu97, MKN7, and IM95 cells were from Japan Health Science Research Resource Bank and were cultured as recommended. YCC cells were a gift from Sun Young Rha (Yonsei Cancer Center) and were grown in MEM supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mmol/L L-glutamine (Invitrogen).

Gene expression profiling. Total RNA was extracted from cell lines using Qiagen RNA extraction reagents (Qiagen) and profiled using Affymetrix Human Genome U133 plus Genechips (HG-U133 Plus 2.0, Affymetrix, Inc.) according to the instructions of the manufacturer. The raw data obtained after scanning the chips were further processed, quality controlled, and analyzed using GeneData Refiner and expressionist software (GeneData).

cDNA synthesis and reverse transcription–PCR. Total RNA was extracted from cell lines using Trizol reagent (Invitrogen) and quantitated using both nanodrop ND-1000 (Nanodrop Technologies) and agarose gel electrophoresis. Equal quantities of RNA were reverse transcribed by SuperScript II reverse transcriptase enzyme using oligo-dT (T<sub>18</sub>) primers, as indicated by the manufacturer (Invitrogen). Reverse transcription–PCR (RT-PCR) was performed using gene specific primers, and the sequences of the oligos are available in the supplementary information.

Western blotting. Western blotting was performed using the following antibodies and dilutions: 1:500 β-catenin (Upstate), 1:1,000 PLA2G2A (Cayman Chemical), 1:500 TCF4 (Upstate), 1:500 T7 epitope tag (Novagen), 1:500 vinculin (Upstate), 1:1,000 β-actin (Santa Cruz), and 1:200 S100A4 (Abcam).

Mutation analysis. PLA2G2A, APC, and CTNNB1 exons were amplified by PCR from genomic DNA of GC cell lines or gastric tumor samples and sequenced using BigDye Terminator 3.1 reagent (ABI) on 3730 DNA Sequence Analyzer (ABI). Primer sequences are available upon request.

Luciferase assay and promoter constructs. Luciferase assays were performed with a luciferase assay kit (Promega), and the results normalized to a parallel internal β-galactosidase activity assay (Galacto Light plus system, Applied Biosystems). All experiments were repeated three independent times, each time in triplicate. To measure TCF activity, Topflash and Fopflash luciferase plasmids (Upstate) were transfected into GC cells and luciferase activities were measured at 48 h after transfection. TCF activities were calculated as fold activity (Topflash/Fopflash) after normalization to β-galactosidase activity.

Promoter activity. PLA2G2A reporter was constructed by amplifying the putative promoter region (∼1,380–0 bp) from human reference genomic DNA (Promega) using high-fidelity DNA polymerase (Roche) and cloning it into a pGL3-enhancer luciferase reporter plasmid (Promega). The PLA2G2A promoter construct was verified by sequencing.

Small interfering RNA and cDNA transfections. SMART pool small interfering RNAs (siRNA) to CTNNB1 and S100A4 were from Dharmacon, whereas PLA2G2A siRNA pool and negative control siRNA were from Ambion. siRNAs (100 pmol) were transfected into 5 × 10<sup>6</sup> AGS cells with Oligofectamine transfection reagent (Invitrogen) in six-well tissue culture plates. Cells were incubated for 72 h before harvesting for Western blotting or RNA analysis. For cDNA constructs, sequence verified full-length PLA2G2A cDNA clones were obtained from Open Biosystems and CTNNB1 cDNA was a gift from Dr. Bert Vogelstein. The full-length coding regions of PLA2G2A and CTNNB1 were cloned into pCDNA6/His plasmid vectors (Invitrogen). The degradation-resistant mutation G34E was introduced into the full-length pCDNA6/His-CTNNB1 plasmid by site-directed mutagenesis using a Quickchange mutagenesis kit (Stratagene) and confirmed by sequencing. Transfections were performed using OptiMEM reduced serum medium (Invitrogen) with the Eugene transfection reagent (Roche) as indicated by the manufacturer.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was performed using the EZ Chip ChIP kit (Upstate) as directed by the manufacturer. Briefly, 5 × 10<sup>6</sup> AGS cells were cross-linked with formaldehyde and lysed and cell lysates were sonicated to an average DNA fragment size of 1,000 bp. The clarified lysates were precleared with bovine serum albumin (BSA) and salmon sperm DNA blocked protein A/G plus agarose (Santa Cruz) and incubated overnight with primary antibodies at 4°C. Immunocomplexes were captured with BSA and salmon sperm DNA–blocked protein A/G plus agarose and washed, and the bead pellet was resuspended in TBE buffer. Formaldehyde cross-links were reversed at 65°C for 6 h, and RNA was digested for 30 min at 37°C. Subsequently, proteins were digested with proteinase K for 2 h at 37°C. The DNA samples were purified with Qiagen clean-up columns, and the eluted DNA was used for PCR screening with PLA2G2A and celson D1 specific promoter oligos. Primer sequences are available in the supplementary information. Antibodies used for ChIP were 5 μg of anti-β-catenin (Upstate), 5 μg of anti-TCF4 (Upstate), 5 μg of rabbit IgG (Santa Cruz), and 5 μg of mouse IgG (Santa Cruz).

