Tumor-Associated Macrophages Promote Epigenetic Silencing of Gelsolin through DNA Methyltransferase 1 in Gastric Cancer Cells

Hao-Chen Wang1,2, Chin-Wang Chen3, Chia-Lung Yang1,2, I-Min Tsai1,2, Ya-Chin Hou1,2, Chang-Jung Chen3, and Yan-Shen Shan1,2

Abstract

Epigenetic repression of the tumor suppressor gelsolin (GSN) is frequently observed in cancers. Chronic inflammation can promote tumor progression via aberrant DNA methylation. In this study, we investigated the role of tumor-associated macrophages (TAMs) in DNA methylation of the GSN gene during gastric cancer progression. Immunofluorescence staining of 121 gastric cancer tissues showed aberrant localization of GSN and DNA methyltransferase 1 (DNMT1) and juxtaposition of DNMT1 and M2 TAMs. Decreased GSN protein and mRNA expression and increased DNA methylation in the GSN promoter were observed in gastric cancer cell lines and clinical specimens. To examine the effect of TAMs on DNA methylation in gastric cancer cells, we performed in vitro coculture assays and found increased DNMT1 expression but decreased GSN expression in gastric cancer cells after coculture with U937 cells. Knockdown of DNMT1 expression in gastric cancer cells could abort U937 coculture-mediated GSN downregulation. Meanwhile, CCL5 was the main chemokine upregulated in coculture medium. Treatment with CCL5 could induce DNMT1 expression in gastric cancer cells via STAT3 signaling. Inhibiting DNMT1 activity with procainamide, inhibiting DNA methylation with 5-AZA, or inhibiting CCL5/CCR5 signaling with maraviroc reduced tumor growth in vivo. In conclusion, upregulation of DNMT1 by CCL5/CCR5/STAT3 signaling is critical for TAM-mediated GSN silencing in gastric cancer. This study identified potential targets for gastric cancer therapy.

Introduction

Gastric cancer is the fifth most common cancer and the third leading cause of cancer death worldwide (1). At diagnosis, over 50% of patients present with locally advanced or metastatic gastric cancer and consequently are ineligible for curative surgery (2). If surgery is not possible, chemotherapy is often given to shrink tumors; however, it offers limited survival benefits for patients (3). Therefore, improvements in gastric cancer treatment depend on a better understanding of the molecular events responsible for the development and progression of this malignancy.

Chronic inflammation that precedes or accompanies cancers is recognized as a key driver of gastric cancer (4). Inflammatory responses can recruit immune cells to the tumor microenvironment. Macrophages, part of the inflammatory infiltrate in tumors, provide a link between inflammation and cancer (5).

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H.-C. Wang, C.-W. Chen, and C.-L. Yang contributed equally to this article.

Corresponding Author: Yan-Shen Shan, Department of Surgery, National Cheng Kung University Hospital, Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan. Phone: 886-6-2353535, 5811. Fax: 886-6-2766676; E-mail: ysshan@mail.ncku.edu.tw
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Tumor-infiltrating monocytes are capable of polarizing into two macrophage types, tumor-suppressive macrophages (M1) and tumor-promoting macrophages (M2) that are also called tumor-associated macrophages (TAMs; ref. 6). In gastric cancer, TAM infiltration is associated with phenotypes such as angiogenesis, depth of invasion, and nodal status that characterize aggressive malignancies. High infiltration of TAMs conferred a poor prognosis in gastric cancer after resection (7, 8). The invasiveness of gastric cancer cells could be enhanced by coculture with TAMs (8). However, the way in which TAMs promote gastric tumorigenesis remains unclear.

Aberrant actin cytoskeleton architecture characterizes of tumor cells and is associated with cell migration and invasion. The mechanisms underlying the dysregulation of actin cytoskeleton organization in cancer cells, however, are not fully elucidated. Gelsolin (GSN) is an actin-binding protein that controls actin filament assembly and disassembly by severing, capping, and nucleating F-actin (9). GSN expression is downregulated in many cancers (10–13) and GSN overexpression leads to reduced cell growth, tumorigenicity, and metastasis in cell lines of human bladder cancer and mouse melanoma (11, 14), suggesting its potential role in tumor suppression. Because no major mutation, rearrangement, and deletions have been identified in the GSN gene, inactivation of GSN in tumors may occur through epigenetic mechanisms, such as DNA methylation and histone modifications (15, 16). However, how GSN expression is epigenetically regulated remains poorly understood.

