Inhibition of Gastric Cancer Invasion and Metastasis by *PLA2G2A*, a Novel β-Catenin/TCF Target Gene

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Abstract

Elevated expression of the *PLA2G2A* phospholipase in gastric cancer (GC) is associated with improved patient survival. To elucidate function and regulation of *PLA2G2A* in GC, we analyzed a panel of GC cell lines. *PLA2G2A* was specifically expressed in lines with constitutive Wnt activity, implicating β-catenin–dependent Wnt signaling as a major upstream regulator of *PLA2G2A* expression. The invasive ability of *PLA2G2A*-expressing AGS cells was enhanced by *PLA2G2A* silencing, whereas cellular migration in non–*PLA2G2A*-expressing N87 cells was inhibited by enforced *PLA2G2A* expression, indicating that *PLA2G2A* is both necessary and sufficient to function as an inhibitor of GC invasion *in vitro*. We provide evidence that antiinvasive effect of *PLA2G2A* occurs, at least in part, through its ability to inhibit the *S100A4* metastasis mediator gene. Consistent with its invasion inhibitor role, *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 *PLA2G2A* promoter methylation status and *PLA2G2A* expression, suggesting that the loss of *PLA2G2A* expression in late-stage cancers may be due to epigenetic silencing. Supporting this, among the non–*PLA2G2A*-expressing lines, pharmacologic inhibition of epigenetic silencing reactivated *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 *PLA2G2A* reactivation. Our results highlight the complexity of *PLA2G2A* regulation and provide functional evidence for *PLA2G2A* as an important regulator of invasion and metastasis in GC. [Cancer Res 2008;68(11):4277–86]

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

We recently reported an association between *PLA2G2A* expression and components of the Wnt signaling pathway, including *CTNNB1* (β-catenin) and the Wnt target gene *EphB2* 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 *PLA2G2A* prosurvival activity by examining *PLA2G2A* activity and regulation in a panel of GC cell lines. Using a variety of experimental approaches, we show that *PLA2G2A* is a direct target of Wnt/β-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 *S100A4* and *NEDD9*. Our results support the notion that in addition to being a prognostic biomarker, *PLA2G2A* plays an intimate functional role in inhibiting GC progression. One implication of our findings is that because *PLA2G2A* is often underexpressed in late-stage and metastatic tumors, it is plausible that the reintroduction of *PLA2G2A* into aggressive tumors, by either gene therapy, administration of *PLA2G2A* protein, or intriguingly via epigenetic reactivation, might constitute a novel therapy for late-stage GC.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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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_{18}) 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, Inc.), 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 folds of activity (Topflash/Fopflash) after normalization to β-galactosidase activity. The *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 5×10^6 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 Fugene 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, 5×10^6 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)/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/salmon sperm DNA–blocked protein A/G plus agarose and washed, and the bead pellet was resuspended in TE 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 *cyclin 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 transfected 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 12 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 5×10^5 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 2×10^6 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 mmol/L, respectively, and incubated

at 50°C for 4 h. The DNA was purified using a DNA purification kit (Qiagen), desulfonated 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 H_2O. The *PLA2G2A* promoter was amplified from bisulfite-modified DNA using JumpStart REDTaq DNA polymerase (Sigma) with the oligos *PLA2G2A*-MSF, TTGTAAAATAGTTTGAAATGATGGG, and *PLA2G2A*-MSR, CAATAATTCCT CCAATAAACAAAAC. 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 LY2119301, 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. S1*A*) 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* transcripts. Other cell lines (SNU1, SNU5, N87, YCC1, YCC2, YCC6, YCC9, YCC16, MKN7, Hs746, 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. 1*A* and Supplementary Fig. S1*B*).

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. 1*A*). 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. 1*A*). 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