Stable transfection of cell lines. To establish stable silencing of PLA2G2A, we transduced AGS cells with either PLA2G2A short hairpin RNA (shRNA) or control nontargeting shRNA lentiviral particles (Sigma) in six-well plates in the presence of hexadimethrine bromide (Sigma). Transduced cells were subjected to selection with 1.8 μg/mL puromycin (AG Scientific, Inc.) from 36 h after transduction. After 7 d of selection, we obtained stable pools of puromycin-resistant cells and were expanded further and assayed for PLA2G2A silencing by RT-PCR and Western blotting. To establish cell lines stably overexpressing PLA2G2A, we transduced N87 cells with pcDNA6/His-PLA2G2A or control pcDNA6/His constructs. After transfection for 48 h, the cells were split into 60-mm tissue culture plates and selected with 5 μg/mL blasticidin (Invitrogen) in RPMI supplemented with 10% FBS (Hyclone). After 3 wk, stable pools of blasticidin-resistant cells were expanded and analyzed for PLA2G2A overexpression by RT-PCR and Western blotting analysis.

Cell proliferation assays. For cell proliferation assay, equal number of cells were plated in triplicate in a six-well tissue culture plate and counted on the following days. AGS-derived cells were counted for 3 d, whereas N87-derived cells were counted until 2 wk. The proliferation of PLA2G2A-modulated cell lines (AGS-shPLA2G2A and N87-PLA2G2A) were represented in percentage by considering the cell count of the control cell lines (AGS-shNSC and N87-pCDNA) as 100% proliferation. The mean values from three different experiments were calculated to represent the proliferation difference.

Invasion assays. Matrigel invasion assays were performed using Biocoat Matrigel invasion chambers (BD Biosciences) as recommended by the manufacturer. Briefly, AGS cells were transfected twice at 24-h intervals with the specified siRNAs in OptiMEM medium (Invitrogen). After 24 h, cells were trypsinized, washed in PBS, resuspended in serum-free RPMI medium, and loaded on the upper well of a Matrigel invasion chamber at a concentration of 5 × 10<sup>5</sup> cells per well in a six-well chamber. The lower side of the separating filter was filled with RPMI medium with 10% FBS. The chamber was incubated in a tissue culture incubator, and after 24 h, cells on the upper surface were removed by scrubbing with a cotton swab, and the cells that successfully migrated through the filter were photographed. At least 15 different fields were counted for each experiment, and the results were averaged over three independent experiments.

Cell migration assays. Aliquots of 2 × 10<sup>5</sup> N87, N87-pCDNA (control), or N87-PLA2G2A cells were plated in individual wells of six-well tissue culture plates in RPMI with 1% FBS. After 48 h, a line of adherent cells was scraped from the bottom of each well with a p-200 pipette tip to generate a wound and the medium was replaced by RPMI that contained 5% FBS and 5 μg/mL blasticidin (Invitrogen). Cells were allowed to proliferate and migrate into the wound for 96 h. The extent of migration of cells into the region from which cells had been scraped was determined from photographs. The experiment was repeated thrice with multiple scratches each time. Because N87-PLA2G2A cells grew very slowly, the assay period was extended for 2 wk.

DNA bisulfite sequencing. Three micrograms of GC cell line genomic DNA was denatured by 0.35 mol/L NaOH at 37°C for 10 min. Sodium bisulfite and hydroquinone were added to the denatured DNA to final concentrations of 3.2 mol/L and 0.61 mol/L, respectively, and incubated...
at 50 °C for 4 h. The DNA was purified using a DNA purification kit (Qiagen), desalted by 0.3 mol/L NaOH at 37 °C for 15 min, and neutralized with 5 mol/L ammonium acetate (pH 7.0). DNA was subsequently precipitated with ethanol, washed with 70% ethanol, and resuspended in H2O. The PLA2G2A promoter was amplified from bisulfite-modified DNA using JumpStart REDTaq DNA polymerase (Sigma) with the oligos PLA2G2A-MSF, TTGTAAATTGTTGAAATGATG6G, and PLA2G2A-MSR, CATAATTCCT CAATAAAACAAAC. PCR products were purified by a PCR DNA purification kit (Qiagen) and sequenced.