DNA methylation is an epigenetic modification that allows gene silencing without changing the DNA sequence. This process is commonly disrupted in neoplastic cells and thus facilitates malignant transformation (17). Increased methylation is
associated with inflammation, dysplasia, and malignant transformation, suggesting the involvement of epigenetic changes in inflammation-associated carcinogenesis (18, 19). In gastric cancer, several tumor suppressor genes, such as p16INK4, E-cadherin, and RUNX3, are hypermethylated in their promoter (20–22). DNA methylation in mammalian cells is catalyzed by enzymes of the DNA methyltransferase (DNMT) family, including DNMT1, DNMT3A, DNMT3B, and DNMT3L (23). DNMT1, the major DNMT in adult cells, is highly expressed in various cancers, including gastric cancer (24, 25). Therefore, in this study, we plan to investigate whether TAMs downregulate GSN in gastric cancer cells via DNMT1-mediated DNA methylation.

Materials and Methods

Cell culture

The human gastric cancer cell lines AGS and NCI-N87 were purchased from Food Industry Research and Development Institute (Hsinchu, Taiwan); AZS21, HR, MKN45, and NUGC3 were gently provided by Prof. Chia-Jui Yen (Department of Internal Medicine, National Cheng Kung University Hospital, Tainan, Taiwan). The SV40-immortalized human normal gastric epithelial cell lineGES-1was provided by Prof. Pei-Jung Lu (Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan). The human leukemic monocyte lymphoma cell line U937 was a kind gift from Prof. Ming-Derg Lai (Department of Biochemistry and Molecular Biology, College of Medicine, National Cheng Kung University, Tainan, Taiwan). All the cell lines were authenticated using STR analysis. Testing for mycoplasma contamination in cell cultures was routinely performed at least 3 months. All the cell lines were maintained in RPMI-1640 medium (HyClone) with 10% fetal bovine serum (HyClone) or 30% human AB serum. The cell cultures were grown in a humidified 5% CO2 atmosphere at 37°C. The next day, 1 × 105 U937 cells were seeded in the lower chamber inserts to attach overnight. The next day, 1 × 105 THP1 cells were seeded in the lower chamber. The cocultures were incubated for 72 hours before further assays.

Reagents and antibodies

Protriamide hydrochloride (PCA; Sigma-Aldrich) was used to treat cells at the indicated concentrations for 24 hours. 5-Aza-2’-deoxycytidine (5-Aza; Sigma-Aldrich) was used at the concentration of 1 μmol/L for 24 hours. CCL5 recombinant protein (Cell Guidance System) was used at the indicated concentrations for 24 hours or at 10 ng/mL for various time points. CCL5-neutralizing antibodies (GeneTex) were used at 2 μg/mL for 72 hours. Maraviroc (Cayman) was used at the indicated concentrations for 24 hours or used at 1 μmol/L for 1 hour before CCL5 treatment. Antibodies against GSN and STAT3 were purchased from BD Biosciences. Antibodies against DNMT1 and CD204 were purchased from Abcam. Antibody against phospho-STAT3 (Tyr705) was purchased from Cell Signaling Technology. Antibody against β-actin was purchased from Sigma-Aldrich. Antibody against GAPDH was purchased from Genetex.

RNA interference and generation of stable cell lines

Short hairpin RNA (shRNA) plasmids for DNMT1 and luciferase were purchased from the National RNAi Core Facility Platform, Academia Sinica (Taipei, Taiwan). The shRNA lentivirus was produced in RNAi Core of Research Center of Clinical Medicine, National Cheng Kung University Hospital. Cells were infected with lentivirus in the presence of polybrene (8 μg/mL Sigma). Puromycin (5 μg/mL Sigma) was used for selection of stable transfectants. Western blotting was performed to determine the knockdown efficiency in the permanent knockdown cell lines.

In vitro coculture assay

A Transwell insert with 0.4-μm pore size (BD Bioscience) was used for the coculture experiments. Gastric cancer cells (1 × 105/mL well) were seeded in the upper chamber inserts to attach overnight. The next day, 1 × 105 THP1 cells were seeded in the lower chamber. The cocultures were incubated for 72 hours before further assays.

DNA extraction, bisulfite conversion, methylation-specific PCR (MSP), and pyrosequencing

CpG islands located in the GSN promoter were identified using the UCSC genome browser. Position of the CpG islands is located at chr9:124061806–124062229, which contains 55 CG dinucleotides in 424 base pairs. Transcription factor binding sites in the sequence of CpG islands were identified by the PROMO and TFSEARCH programs.