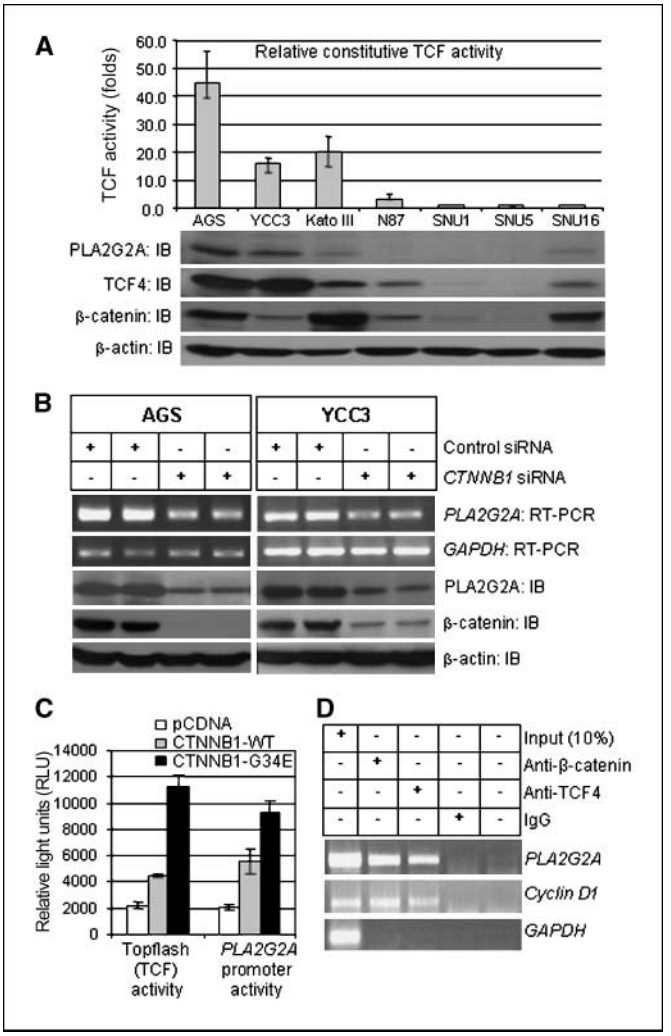

Figure 1. *PLA2G2A* is expressed in Wnt hyperactive GC cell lines and is a direct target gene of Wnt/β-catenin pathway. *A,* TCF transcriptional activity in GC cell lines measured by a Topflash reporter. TCF activity is represented as a relative fold measurement (Topflash/Fopflash activity). Immunoblots show PLA2G2A, TCF4, and β-catenin protein expression in the GC cell lines, normalized against a β-actin antibody. *B,* β-catenin knockdown by siRNA-mediated silencing in AGS and YCC3 cells results in decreased *PLA2G2A* mRNA and protein expression, revealed by semiquantitative RT-PCR and immunoblots. The anti–β-catenin immunoblot confirms efficient β-catenin silencing, whereas the anti–β-actin immunoblot and GAPDH RT-PCR shows equal sample loading. The two lanes for each cell line represent two independent experiments. *C,* 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 pulled down DNA or input DNA with specific primers to the *PLA2G2A* promoter, *cyclin D1* promoter, and GAPDH coding region.

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

cells, explaining the relative lack of Wnt activity in this line (11). These observations support our hypothesis that *PLA2G2A* expression in GC is highly correlated with constitutively activated Wnt signaling.

***PLA2G2A* is a direct target of the Wnt signaling pathway in GC cells.** We then asked if β-catenin–mediated Wnt signaling might directly regulate *PLA2G2A* expression in GC. Using siRNAs, we silenced β-catenin in AGS, YCC3, and Kato III cells. *PLA2G2A* gene and protein expression were considerably reduced upon β-catenin silencing (Fig. 1*B* and Supplementary Fig. S3*A*). A quantitative analysis revealed a 50% reduction of *PLA2G2A* expression in AGS and YCC3 cells at 72 hours after β-catenin silencing (Supplementary Fig. S3*B*). This result indicates that β-catenin is necessary for *PLA2G2A* expression in GC cells, suggesting that *PLA2G2A* 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 *PLA2G2A* being a direct β-catenin/TCF target gene, we identified multiple TCF/LEF transcription factor binding sites in the *PLA2G2A* 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 *PLA2G2A* 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). To investigate the Wnt inducibility of this region, we cloned the 1.3-kb *PLA2G2A* promoter fragment containing the TCF consensus sites into a luciferase reporter plasmid and transfected it into YCC3 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 *PLA2G2A* reporter exhibited increasing 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 *PLA2G2A* promoter fragment (Fig. 1*C*). Thus, activation of Wnt signals through β-catenin is sufficient to enhance *PLA2G2A* expression, arguing that *PLA2G2A* 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 *PLA2G2A* promoter, we performed ChIP experiments with β-catenin and TCF4 antibodies in AGS cells, which express high quantities of β-catenin, TCF4, and PLA2G2A 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. 1*D*; 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 *PLA2G2A* promoter in both β-catenin and TCF4 ChIP DNA, thus demonstrating that β-catenin and TCF4 proteins are likely to directly interact with the *PLA2G2A* 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 *PLA2G2A* promoter, all show that *PLA2G2A* is a direct target of the Wnt signaling pathway. To our knowledge, this is the first time *PLA2G2A* has been established as a direct Wnt target.