5-Aza-2'-deoxycytidine treatment. GC cell lines were treated with 5 μmol/L 5-aza-2'-deoxycytidine (5-azadC; Sigma) for 72 h, and RNA isolated from 5-azadC and vehicle (DMSO)-treated cells were analyzed for the expression of PLA2G2A and SFRP1 by semiquantitative RT-PCR. SFRP1 served as a positive control gene that is methylated in GC (12). N87 and YCC1 cells were treated with 5 μmol/L 5-azadC for 48 h followed by 24 h of combined treatment with 5 μmol/L 5-azadC and 5 μmol/L 1,2-Di(2-aminooxy)ethane-N,N,N',N’-tetraacetate (EGTA), a GSK3β small molecule inhibitor.

Results

PLA2G2A expression is positively correlated with Wnt pathway activation in GC cells. To identify a suitable cell line model for studying PLA2G2A, we screened the microarray gene expression profiles of 17 cell lines. PLA2G2A was heterogeneously expressed across the cell lines (Supplementary Fig. S1A) similar to its in vivo expression pattern in primary tumors where high variability in PLA2G2A expression has been observed (5). Specifically, AGS and YCC3 cells expressed abundant levels of PLA2G2A mRNA, KATO III cells expressed relatively moderate levels, and SNU16 and YCC10 cells expressed very low but detectable quantities of PLA2G2A levels, and SNU16 and YCC10 cells expressed very low but detectable quantities of PLA2G2A. Other cell lines (SNU1, SNU5, N87, YCC1, YCC2, YCC6, YCC9, YCC16, MK7, Hu746, Fu97, and IM95) did not express PLA2G2A. This expression pattern was further confirmed by semiquantitative RT-PCR with PLA2G2A-specific oligonucleotides and Western blotting with PLA2G2A antibodies on the PLA2G2A-expressing and nonexpressing lines (Fig. 1A and Supplementary Fig. S1B).

Motivated by our previous observation that PLA2G2A is coexpressed with components of the Wnt signaling pathway, including CTNNB1 (β-catenin; ref. 11), we hypothesized that PLA2G2A expression in the GC lines might be related to activation of the Wnt signaling pathway. To explore this possibility, we analyzed (a) the expression of Wnt pathway components, (b) TCF transcriptional activity, and (c) the mutational and genomic status of Wnt pathway genes in PLA2G2A-expressing and nonexpressing cell lines. β-Catenin, a core member of the canonical Wnt signaling pathway, was expressed in all of the PLA2G2A-expressing cell lines (AGS, YCC3, and Kato III), but was not expressed in some non–PLA2G2A-expressing lines (SNU1, SNU5). Likewise, the TCF/LEF transcription factor TCF4, another major component of the Wnt pathway, also showed high expression in AGS and YCC3 cells and detectable expression in Kato III cells (Fig. 1A). To directly assay Wnt pathway activity, we determined TCF/LEF transcriptional activity in the cell lines using Topflash, a luciferase expressing plasmid containing multimerized TCF binding sites. The Topflash assay revealed constitutive TCF/LEF transcriptional activity in PLA2G2A-expressing cell lines (AGS, YCC3, and Kato III) but minimal or no Topflash activity in non–PLA2G2A-expressing lines (Fig. 1A). Thus, PLA2G2A expression in GC cells seems to be highly correlated with constitutive activation of the Wnt signaling pathway. Furthermore, supporting this model, AGS cells possess a gain of function phosphorylation site mutation in the CTNNB1 (β-catenin) gene (13), and Kato III cells have both a genomic amplification of the CTNNB1 gene and a promoter methylation of APC, a negative regulator of Wnt signaling (14). Similarly, we found that YCC3 cells exhibited CTNNB1 gene amplification and high CTNNB1 mRNA expression levels (Supplementary Fig. S2). Notably, although SNU16 cells express both β-catenin and TCF4, we have previously shown that β-catenin is membrane-localized and nonnuclear in SNU16

Figure 1. PLA2G2A is expressed in Wnt hyperactive GC cell lines and is a direct target gene of Wnt(β)-catenin pathway. A, TCF transcriptional activity in GC cell lines measured by a Topflash reporter. TCF activity is represented as a relative fold measurement (Topflash/Topflash activity). Immunoblots show PLA2G2A, TCF4, and β-catenin protein expression, revealed by semiquantitative RT-PCR and immunoblots. The anti–β-catenin immunoblot confirms efficient β-catenin silencing, whereas the anti–β-catenin immunoblot and GAPDH RT-PCR shows equal sample loading. The two lanes for each cell line represent two independent experiments. B, hyperactivation of Wnt(β)-catenin signaling by wild-type and mutant CTNNB1 (G34E) transactivates the PLA2G2A promoter (−1,380 to 0 bp) in YCC3 cells. Plasmids (pCDNA or CTNNB1-WT or CTNNB1-G34E) were cotransfected with Topflash or PLA2G2A promoter-luciferase plasmids and cytomegalovirus−β-galactosidase plasmid. At 48 h after transfection, the cell lysates were assayed for luciferase and β-galactosidase activities. Left columns, enhancement of TCF activity in CTNNB1-transfected YCC3 cells; right columns, PLA2G2A promoter activation. Results were from three independent experiments. Luciferase activity measurements were normalized with β-galactosidase activities. D, ChIP with anti–β-catenin and anti-TCF4 antibodies in AGS cells enrich the PLA2G2A promoter region. PCR performed from ChIP pulsed down DNA or input DNA with specific primers to the PLA2G2A promoter, cyclin D1 promoter, and GAPDH coding region.