Genomic DNA was extracted by genomic DNA Mini Kit (Gen-eaid), and bisulfite conversion of 500 ng genomic DNA samples was performed using the EZ DNA Methylation Gold kit (Zymo). Converted genomic DNA was amplified by the KAPA2G Fast HotStart ReadyMix PCR Kit (KAPABIOSYSTEM) with GSN unmethylated (U) primers (F: 5’-TGGGAATTTAAGTTTAAAGTT-3’; R: 5’-AACAAACAAAAACCCCTCAGT-3’) and methylated (M) primers (F: 5’-TGGGAATTTAAGTTTAAAGTT-3’; R: 5’-AAGCGAAAAAAACCCCTCAGT-3’). The PCR program included an initial denaturation at 95°C for 3 minutes followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 45.1°C (U)/43.8°C (M) for 15 seconds, and extension at 72°C for 5 seconds. The length of the extension step in the final cycle was increased to 5 minutes, and the final temperature was held at 4°C. For pyrosequencing, bisulfite converted DNA was PCR-amplified with GSN forward primers (5’-CTGGGGGTTAGCCTTTGTG-3’) and biotinylated GSN reverse primers (5’-biotin-ACCTTAAATTACTCCCCCTTCTTCT-3’). The products were captured with Streptavidin Sepharose beads (GE Healthcare Biosciences) and pyrosequenced with GSN sequencing primers (5’-CTGGGAATTTAAGTTTAAAGTT-3’) using PyroMark 24 (Qiagen). Individual methylation frequencies of CpG sites were determined by the Pyro-Q-CpG software (Qiagen).

Immunohistochemical (IHC) staining, immunofluorescence (IF) staining, and analysis of clinical samples

A total of 121 gastric tumor tissues were collected from patients who underwent resection in National Cheng Kung University Hospital (NCKUH) between 1998 and 2008. The tumors were staged according to the 7th edition of the American Joint Committee on Cancer staging system and were classified into two groups: “early-stage” including stage Ia and “late-stage” including stages Ib, II, III, and IV. The protocol was reviewed and approved by the institutional review board of NCKUH. The formalin-fixed paraffin-embedded tissues were cut into 5-μm-thick sections and stained with primary antibodies at 4°C overnight followed by incubation with HRP-conjugated secondary antibodies (DAKO) for IHC or Alexa Fluor-conjugated secondary antibodies (Molecular Probes) for IF at room temperature for 1 hour. Immunoreaction products of IHC were visualized by the DAB chromogen.
system (DAKO). Fluorescence imaging was performed using a laser scanning confocal microscope (Fluoview FV1000). The signals were quantified using the Tissue-Quest software (TissueGnostics GmbH). The percentage of protein staining in tumor specimen was classified into two staining grades according to the mean value of protein expression (the high grade represents ≥ mean; the low grade represents < mean).

Protein extraction and Western blotting
Cell pellets and homogenized tissues were lysed with M-PER buffer (Pierce) containing protease inhibitor cocktail (Roche) at 4°C for 30 minutes. After centrifuged at 14,000 rpm at 4°C for 30 minutes, the supernatant was collected and stored at −80°C. Protein concentration was determined by the BCA protein assay (Pierce). For Western blotting, 30 μg of protein was heated at 95°C for 5 minutes with sample buffer. The samples were loaded onto SDS-polyacrylamide gels for electrophoresis and then transferred to PVDF membrane (Millipore). After blocking with 5% non-fat milk at room temperature for 1 hour, the membrane was incubated with primary antibodies at 4°C overnight followed by incubation with secondary antibodies at room temperature for 1 hour. The blot signals were developed using Immobilon Western Chemiluminescent HRP Substrate (Millipore), captured by the Biospectrum Imaging System (UVI), and quantified by the Gel-Pro Analyzer software (MediaCybernetics). GAPDH and β-actin were used as loading controls for tumor tissues and cell lines, respectively.

RNA preparation and quantitative real-time polymerase chain reaction (qPCR)
Total RNA extraction from gastric cancer cell lines or tissues was performed using the Total RNA Miniprep Purification Kit (GeneMark Technology) according to the manufacturer’s protocol. One microgram of total RNA was reverse transcribed for each cDNA. First-strand cDNA was synthesized using Oligo-dT primers (Promega) and M-MLV reverse transcriptase (Promega) and was used as a template for analysis of gene expression by qPCR. For qPCR, 1 μL cDNA template was added to 9 μL of mixed reagent (1 μL forward primer (10 μmol/L), 1 μL reverse primer (10 μmol/L), 5 μL 2X GoTaq qPCR Master Mix (Promega), and 2 μL nuclease-free water) in a 96-well plate (Roche). The sequences of qPCR primers were as follows: GSN forward primer, 5′-TTACCGTGCTGCAGGCTGATGACTACTA-T3′, GSN reverse primer, 5′-CCTGTTTGGCTGCTGGATTTCC-3′, IL1β forward primer, 5′-TACCTGTCGCTGCGTGGATGA-3′, IL1β reverse primer, 5′-TCTTTGGTAATTTTTGGCCTCTT-3′, IL10 forward primer, 5′-GATGCCCTTCCAGCAGATGTGAA-3′, IL10 reverse primer, 5′-GCAACCCAGGTACCCCTTAAA−3′, CCL2 forward primer, 5′-AGTCTCTGCCGGCCCTTCTT-3′, CCL2 reverse primer, 5′-GTGACCGGAGGTATTTGTTGG-3′, GAPDH forward primer, 5′-GGGGCTCTCCCAGAACATCAT-3′, GAPDH reverse primer, 5′-GTCGTTGAGGGCAATGCCAG-3′, ACTB (β-actin) forward primer, 5′-CTCTACCGCAACACAGTGGC-3′, and ACTB reverse primer 5′-ATACCTGCTGCTGCTGATCC-3′. The reaction was performed with a LightCycler 480 Real-Time PCR system (Roche), and the protocol was set as follows: an initial denaturation at 95°C for 10 minutes followed by 50 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 30 seconds. The length of the extension step in the final cycle was increased to 10 minutes, and the final temperature was held at 4°C. GAPDH and β-actin were used as internal controls for tumor tissues and cell lines, respectively.