***PLA2G2A* inhibits GC invasion and migration.** The relationship between *PLA2G2A* expression and clinical outcome (5) may be merely associative or *PLA2G2A* may functionally act to inhibit cancer aggressiveness. To distinguish between these possibilities, we silenced *PLA2G2A* in AGS cells and examined the effects of *PLA2G2A* silencing on cellular invasion using an *in vitro* Matrigel assay. We found that *PLA2G2A* silenced AGS cells exhibited enhanced invasion compared with either control untransfected cells or cells treated with control siRNAs, with *PLA2G2A*-silenced cells being ∼2-fold to 3-fold more invasive (Fig. 2*A* and Supplementary Fig. S5). This result suggests that *PLA2G2A* 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 *PLA2G2A* in AGS cells by lentiviral-mediated transduction of *PLA2G2A* shRNA-producing constructs. After screening several distinct shRNA-delivering plasmids, we identified stable clones of cells where *PLA2G2A* was efficiently silenced (AGS-shPLA2G2A cells; Fig. 2*B* and Supplementary Fig. S6). Similar to the siRNA experiment, the invasive abilities of AGS-shPLA2G2A cells were significantly enhanced compared with parental AGS cells or control AGS-shNSC cells expressing nonspecific control shRNAs (Fig. 2*B*). Notably, the *PLA2G2A* siRNA and shRNA reagents used for these experiments target entirely different regions of the *PLA2G2A* 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 *PLA2G2A*-silenced cells. Despite being more invasive, *PLA2G2A*-silenced cells exhibited a modest but significantly decreased proliferation rate compared with control cells (Fig. 2*C*), suggesting that the enhancement of invasion is unlikely to be due to an increase in cell proliferation rate. Thus, endogenous *PLA2G2A* expression seems to be necessary to suppress cellular invasion in GC cells.

To ask if *PLA2G2A* overexpression might be sufficient to suppress cellular invasion in GC, we then performed the reciprocal experiment and ectopically expressed *PLA2G2A* in N87 cells, which do not normally express endogenous *PLA2G2A* (Fig. 3*A*). Strikingly, the ectopic expression of *PLA2G2A* dramatically modified the cellular appearance of N87 cells (N87-PLA2G2A; Fig. 3*A*), resulting in an expanded and differentiated morphology compared with control N87 cells. N87-PLA2G2A cells were potentially postmitotic, as their proliferation rate was significantly decreased compared with control cells (Fig. 3*B*). We compared the migratory capacities of parental N87, N87-pCDNA, and N87-PLA2G2A 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-PLA2G2A cells, whereas >95% of the scratched areas were occupied by control N87 cells (Fig. 3*C* and Supplementary Fig. S7). Notably, unlike AGS cells, N87 cells do not exhibit significant TCF4 activity (Fig. 1*A*). This result indicates that the antiinvasive effect of *PLA2G2A* is not cell line specific, suggesting that *PLA2G2A* may prove capable of acting as a general suppressor of cellular migration in GC.

***PLA2G2A* inhibits GC invasion by regulating *S100A4*.** To explore possible molecular mechanisms underlying the antiinvasive