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cells, explaining the relative lack of Wnt activity in this line (11). These observations support our hypothesis that PL2G2A expression in GC is highly correlated with constitutively activated Wnt signaling.

**PL2G2A is a direct target of the Wnt signaling pathway in GC cells.** We then asked if β-catenin–mediated Wnt signaling might directly regulate PL2G2A expression in GC. Using siRNAs, we silenced β-catenin in AGS, YC3, and Kato III cells. PL2G2A gene and protein expression were considerably reduced upon β-catenin silencing (Fig. 1B and Supplementary Fig. S3A). A quantitative analysis revealed a 50% reduction of PL2G2A expression in AGS and YC3 cells at 72 hours after β-catenin silencing (Supplementary Fig. S3B). This result indicates that β-catenin is necessary for PL2G2A expression in GC cells, suggesting that PL2G2A is likely to be a downstream target of Wnt pathway in this tissue type.

Transcription of Wnt responsive genes is generally mediated through the activity of high-mobility group box transcription factors TCF/LEF, and β-catenin/TCF target genes usually possess TCF binding site in their promoters (15, 16). Consistent with PL2G2A being a direct β-catenin/TCF target gene, we identified multiple TCF/LEF transcription factor binding sites in the PL2G2A promoter, three of which were in the region −1,340 to −920 bp from the transcription start site (Supplementary Fig. S4). The three predicted TCF binding sites in the PL2G2A promoter are CTTTGAA, CTTTGTT, and CTTTGAT, which are strong matches to the CTTTG(A/T)(A/T) TCF-binding consensus motif, as revealed in a recent genome-wide DNA binding study (17). Notably, the PL2G2A promoter fragment containing the TCF consensus sites into a luciferase reporter plasmid and transfected it into YC3 cells. To ask if an enhancement of Wnt/β-catenin signaling might be sufficient to transcriptionally activate this reporter, we cotransfected the reporter constructs with companion plasmids expressing high levels of either a wild-type β-catenin (WT) or a gain-of-function version (G34E), which is resistant to degradation. The PL2G2A reporter exhibited increased levels of transcriptional activation comparable with a positive control Topflash reporter plasmid in both WT and G34E β-catenin–transfected cells, thus demonstrating the Wnt/β-catenin inducibility of the 1.3-kb PL2G2A promoter fragment (Fig. 1C). Thus, activation of Wnt signals through β-catenin is sufficient to enhance PL2G2A expression, arguing that PL2G2A is a target gene of the Wnt pathway in GC cells.

To show the in vivo occupancy of β-catenin and TCF4 proteins on the PL2G2A promoter, we performed ChIP experiments with β-catenin and TCF4 antibodies in AGS cells, which express high quantities of β-catenin, TCF4, and PL2G2A proteins. The quality of the ChIP DNA was confirmed using the cyclin D1 promoter, a well-known Wnt/β-catenin target gene, as a positive control (Fig. 1D; ref. 18), and a region in the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) coding region as a negative control. Cyclin D1 mRNA is also down-regulated upon β-catenin silencing in AGS cells (data not shown). We observed a specific enrichment of the PL2G2A promoter in both β-catenin and TCF4 ChIP DNA, thus demonstrating that β-catenin and TCF4 proteins are likely to directly interact with the PL2G2A promoter. Taken collectively, the occurrence of TCF binding sites, the β-catenin and TCF transcriptional activity–dependent regulation and binding of β-catenin and TCF4 proteins with the PL2G2A promoter, all show that PL2G2A is a direct target of the Wnt signaling pathway. To our knowledge, this is the first time PL2G2A has been established as a direct Wnt target.

**PL2G2A inhibits GC invasion and migration.** The relationship between PL2G2A expression and clinical outcome (5) may be merely associative or PL2G2A may functionally act to inhibit cancer aggressiveness. To distinguish between these possibilities, we silenced PL2G2A in AGS cells and examined the effects of PL2G2A silencing on cellular invasion using an in vitro Matrigel assay. We found that PL2G2A silenced AGS cells exhibited enhanced invasion compared with either control untransfected cells or cells treated with control siRNAs, with PL2G2A-silenced cells being 2- to 3-fold more invasive (Fig. 2A and Supplementary Fig. S5). This result suggests that PL2G2A may function to suppress cellular invasion in GC cells, which is consistent with its expression being positively correlated with improved survival in GC patients (5).