DNMT1 activity assay
DNMT1 activity was measured using a colorimetric DNMT Activity/Inhibition Assay Kit (Active Motif). The assay was initiated by adding 10 μg of nuclear protein extracts to a cytosine-rich DNA substrate coated ELISA plate. After incubation at 37°C for 2 hours, DNA methylation was detected with anti-methyl CpG binding domain 2b antibodies and quantified by reading the absorbance in a microplate spectrophotometer (BioTek) at 450 nm. Enzyme activity was calculated using the following formula: DNMT activity (OD/h/μg) = (Sample OD − Blank OD)/(10 μg × 2 hour).

Migration assay
Cell migration assays were performed using 8-μm pore size transwells (Corning). The lower chamber was filled with 0.6 ml of growth medium containing 20 μg/ml fibronectin (Sigma). Cells (1 × 105/well) were seeded in the upper chamber containing serum-free medium and were incubated at 37°C for 24 hours. Cells remaining in the upper side of the membrane were removed. Cells migrating to the bottom of the membrane were fixed with 4% paraformaldehyde for 20 minutes and stained with hematoxylin (MERCK). After being cut out and mounted, the membrane was viewed underneath an inverted microscope and the cells that have reached the underside of the membrane were counted in 10 randomly chosen fields of view to get an average sum of migrating cells.

Cytokine array
Gastric cancer AGS cells were monocultured or cocultured with U937 cells for 3 days. The conditioned medium was collected and centrifuged to remove debris. The supernatants (500 μL) were used for cytokine detection using the Human Cytokine Array Panel A Array Kit (R&D) according to the manufacturer’s instruction.

Xenograft animal model
AGS (1 × 106) and U937 (1 × 106) cells were subcutaneously coinjected into 6-week-old male nude mice, which were obtained from the NCKLI Laboratory Animal Center (Tainan, Taiwan) with the approval of the Institutional Animal Care and Use Committee of NCKLI. Tumor volume in mm3 was calculated by the formula: volume = width2 × length/2. Intratumoral injection of drugs began when tumor volume reached 125 mm3. Tumor-bearing mice were treated with 5-AZA (2.5 mg/kg), PCA (250 mg/kg), and maraviroc (10 mg/kg) twice weekly. After 24 days of treatment, the tumors were collected for further analysis.

Statistical analysis
Values are expressed as mean ± SEM of triplicate determinations from three independent experiments. Statistical analysis of data was performed with the Student t test and ANOVA. The survival probability was calculated with the Kaplan–Meier method. P values less than 0.05 were considered to be statistically significant (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

Results
GSN was downregulated in human gastric cancer cells
To determine whether GSN is downregulated in gastric cancer cells, we analyzed GSN expression in human gastric cancer tissues by IHC staining. GSN was widely expressed in the early-stage
Figure 1.

GSN is downregulated in human gastric cancer. 

A, IHC staining of GSN was performed on the early-stage and the advanced-stage gastric tumors. Magnification: 100× (top). Images on the lower panel are high-magnification (400×) of areas outlined by black squares. 

B, GSN expression in 3 paired gastric normal and tumor specimens was analyzed by Western blotting (left) and qPCR (right). GSN mRNA level was calculated as fold change in gastric tumors relative to their corresponding normal tissues, normalized to GAPDH. N: corresponding normal tissue; T: tumor tissue; *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus respective corresponding normal tissue, Student t test. 

C, GSN expression in human gastric cancer cell lines was measured by Western blotting (left) and qPCR (right). GSN mRNA expression was calculated as fold change in the gastric cancer cell lines relative to the normal gastric cell line GES-1, normalized to β-actin. *, P < 0.05; ***, P < 0.001 versus GES-1 cells, one-way ANOVA. 