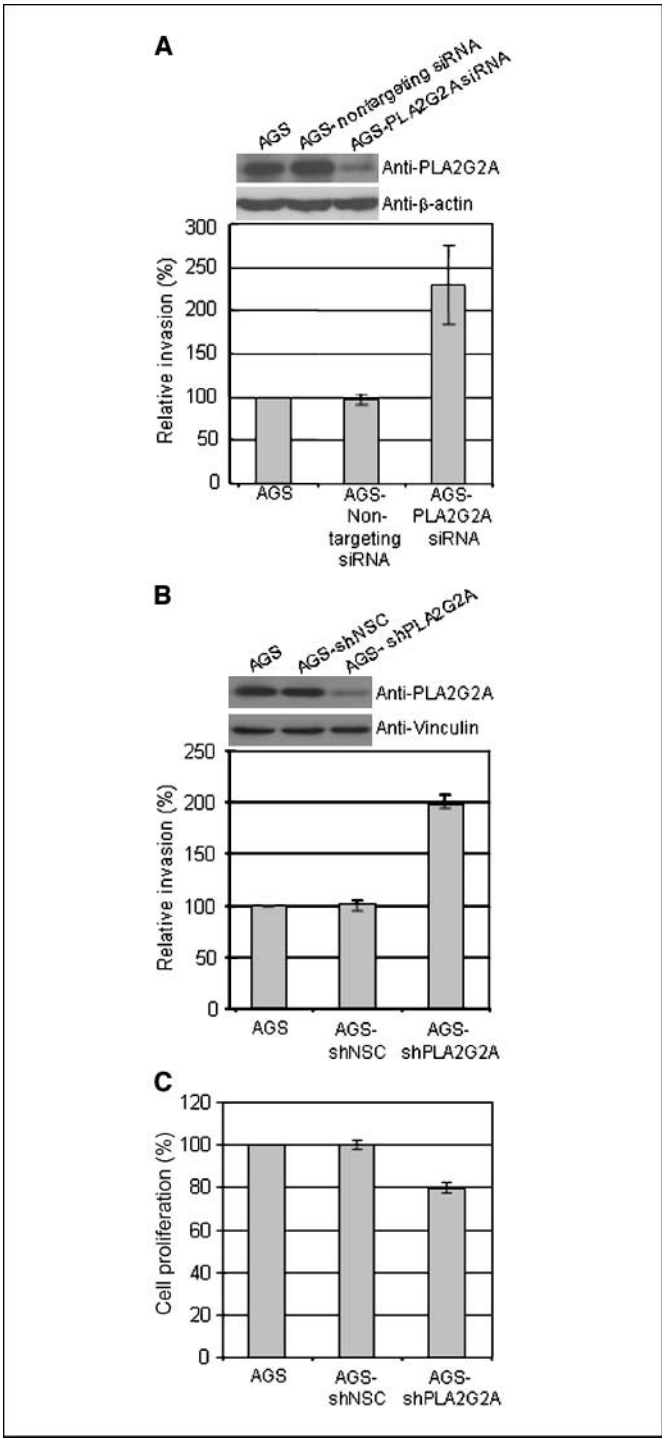

Figure 2. *PLA2G2A* inhibits cellular invasion in AGS cells. *A, top,* Western blots showing efficient silencing of PLA2G2A in AGS cells at 72 h after the siRNA transfection. *Bottom,* graph showing enhanced invasion of *PLA2G2A*-silenced AGS cells in Matrigel invasion assays. The relative invasive ability 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.

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

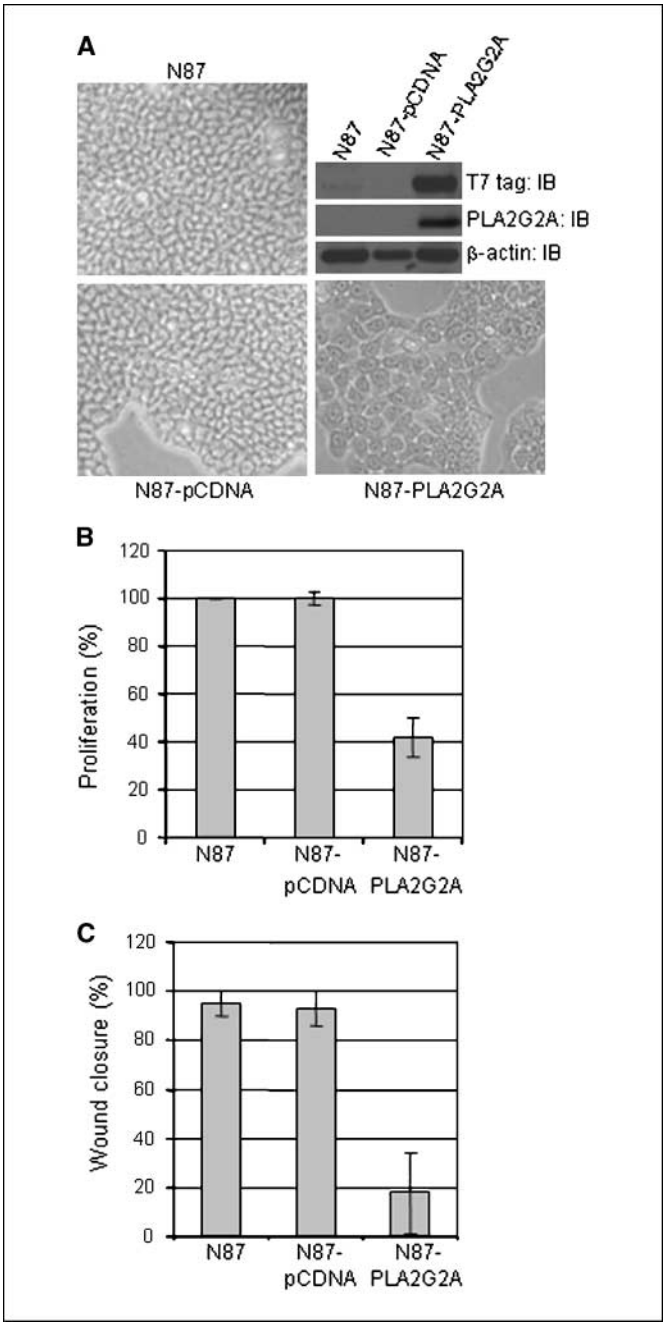

Figure 3. *PLA2G2A* inhibits cellular migration in N87 cells. *A,* Western blots with anti-T7 and anti-PLA2G2A antibodies showing the ectopic expression of T7-PLA2G2A fusion protein in N87-PLA2G2A cells. All photographs were taken at the same magnification. N87 cells grow as small tightly aggregated 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. 4*A*). Second, in *PLA2G2A*-overexpressing N87 cells, we observed the opposite pattern, wherein both *S100A4* and *NEDD9* were down-regulated (Fig. 4*A*). 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. 4*B*). 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. 4*C* 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 antiinvasive 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. 5*A* 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 prostrate cancers compared with primary tumors (Fig. 5*C* 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.