To confirm the siRNA results, we then stably silenced PL2G2A in AGS cells by lentiviral-mediated transduction of PL2G2A shRNA-producing constructs. After screening several distinct shRNA-delivering plasmids, we identified stable clones of cells where PL2G2A was efficiently silenced (AGS-shPL2G2A cells; Fig. 2B and Supplementary Fig. S6). Similar to the siRNA experiment, the invasive abilities of AGS-shPL2G2A cells were significantly enhanced compared with parental AGS cells or control AGS-shNSC cells expressing nonspecific control shRNAs (Fig. 2B). Notably, the PL2G2A siRNA and shRNA reagents used for these experiments target entirely different regions of the PL2G2A gene, thus any enhancement of invasiveness seen in these cells is unlikely to be due to off-target effects. To ask if the increase in invasive ability might be related to alterations in cell proliferation, we conducted cell proliferation assays on PL2G2A-silenced cells. Despite being more invasive, PL2G2A-silenced cells exhibited a modest but significantly decreased proliferation rate compared with control cells (Fig. 2C), suggesting that the enhancement of invasion is unlikely to be due to an increase in cell proliferation rate. Thus, endogenous PL2G2A expression seems to be necessary to suppress cellular invasion in GC cells.

To ask if PL2G2A overexpression might be sufficient to suppress cellular invasion in GC, we then performed the reciprocal experiment and ectopically expressed PL2G2A in N87 cells, which do not normally express endogenous PL2G2A (Fig. 3A). Strikingly, the ectopic expression of PL2G2A dramatically modified the cellular appearance of N87 cells (N87-PL2G2A; Fig. 3A), resulting in an expanded and differentiated morphology compared with control N87 cells. N87-PL2G2A cells were potentially postmitotic, as their proliferation rate was significantly decreased compared with control cells (Fig. 3B). We compared the migratory capacities of parental N87, N87-pCDNA, and N87-PL2G2A cells in a wound healing assay (the previously used Matrigel assay was not used because N87 cells are not appropriate for this assay). Here, a series of scratches were made on confluent cells, and the time taken for the “wound closure” was monitored. After 15 days, 80% of the scratched areas were not filled in by N87-PL2G2A cells, whereas >95% of the scratched areas were occupied by control N87 cells (Fig. 3C and Supplementary Fig. S7). Notably, unlike AGS cells, N87 cells do not exhibit significant TCF4 activity (Fig. 1A). This result indicates that the antiinvasive effect of PL2G2A is not cell line specific, suggesting that PL2G2A may prove capable of acting as a general suppressor of cellular migration in GC.

**PL2G2A inhibits GC invasion by regulating S100A4.** To explore possible molecular mechanisms underlying the antiinvasive
activity of PLA2G2A, we then performed a microarray expression analysis of PLA2G2A-silenced cells to identify potential PLA2G2A-regulated genes. We identified two metastasis mediator genes, S100A4 and NEDD9, whose expression was increased upon

Figure 2. PLA2G2A inhibits cellular invasion in AGS cells. A, top, Western blots showing efficient silencing of PLA2G2A in AGS cells at 72 h after the siRNA transfection. Bottom, graph showing enhanced invasion of PLA2G2A-silenced AGS cells in Matrigel invasion assays. The relative invasive ability of control and PLA2G2A siRNA-transfected cells was compared against untransfected AGS cells (100%). Three independent experiments were performed.

B, top, Western blots showing reduced expression of PLA2G2A in stably silenced AGS cells (AGS-shPLA2G2A) compared with parental and control AGS-shNSC (nontargeting shRNA) cells. Bottom, enhancement of invasion in AGS-shPLA2G2A cells compared with parental AGS and nontargeting shRNA producing stable cells (AGS-shNSC). Three independent experiments were performed. C, parental AGS and AGS-shNSC cells are relatively more proliferative compared with AGS-shPLA2G2A cells. Graphs show cell proliferation compared against parental AGS cells (100%). SE was calculated from three independent experiments.

Figure 3. PLA2G2A inhibits cellular migration in N87 cells. A, Western blots with anti-T7 and anti-PLA2G2A antibodies showing the ectopic expression of T7-PLA2G2A fusion protein in N87-PLA2G2A cells. All photographs were taken at the same magnification. N87 cells grow as small tightly aggregated cells, whereas constitutive expression of PLA2G2A in N87 cells resulted in larger cells. B, stable expression of PLA2G2A in N87 cells retards cell proliferation. Proliferation differences between PLA2G2A-overexpressing and either parental or control transfected N87 cells. Measurements were performed on the 9th day from seeding. C, wound healing/cell migration assays performed on monolayer cells reveal PLA2G2A-mediated inhibition of cellular migration in N87 cells. Graph showing the percentage of wound closure on the 15th day after wounding, derived from six independent experiments. N87-PLA2G2A cells were compared with parental N87 and N87-pCDNA cells.
PLA2G2A silencing (data not shown). S100A4 is expressed in highly motile cells and contributes to metastatic progression in many cancer types, and NEDD9 is a melanoma metastasis gene (19, 20). Three lines of evidence further support the hypothesis that S100A4 and NEDD9 are bona fide PLA2G2A-regulated genes. First, we confirmed the up-regulation of S100A4 and NEDD9 in PLA2G2A-silenced cells by semiquantitative RT-PCR and in the case of S100A4 up-regulation also by immunoblotting (Fig. 4A). Second, in PLA2G2A-overexpressing N87 cells, we observed the opposite pattern, wherein both S100A4 and NEDD9 were down-regulated (Fig. 4A). Third, across the GC cell lines, we observed that S100A4 expression was relatively less in PLA2G2A expressing cells (especially AGS and YCC3) compared with non-PLA2G2A expressing lines (Fig. 4B). These results strongly suggest that PLA2G2A is likely to negatively regulate both S100A4 and NEDD9 expression in GC cells.