D, The gray box represents CpG islands of the GSN promoter. Regions analyzed by MSP and pyrosequencing were indicated by bidirection arrows. Four CG units containing two predicted SP1 binding sites were targets for pyrosequencing. 

E, Methylation levels in the GSN promoter were analyzed by MSP with unmethylated (U) and methylated (M) primers. 

F, Methylation levels of the four CG units in CpG islands of the GSN promoter were analyzed by pyrosequencing.
tumors but not in the advanced-stage tumors (Fig. 1A). Grading according to the percentage of GSN staining in the 121 gastric tumors showed that high GSN expression more frequently occurred in the early-stage gastric cancer compared with the advanced-stage gastric cancer (Supplementary Table S1). We next analyzed GSN expression in 3 matched pairs of normal and tumor tissues (GC1, GC2, and GC3) and 6 gastric cancer cell lines. Both protein and mRNA levels of GSN were lower in the tumor parts than in their corresponding noncancerous parts (Fig. 1B). Most of the gastric cancer cell lines also showed decreased expression of GSN protein and mRNA as compared with the SV40-immortalized human normal gastric epithelial cell line GES-1 (Fig. 1C). The results suggest that GSN is transcriptionally suppressed in gastric cancer cells.

The decrease in GSN expression was mediated by promoter methylation

Because GSN expression can be epigenetically regulated, we analyzed methylation status of the GSN promoter (Fig. 1D). MSP assay showed that CpG island methylation in the GSN promoter could be frequently detected in gastric cancer cells, particularly AGS and AZ521 cells (Fig. 1E). Using pyrosequencing, we also observed methylation in four CpG dinucleotides within the GSN promoter region containing two predicted binding sites of specific protein 1 (SP1), a transcription activator of GSN (26), confirming the MSP results (Fig. 1F). To determine the effect of methylation on GSN expression, gastric cancer cells were treated with the demethylating agent 5-AZA. We observed that 5-AZA reduced methylation of the GSN promoter, specifically the four CpG

Figure 2.
DNA demethylation reactivates GSN expression in gastric cancer cells. Six gastric cancer cell lines were treated with 5-AZA for 24 hours. A, Methylation status of the GSN promoter was evaluated by MSP using unmethylated (U) and methylated (M) primers. B, Methylation levels of four CG units in CpG islands in the GSN promoter were analyzed by pyrosequencing. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus controls, two-way ANOVA. C, GSN protein expression was detected by Western blotting analysis. β-actin was used as a loading control. D, GSN mRNA expression was analyzed by qPCR. Data were normalized relative to the untreated control. *, P < 0.05; **, P < 0.01 versus controls, two-way ANOVA.
dinucleotides within the region containing two SP1 binding sites (Fig. 2A and B). Western blotting and qPCR showed that 5-AZA-mediated demethylation could restore GSN protein and mRNA expression (Fig. 2C and D), suggesting that promoter methylation is a cause of GSN silencing.

**Low GSN expression and high DNMT1 expression predicted poor survival**

DNMT1, which directs DNA methylation in adult somatic cells, is overexpressed in various cancers (24). To identify the clinical correlation between GSN and DNMT1, IHC staining for DNMT1 in 121 human gastric tumors was performed. Higher DNMT1 expression was observed more often in the advanced-stage tumors than in the early-stage tumors (Fig. 3A and Supplementary Table S2). Double IF staining for GSN and DNMT1 in tumor tissues showed that GSN was frequently detected in areas without DNMT1 expression but rarely in areas exhibiting abundant DNMT1 (Fig. 3B), suggesting an inverse correlation between GSN and DNMT1 expression in gastric cancer. To correlate GSN and DNMT1 expression status with clinical outcomes, Kaplan-Meier analysis was performed. The median survival times for the groups of DNMT1LowGSNHigh, DNMT1LowGSNLow, DNMT1HighGSNHigh, and DNMT1HighGSNLow were estimated to be 121, 68, 51, and 34 months, respectively (Fig. 3C), indicating that low GSN expression in combination with high DNMT1 expression conferred the worst prognosis in gastric cancer patients.