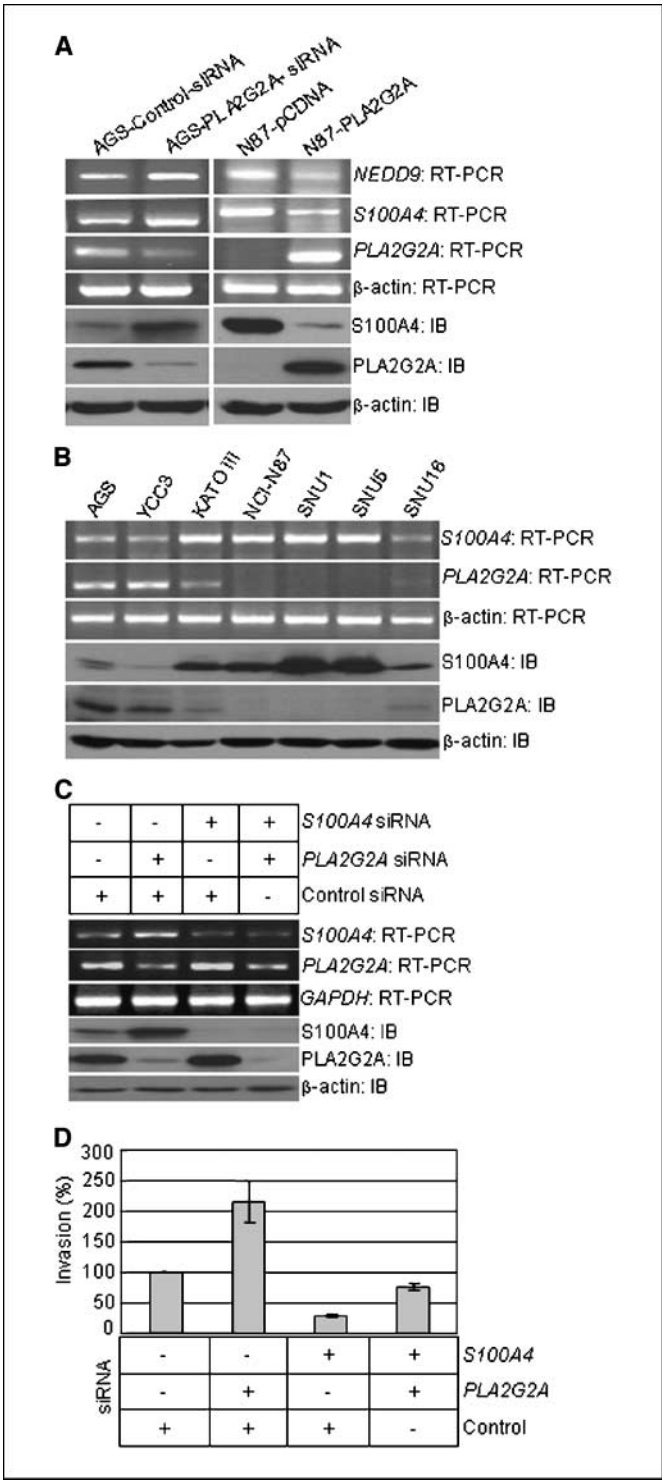

Figure 4. Inhibition of invasion by *PLA2G2A* is mediated through *S100A4* and *NEDD9* expression. *A, left,* RT-PCR showing increased expression of *S100A4* and *NEDD9* in *PLA2G2A*-silenced AGS cells. Immunoblots are for PLA2G2A and S100A4 proteins. *Right,* RT-PCR showing down-regulation of *S100A4* and *NEDD9* upon induced expression of *PLA2G2A* in N87 cells (N87-PLA2G2A stable cells). RT-PCR and immunoblot for β-actin confirm that equal quantities of cDNA and proteins were used. *B,* RT-PCRs and immunoblots showing reduced expression of *S100A4* in *PLA2G2A*-expressing cell lines. *C,* representative RT-PCRs and immunoblots showing efficient knockdown of *PLA2G2A* and *S100A4* in samples parallel to an experiment in *D*. *D,* Matrigel invasion assays show that *PLA2G2A*-mediated invasion inhibition is executed through *S100A4*. Combined silencing of *PLA2G2A* along with *S100A4* abolished the enhanced invasion observed in *PLA2G2A* silenced cells.