We then asked if the enhanced invasion observed in PLA2G2A silenced AGS cells might be due to S100A4 up-regulation. To address this, we silenced both PLA2G2A and S100A4 and compared the invasive capacities of PLA2G2A-silenced, S100A4-silenced, and PLA2G2A/S100A4-silenced cells. We found that S100A4-silenced cells exhibited significantly lower invasive ability compared with control cells, and importantly, the enhanced invasion observed after silencing PLA2G2A was almost entirely abrogated when S100A4 was silenced along with PLA2G2A (Fig. 4C and D). These results show that S100A4 is necessary for the enhanced invasion observed in PLA2G2A silenced cells, suggesting that PLA2G2A may affect metastasis in GC by regulating downstream metastasis mediator genes, such as S100A4 and possibly NEDD9.

PLA2G2A is expressed in early-stage and primary cancers but is significantly reduced in late-stage and metastatic tumors. To extend our results to the clinical setting, we then asked if the antinvasive effects of PLA2G2A observed in vitro were consistent with its in vivo expression in primary tumors. To examine the expression patterns of PLA2G2A in primary gastric tumors, we queried mRNA expression data of two independent GC patient cohorts from Hong Kong (5) and United Kingdom. In both cohorts, we observed high levels of PLA2G2A expression in early-stage tumors, wherein the cancer cells are largely localized to the primary site of origin. However, we also observed a stage-wise gradual loss of PLA2G2A expression during GC progression, with markedly diminished expression in late-stage GCs compared with early-stage disease (Fig. 5A and B). This finding is consistent with the possibility that the loss of PLA2G2A activity may functionally contribute to the development and progression of advanced stage GC.

Besides GC, PLA2G2A has also been reported to be expressed in other cancer types, including colon and prostate cancer (9, 21, 22), wherein Wnt/β-catenin signaling has also been implicated (12, 23, 24). For these cancers, data sets containing gene expression profiles from primary and metastatic tumors were available, thereby providing an opportunity to directly compare PLA2G2A levels between primary and metastatic tumors (25). We analyzed PLA2G2A expression patterns in one colon cancer data set and five prostate cancer data sets and found that PLA2G2A was repeatedly and significantly underexpressed in metastatic colon and prostate cancers compared with primary tumors (Fig. 5C and D). Thus, PLA2G2A expression seems to be decreased or lost in metastatic cancers compared with primary lesions, consistent with the possibility that PLA2G2A activity loss in vivo may contribute to the development of more aggressive tumors.
Epigenetic silencing and allelic loss of PLA2G2A in GC cells.

Finally, we considered three potential mechanisms to explain the loss or reduction of PLA2G2A expression in advanced cancers, including (a) mutational inactivation, (b) epigenetic silencing, and (c) loss of heterozygosity (LOH). To investigate the relative contribution of these three mechanisms, we sequenced the PLA2G2A coding regions in all 17 gastric cell lines and found that the coding regions were intact, thereby arguing against DNA mutation as a PLA2G2A inactivation mechanism. To investigate if PLA2G2A might be epigenetically regulated, we scanned the PLA2G2A promoter for potential regions of DNA methylation. Although no distinct CpG islands were seen in the PLA2G2A promoter, we detected a handful of CpG sites in the promoter region between −1,340 and −900 bp, a region containing the TCF consensus binding sites (Supplementary Fig. S8). Because methylation of specific CpG sites with functional implications on gene expression have been reported (26), we decided to analyze the methylation status of these CpG sites. The PLA2G2A promoter region was subjected to bisulfite sequencing in a set of PLA2G2A expressing and nonexpressing GC cell lines. We found that the PLA2G2A CpG sites were methylated at both alleles in cell lines N87, YCC1, and YCC11, which do not express PLA2G2A (Fig. 6A), whereas among the PLA2G2A-expressing cell lines, one or all the four sites always remained unmethylated or only partially methylated, thereby raising the possibility that PLA2G2A could be epigenetically regulated. Supporting this, treatment of GC cells with 5-azadC, an inhibitor of DNA methyltransferases, resulted in the transcriptional reactivation of PLA2G2A in Kato III, YCC10, and YCC11 cells, where the Wnt pathway is active (Fig. 6A and B). However, a similar 5-azadC treatment did not restore PLA2G2A expression in N87 and YCC1 cells, which exhibit a minimal level or no TCF activity, despite these cells also containing a methylated PLA2G2A promoter (Fig. 6A and D). These results suggest that in GC, promoter demethylation alone may be insufficient to reactivate PLA2G2A without the simultaneous presence of an activated Wnt pathway. To directly test this possibility, we simultaneously treated N87 and YCC1 cells with 5-azadC and LY2119301, a small molecule GSK-3β inhibitor (27). Inhibition of GSK-3β by LY2119301 blocks