**DNMT1 mediated transcriptional repression of the GSN gene**

To confirm that DNMT1 is responsible for GSN silencing, we knocked down DNMT1 in AGS and AZ521 cells and established stable clones. We showed that DNMT1 expression was silenced

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**Figure 3.**

GSN and DNMT1 expression are inversely correlated in a pattern associated with patient survival. **A**, IHC staining of DNMT1 was performed in early-stage and advanced-stage gastric cancer. Magnification: 100× (top). Images on the lower panel are high-magnification (400×) of the areas outlined by black squares. **B**, IF staining of GSN (green) and DNMT1 (red) was performed in early-stage and advanced-stage tumors. Original magnification: 100× (top). Images on the lower panel are high-magnification (400×) of areas outlined by white squares. **C**, Kaplan-Meier survival curves showed that the DNMT1HighGSNLow group had the worst patient survival than the other groups (P = 0.0406).
Figure 4.
Inactivation of DNMT1 increases GSN transcription. A, GSN expression in both AGS and AZ521 cells permanently expressing shRNA against DNMT1 (shDNMT1) and luciferase (shLuc) was detected by Western blotting. β-actin was used as a loading control. B, GSN mRNA expression in the knockdown clones was measured by qPCR and is depicted as fold changes relative to parental cells. **P < 0.01, significant differences between groups, two-way ANOVA. C, Cell motility of the knockdown clones was determined by transwell migration assay. The bar graph represents the fold changes in the number of migrating cells relative to parental cells. Original magnification: 100×. ***P < 0.001, significant differences between groups, two-way ANOVA. D, AGS and AZ521 cells were treated with PCA at the indicated concentrations. Total methyltransferase enzyme activity was detected by the DNMT activity assay. The percentages of enzyme activity were calculated relative to untreated control cells. *, P < 0.05; ***, P < 0.001 versus controls, one-way ANOVA. E and F, After PCA (10 mmol/L) treatment, GSN protein and mRNA levels were analyzed by Western blotting and qPCR, respectively. Fold changes in mRNA expression were calculated relative to untreated control cells. *, P < 0.05; **, P < 0.01 versus controls, one-way ANOVA.
Figure 5.

Coculture with U937 cells enhances DNMT1 expression in gastric cancer cells. A, The M2 marker CD204 (left) and the M1 marker CD86 (right) expression in early- and advanced-stage gastric cancer was analyzed by IHC staining. Magnification: 100× (top). Images on the bottom panel are high-magnification (400×) of areas outlined by black squares. (Continued on the following page.)
by RNAi in stable knockdown cell lines. AGS

DNMT1 and AZ521

DNMT1 downregulation increased both protein and mRNA expression of GSN in AGS and AZ521 cells (Fig. 4A and B). Because GSN may function as a metastasis suppressor, the cell migration assay was performed to determine whether DNMT1 suppresses GSN expression and thus affects cell motility. As compared with control cells, cell migration of the DNMT1-knockdown cells was reduced by over 50% in AGS and AZ521 cells (Fig. 4C).

In addition to genetic inhibition, we also examined effect of pharmacological inhibition of DNMT1 activity on GSN expression. After treatment with PCA, an inhibitor specific to DNMT1 rather than other DNMTs (27), at various concentrations, total DNMT activity in AGS and AZ521 cells was reduced in a dose-dependent manner (Fig. 4D). At the dose of 10 mmol/L, PCA increased both mRNA and protein expression of GSN (Fig. 4E and F). Collectively, our results demonstrated that DNMT1 contributes to transcription inhibition of the GSN gene in gastric cancer cells.

Infiltration of TAMs correlated with DNMT1 expression in tumor tissues

Increased DNMT1 expression has been associated with DNA hypermethylation in inflammation-induced carcinogenesis (28), suggesting that inflammation may affect DNMT1 expression. Because TAMs represent the most abundant inflammatory cells in the tumor microenvironment, we determined the role of TAMs in DNMT1 upregulation in gastric cancer cells. IHC staining for the putative M2 marker CD204 (29) and the macrophage marker CD163 in 121 human gastric cancer samples showed that M2 TAMs were detected in both the early- and advanced-stage gastric tumors (Supplementary Tables S3 and S4), but the density of M2 TAM infiltration was higher in the advanced-stage tumors than in the early-stage tumors (Fig. 5A and Supplementary Fig. S1). By contrast, IHC staining for the M1 marker CD86 did not show a difference in M1-polarized macrophage levels between the early- and advanced-stage gastric tumors (Fig. 5A). Double IF staining for CD204 and DNMT1 revealed that DNMT1-expressing tumor cells were surrounded by TAMs in close juxtaposition (Fig. 5B), suggesting a positive correlation between TAM infiltration and DNMT1 expression in gastric cancer.