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. 6*A*), 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. 6*A* 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. 6*A* 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

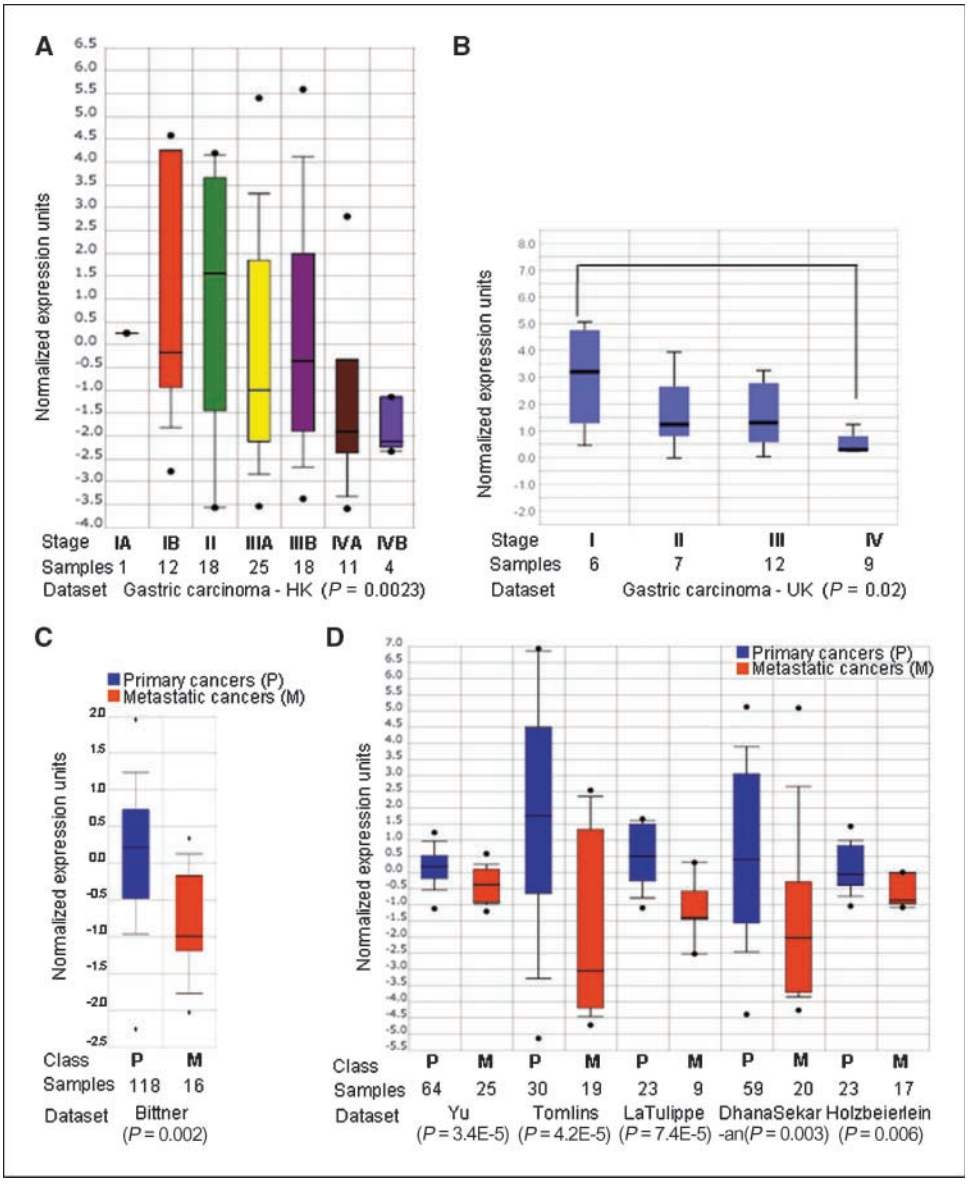

Figure 5. *PLA2G2A* is underexpressed in late-stage and metastatic cancers. *A, PLA2G2A* expression in gastric tumors from Hong Kong. *B, PLA2G2A* expression in the U.K. cohort of gastric tumors. *C, PLA2G2A* expression in primary and metastatic colon cancers. *D, PLA2G2A* expression in multiple prostate 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.