Figure 5. PLA2G2A is underexpressed in late-stage and metastatic cancers. A, PLA2G2A expression in gastric tumors from Hong Kong. B, PLA2G2A expression in the U.K. cohort of gastric tumors. C, PLA2G2A expression in primary and metastatic colon cancers. D, PLA2G2A expression in multiple prostate cancer datasets comprising primary and metastatic prostate cancers. The PLA2G2A mRNA expression analyses in A, C and D were performed using the Oncomine database (25). The numbers of samples in each group and the datasets are mentioned in the bottom. P and M symbols depict primary and metastatic tumors, respectively.
h-catenin degradation, leading to the accumulation of the latter and consequent Wnt pathway hyperactivation (28). In N87 and YCC1 cells, LY2119301 treatment induced a striking accumulation of h-catenin protein and activated TCF transcriptional activity (Fig. 6C and Supplementary Fig. S9). Importantly, promoter demethylation with 5-azadC and Wnt hyperactivation with LY2119301 restored PLA2G2A gene and protein expression in both N87 and YCC1 cells after 5-azadC treatment. Restoration of SFRP1 gene expression is shown as a positive control for the treatment. C, RT-PCR and immunobLOTS showing enhanced expression of PLA2G2A in N87 and YCC1 cells after combined treatment with 5-azadC and a GSK3β inhibitor (LY2119301). ImmunobLOTS also show accumulation of β-catenin protein in N87 and YCC1 cells upon treatment with LY2119301. D, schematic model summarizing the role of PLA2G2A in GC progression. In early-stage gastric tumors, Wnt dysregulation results in PLA2G2A expression which in turn suppresses the expression of metastasis gene S100A4 and NEDD9. The reduced expression of metastasis genes keeps the tumor cells less invasive and aggressive. In late-stage tumors, loss of PLA2G2A expression, either due to epigenetic silencing or genomic deletion, results in abundant expression of metastasis genes S100A4 and NEDD9, and this adds aggressive and highly invasive capability to the gastric tumor cells.

Discussion

In this study, we investigated if PLA2G2A plays an important functional role in GC progression by identifying its upstream regulators and its downstream target genes and characterizing its phenotypic effects on GC cells. Our results strongly suggest that PLA2G2A is a direct target gene of Wnt signaling in GC cells, for the following reasons: (a) PLA2G2A is expressed in GC cell lines with constitutive TCF transcriptional activity, (b) silencing of β-catenin in AGS, YCC3, and Kato III cell lines resulted in significant reductions of PLA2G2A mRNA, (c) the PLA2G2A promoter contains multiple TCF binding sites and shows Wnt/β-catenin inducibility in a reporter promoter and genomic deletion may both contribute toward the reduction of PLA2G2A expression in late-stage and metastatic GCs.
assay, and (d) binding of TCF4 and β-catenin to the PL2G2A promoter could be observed in a ChIP experiment. To our knowledge, this is the first report demonstrating PL2G2A as a direct Wnt target gene. PL2G2A thus joins a number of other previously identified TCF/LEF target genes, including c-myc, cyclin D1, Axin2, Minp7, EphB2, ITF2, CD44, and gastrin as potential downstream mediators of Wnt activity. It is intriguing that the Wnt pathway, normally thought of as prooncogenic, may also cause up-regulation of potent invasion suppressor genes, like PL2G2A. One possibility might be that the final cellular output of Wnt signaling may be determined not simply by its upstream activation status, but also through a complex interplay of both positive and negative downstream effector target genes. There is some precedent for this, as some Wnt-regulated genes, such as Dickkopf, Axin2, β-TrCP, and TCF1, have also been shown to cause inhibition of Wnt signaling itself (15).