M2-like macrophages suppressed GSN expression by upregulating DNMT1

We next utilized an in vitro coculture system of gastric cancer cells and human leukemic monocyte lymphoma U937 cells that can differentiate into mature macrophages (30) to clarify whether DNMT1 in gastric cancer cells is upregulated by TAMs (Supplementary Fig. S2A). Coculture with AGS or HR cells increased CD204 protein expression by 2-fold in U937 cells (Supplementary Fig. S2B). In addition to surface marker detection, changes in mRNA expression of M1 and M2 cytokines were also evaluated by qPCR. After cocultured with gastric cancer cells, mRNA expression of interleukin 1β (IL1β), a cytokine released by M1 macrophages (31), was decreased in U937 cells (Supplementary Fig. S2C). By contrast, mRNA expression of interleukin 10 (IL10) and chemokine (C-C motif) ligand 2 (CCL2), two definite M2 cytokines (31), was increased in U937 cells cocultured with gastric cancer cells (Supplementary Fig. S2C). These results reveal that coculture with gastric cancer cells enables U937 cells to differentiate into M2-like macrophages.

We further analyzed DNMT1 and GSN expression in gastric cancer cells after coculture with M2-like macrophages. DNMT1 protein expression in AGS and HR cells was induced by M2-like macrophages (Fig. 5C). In contrast to DNMT1, GSN was downregulated in both mRNA and protein levels after M2-like macrophage coculture (Fig. 5C), suggesting that M2-like macrophages reduced GSN expression via transcription inhibition. To clarify whether M2-like macrophages attenuate GSN expression by increasing DNMT1 expression, DNMT1 knockdown AGS

DNMT1 cells were applied to be cocultured with U937 cells. M2-like macrophage coculture decreased GSN expression, whereas this effect was prevented by DNMT1 knockdown (Fig. 5D), suggesting that DNMT1 is required for M2-like macrophages-mediated downregulation of GSN. Gastric cancer cell migration induced by M2-like macrophages was also retarded when DNMT1 was knocked down (Fig. 5E). In addition to protein expression, M2-like macrophages enhanced DNMT1 enzyme activity. M2-like macrophages-induced DNMT1 activity and M2-like macrophage-mediated GSN suppression could be counteracted by the DNMT1 inhibitor PCA (Fig. 5F). Taken together, these results demonstrate that M2-like macrophages inhibit transcription of GSN through DNMT1-dependent methylation.

CCL5 secreted by M2-like macrophages stimulated DNMT1 expression

To identify paracrine factors that contribute to DNMT1 upregulation in gastric cancer cells, the coculture medium was collected for cytokine arrays. Levels of four chemokines CCL2, CCL5, IL8, and CXCL10 were increased in the coculture medium of AGS and U937 cells compared with the monoculture medium of AGS cells (Supplementary Fig. S3A). After treatment with these 4 chemokines in 7 gastric cancer cell lines, only CCL5 increased DNMT1 expression in almost all of the cell lines (Supplementary Fig. S3B). In AGS cells and HR cells, CCL5 induced DNMT1 expression in a dose- and time-dependent manner (Fig. 6A and B). To confirm whether CCL5 is the effector of M2-like macrophage-mediated DNMT1 upregulation, CCL5-neutralizing antibodies were used in the coculture. Induction of DNMT1 was blocked by CCL5 neutralization (Fig. 6C). CCL5 interacts with CCR1, CCR3, and CCR5 (32). In gastric cancer, the CCL5/CCR5 axis seems to be associated with tumor growth and metastasis.
Figure 6.
TAMs upregulate DNMT1 in gastric cancer cells through the CCL5/CCR5/STAT3 pathway. A, AGS cells and HR cells were treated with CCL5 at different concentrations for 24 hours. DNMT1 expression was measured by Western blotting. Graphs below each blot show the quantification of DNMT protein. \( P < 0.05 \) versus untreated cells, one-way ANOVA. B, After treatment with CCL5 at the fixed dose of 10 ng/mL for the indicated times in AGS cells and HR cells. DNMT1 expression was analyzed by Western blotting and was quantified. \( P < 0.05; P < 0.01 \) versus the 0 hour time point, one-way ANOVA. C, During coculture with U937 cells for 72 hours, AGS cells and HR cells were treated with neutralizing CCL5 antibodies (\( \alpha \)-CCL5). DNMT1 expression was analyzed by Western blotting and was quantified. \( P < 0.05; P < 0.01 \), significant differences between groups, one-way ANOVA. (Continued on the following page.)
Therefore, to verify whether CCL5 induces DNMT1 expression by binding to CCR5, the CCR5 antagonist maraviroc was used. The result showed that treatment with maraviroc prevented DNMT1 upregulation by CCL5 stimulation (Fig. 6D), suggesting the requirement of CCR5 for CCL5-induced DNMT1 expression. Given that the JAK2/STAT3 signaling pathway is downstream of CCL5 and also an inducer of DNMT1 gene transcription (35–37), we next determined the involvement of the JAK2/STAT3 pathway in CCL5-mediated DNMT1 upregulation. The result showed that CCL5 could stimulate tyrosine phosphorylation of STAT3 in AGS and HR cells (Fig. 6E), whereas this effect could be prevented by maraviroc and the Jak2 inhibitor AG490 (Fig. 6F). Also, upregulation of DNMT1 induced by CCL5 could be abrogated by AG490 (Fig. 6G). Collectively, we demonstrate that M2-like macrophage-secreted CCL5 can induce DNMT1 expression by activating the JAK2/STAT3 pathway.