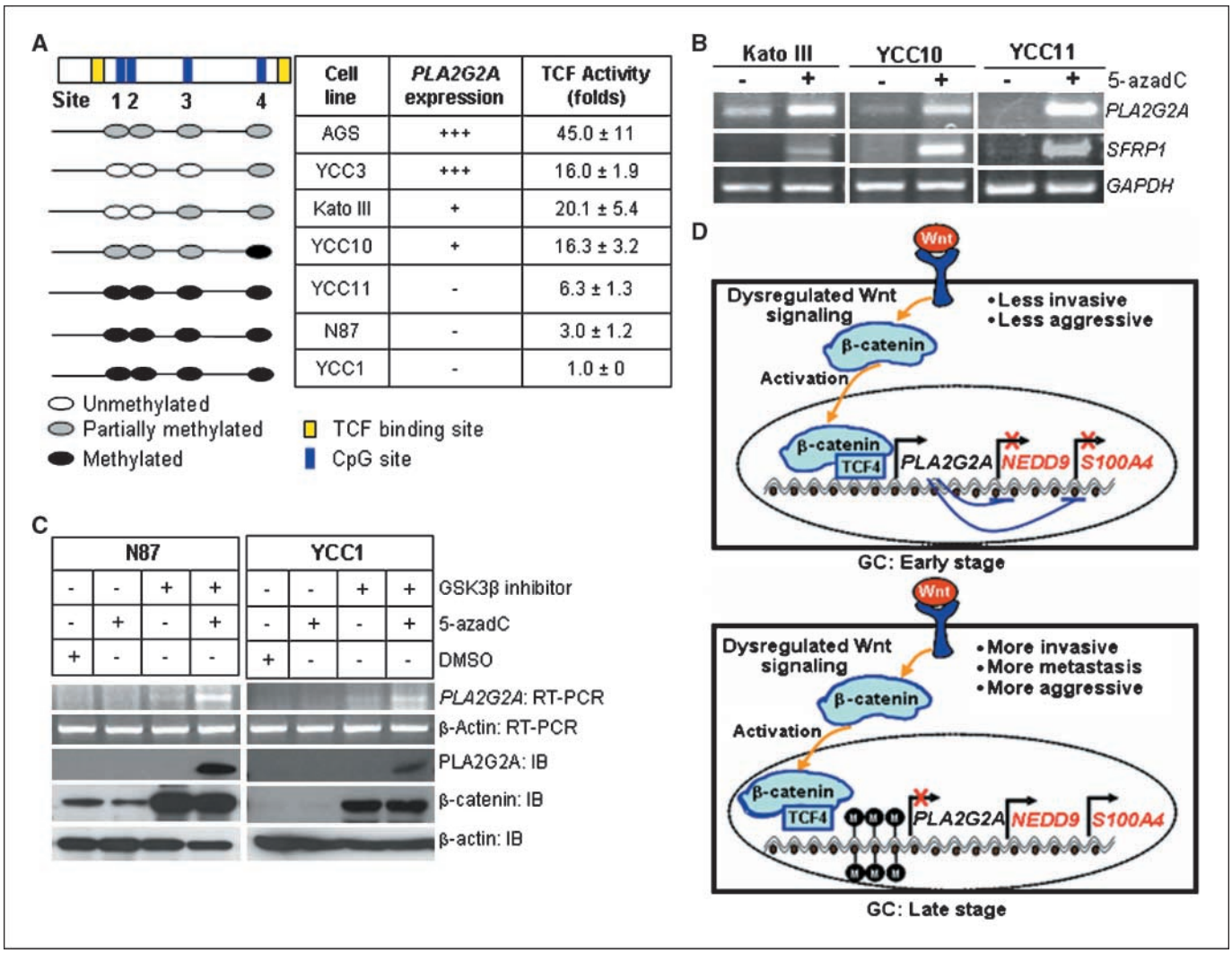

Figure 6. Epigenetic regulation of *PLA2G2A* expression in GC cell lines. *A,* schematic representation of the methylation status of four CpG sites in the *PLA2G2A* promoter, sequenced from bisulfite-modified genomic DNA of GC cells. In the *PLA2G2A*-expressing cell lines, the four sequenced CpG sites are either partially methylated or remain unmethylated. In non–*PLA2G2A*-expressing cell lines (YCC1, N87, and YCC11) the CpG sites are fully methylated. *PLA2G2A* expression and relative TCF transcriptional activity in the cell lines are listed in the right side. *B,* RT-PCR showing enhanced expression of *PLA2G2A* in Kato III, YCC10, and YCC11 cell lines 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.

β-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 β-catenin protein and activated TCF transcriptional activity (Fig. 6*C* 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, which are non-Wnt hyperactive cell lines with *PLA2G2A* promoter methylation (Fig. 6*D*). This clearly illustrates that *PLA2G2A* promoter is a target of both β-catenin/TCF signaling and epigenetic silencing in GC cells.