In GCs, PL2G2A is expressed in primary tumors where its high expression is associated with improved survival (5). Before this study, however, it was not known if PL2G2A expression in GC is merely associated with survival or if PL2G2A plays an active functional role in regulating GC progression. Here, we found that PL2G2A is also heterogeneously expressed in GC cell lines, and identified several PL2G2A-expressing lines (AGS, YCC3) as appropriate experimental models to study PL2G2A function. PL2G2A encodes a secreted phospholipase and is part of the 19-member mammalian phospholipase A2 super family. A major biochemical function of PL2G2A is to hydrolyze the fatty acids of membrane phospholipids (6). Although primarily known for its role in inflammation and antibacterial defense, the first insight linking PL2G2A to cancer was in the APCMin colon cancer mouse model, where the multiple intestinal neoplasia (8) phenotype caused by the APCMin mutation was strongly enhanced by the Mom1 allele, which was revealed to be a loss-of-function mutation in murine PL2G2A (10). Subsequently, it was shown that overexpression of wild-type Mom1/PL2G2A caused a reduction in tumor multiplicity and size (8) in APCMin mice, leading to proposals that PL2G2A might function as a tumor suppressor gene in colon cancer (10). However, the model of PL2G2A as a tumor suppressor, has been subsequently challenged by numerous studies showing a lack of genetic mutations of PL2G2A in human colorectal carcinomas, neuroblastoma, and melanoma cell lines (29, 30) and elevated rather than decreased expression of PL2G2A in cancers, such as small bowel adenocarcinoma and prostate tumors (9, 22, 31). In this study, we found that PL2G2A is quite capable of functionally inhibiting cancer invasion—PL2G2A-silenced AGS cells were more invasive in vitro than control cells and overexpression of PL2G2A in N87 cells suppressed cellular migration and proliferation. We also defined a potential mechanism for this antiinvasive effect by showing that PL2G2A negatively regulates two important downstream metastasis mediator genes, S100A4 and NEDD9. S100A4, a protein that activates nonmuscle myosin is well known to be involved in tumor progression and metastasis (19), and in GC patients, increased S100A4 expression in tumors is associated with advanced stage, lymph node positivity, metastasis, peritoneal dissemination, and aggressiveness (32–35). NEDD9 is another recently identified metastasis gene that may function as a metastatic hub among cancer signaling pathways (20, 36, 37). These results thus strongly argue that PL2G2A likely plays an important role in inhibiting GC progression.

Importantly, the antiinvasive effects of PL2G2A observed in vitro are highly consistent with its in vivo expression pattern in primary human tumors. Revisiting earlier studies reporting elevated expression levels of PL2G2A in prostate and colon cancers (9, 22), we found that whereas PL2G2A was indeed highly expressed in early-stage cancers, it was significantly underexpressed in late-stage tumors and in metastatic cancers in two GC, one colon cancer, and five prostate cancer datasets (Fig. 5A–D). We also explored the possible mechanism underlying the reduced expression of PL2G2A in advanced disease. It is unlikely that this underexpression is caused by PL2G2A somatic mutations, as we have not identified PL2G2A loss-of-function mutations in either GC cell lines or GC primary tumors in a preliminary screen (data not shown). However, epigenetic silencing of PL2G2A may be one possible cause for the observed loss of PL2G2A expression in late-stage disease. In cell lines, there was a striking correlation between the methylation of CpG sites in the PL2G2A promoter and the basal expression of PL2G2A, and PL2G2A transcription could be reactivated after treatment with the demethylating drug 5-aza2dC in Wnt-hyperactive GC cell lines. Moreover, PL2G2A expression could also be reactivated in non–Wnt-hyperactive GC cell lines N87 and YCC1 by combined modulation of Wnt signaling and epigenetic silencing, showing that PL2G2A expression is determined by a complex interaction between signaling and epigenetic pathways. Besides epigenetics, other mechanisms, such as LOH (PL2G2A is localized at 1p35-36, a well known LOH region in various cancers and cell lines; ref. 30), may also contribute to PL2G2A undereexpression. Clarifying the epigenetic and copy number status of PL2G2A in late-stage primary tumors will be an important future area of research.

Taken collectively, we propose that the following working model for GC progression (Fig. 6D). In early-stage tumors, Wnt signaling is active, causing up-regulation of genes with prooncogenic activity, such as c-myc and cyclin D1, thereby driving cell proliferation and dedifferentiation. However, disease progression in these tumors is held in check due to the simultaneous up-regulation of invasion suppressor genes, such as PL2G2A. In late-stage tumors, Wnt signaling is still active driving expression of prooncogenes; however, PL2G2A expression is decreased possibly by epigenetic inactivation and/or genomic deletions, relieving the inhibition of metastasis-related genes, such as S100A4 and NEDD9. The end result is a tumor with a highly aggressive clinical phenotype (38–40). Whereas it is obviously necessary to test this working model through further experimentation, one implication of this model is that complex interactions between positive and negative downstream effectors of Wnt signaling may profoundly influence the course of tumor behavior in individual patients. It may thus be interesting to ask if similar paradigms might also hold for other oncogenic signaling networks.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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http://www.stanford.edu/~musse/wntwindow.html
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