Finally, an examination of array-CGH profiles of the GC cell lines also revealed *PLA2G2A* hemizygous gene deletions in at least two cell lines (SNU-5 and YCC9; Supplementary Fig. S10). These experiments suggest that both epigenetic silencing of the *PLA2G2A* promoter and genomic deletion may both contribute toward the reduction of *PLA2G2A* expression in late-stage and metastatic GCs.

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 cells 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

assay, and (*d*) binding of TCF4 and β-catenin to the *PLA2G2A* promoter could be observed in a ChIP experiment. To our knowledge, this is the first report demonstrating *PLA2G2A* as a direct Wnt target gene. *PLA2G2A* thus joins a number of other previously identified TCF/LEF target genes, including *c-myc, cyclin D1, Axin2, Mmp7, EphB2, ITF2, CD44*, and *gastrin*[8] 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 *PLA2G2A*. 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, *PLA2G2A* is expressed in primary tumors where its high expression is associated with improved survival (5). Before this study, however, it was not known if *PLA2G2A* expression in GC is merely associated with survival or if *PLA2G2A* plays an active functional role in regulating GC progression. Here, we found that *PLA2G2A* is also heterogeneously expressed in GC cell lines, and identified several *PLA2G2A*-expressing lines (AGS, YCC3) as appropriate experimental models to study *PLA2G2A* function. *PLA2G2A* encodes a secreted phospholipase and is part of the 19-member mammalian phospholipase A2 super family. A major biochemical function of *PLA2G2A* 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 *PLA2G2A* to cancer was in the APC^{Min} colon cancer mouse model, where the multiple intestinal neoplasia (8) phenotype caused by the APC^{Min} mutation was strongly enhanced by the *Mom1* allele, which was revealed to be a loss-of-function mutation in murine *PLA2G2A* (10). Subsequently, it was shown that overexpression of wild-type *Mom1/PLA2G2A* caused a reduction in tumor multiplicity and size (8) in APC^{Min} mice, leading to proposals that *PLA2G2A* might function as a tumor suppressor gene in colon cancer (10). However, the model of *PLA2G2A* as a tumor suppressor, has been subsequently challenged by numerous studies showing a lack of genetic mutations of *PLA2G2A* in human colorectal carcinomas, neuroblastoma, and melanoma cell lines (29, 30) and elevated rather than decreased expression of *PLA2G2A* in cancers, such as small bowel adenocarcinoma and prostrate tumors (9, 22, 31). In this study, we found that *PLA2G2A* is quite capable of functionally inhibiting cancer invasion—*PLA2G2A*-silenced AGS cells were more invasive *in vitro* than control cells and overexpression of *PLA2G2A* in N87 cells suppressed cellular migration and proliferation. We also defined a potential mechanism for this antiinvasive effect by showing that *PLA2G2A* 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–positive 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 *PLA2G2A* likely plays an important role in inhibiting GC progression.

Importantly, the antiinvasive effects of *PLA2G2A* observed *in vitro* are highly consistent with its *in vivo* expression pattern in primary human tumors. Revisiting earlier studies reporting elevated expression levels of *PLA2G2A* in prostrate and colon cancers (9, 22), we found that whereas *PLA2G2A* 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. 5*A–D*). We also explored the possible mechanism underlying the reduced expression of *PLA2G2A* in advanced disease. It is unlikely that this underexpression is caused by *PLA2G2A* somatic mutations, as we have not identified *PLA2G2A* loss-of-function mutations in either GC cell lines or GC primary tumors in a preliminary screen (data not shown). However, epigenetic silencing of *PLA2G2A* may be one possible cause for the observed loss of *PLA2G2A* expression in late-stage disease. In cell lines, there was a striking correlation between the methylation of CpG sites in the *PLA2G2A* promoter and the basal expression of *PLA2G2A*, and *PLA2G2A* transcription could be reactivated after treatment with the demethylating drug 5-azadC in Wnt-hyperactive GC cell lines. Moreover, *PLA2G2A* 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 *PLA2G2A* expression is determined by a complex interaction between signaling and epigenetic pathways. Besides epigenetics, other mechanisms, such as LOH (*PLA2G2A* is localized at 1p35-36, a well known LOH region in various cancers and cell lines; ref. 30), may also contribute to *PLA2G2A* underexpression. Clarifying the epigenetic and copy number status of *PLA2G2A* 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. 6*D*). 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 *PLA2G2A*. In late-stage tumors, Wnt signaling is still active driving expression of prooncogenes; however, *PLA2G2A* 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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[8] http://www.stanford.edu/~rnusse/wntwindow.html

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