**Pharmacological antagonism of DNMT1 inhibits gastric tumor growth**

To confirm the in vitro results, we performed animal studies to observe gastric tumor growth after inhibiting DNMT1 or CCL5 signaling. We found that the final tumor size of AGS xenograft tumors receiving 5-AZA, PCA, or maraviroc was smaller than that of the control group (Fig. 6H). Treatment with 5-AZA, PCA, or maraviroc slowed tumor growth, revealing the anti-tumor effects of DNMT1 suppression in gastric cancer (Fig. 6H, right). IHC and qPCR results confirmed that GSN could be reexpressed after all the treatments and DNMT1 expression was inhibited by maraviroc in the tumor cells (Fig. 6I).

**Discussion**

GSN, downregulated in various cancers such as ovarian cancer, skin cancer, breast cancer, and bladder cancer, is a tumor suppressor (11, 13–15). In breast and ovarian cancer, loss of GSN is likely caused by an epigenetic-related mechanism (15, 16); however, ubiquitin-mediated degradation may decrease GSN expression in pancreatic cancer (38), suggesting that GSN dysregulation is a convergent consequence from different mechanisms in various cancers. In this study, we have provided evidence that GSN is downregulated in gastric cancer cells, and promoter DNA methylation is involved in this process. The binding sites of the transcription activator SP1 within Cpg islands of the GSN promoter were found to be excessively methylated, which may disturb the binding of SP1 to the GSN promoter. In addition, we also demonstrated that TAM-secreted CCL5 mediates GSN promoter hypermethylation by upregulating DNMT1 in gastric cancer cells.

Aberrant methylation has been associated with chronic inflammation, suggesting that inflammatory cells in the tumor microenvironment play a role in DNA methylation dysregulation (18, 19). In this study, high density of TAMs was found in DNMT1-expressing gastric tumor tissues. Coculture of U937 and gastric cancer cells showed that U937 cells could differentiate into M2-like macrophages, in agreement with previous findings showing the influence of the tumor microenvironment on macrophage polarization (39). On the other hand, reduced GSN transcription and increased DNMT1 protein expression was observed in gastric cancer cells after coculture. Inhibiting DNMT1 by shRNA or its inhibitor PCA blocked coculture-mediated GSN downregulation, demonstrating that M2-like macrophages can mediate GSN silencing in gastric cancer cells by increasing DNMT1 expression and activity.

Besides DNMT1, other factors may also influence GSN silencing. DNMT3a and DNMT3b not only catalyze de novo methylation but also regulate DNMT1 activity in cancer (24, 40, 41). Histone-modifying enzymes that mediate epigenetic histone modifications and DNA methyltransferases can work synergistically to regulate target gene expression (41). Not only demethylating agents but also histone deacetylase (HDAC) inhibitors can restore GSN expression in human cancer cell lines (26, 42, 43). Taken together, the previous findings indicate that GSN silencing may not be simply mediated only by DNMT-induced DNA methylation. Further studies are needed to determine the DNMT1-independent mechanisms responsible for GSN repression in gastric cancer.

Our results showed that decreased GSN expression in gastric cancer cell cocultured with M2-like macrophages enhanced gastric cancer cell motility, whereas GSN restoration after DNMT1 knockdown prevented this effect. Genes encoding other tumor suppressors, for example, E-cadherin, can be methylated by DNMT1 (44). DNMT1 may promote cancer progression through methylation of multiple tumor suppressors. Therefore, inhibiting DNMT1 such that expression of tumor suppressor genes is permitted may be a reasonable strategy for cancer treatment. A previous study has reported that inoculation of AGS cells alone into nude mice was insufficient to develop tumors (45); however, we found that coinjection with U937 cells enhanced the tumorigenicity of AGS cells. In this study, we found that antagonizing M2-like macrophages-induced DNMT1 activity with PCA attenuated gastric tumor formation. These results demonstrate that DNMT1 is required for inflammation-associated gastric carcinogenesis and that inactivation of DNMT1 represents an option for antitumor therapy.

In conclusion, we provide mechanistic insight into how inflammation facilitates gastric cancer progression (Fig. 6I). TAMS...
increase DNMT1 expression in gastric cancer cells through the CCL5/CCR5/STAT3 signaling pathway. DNMT1 upregulation mediates G2/N silencing and thus potentiates gastric cancer progression. These findings identify potential therapeutic targets for inflammation-driven tumorigenesis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: H.-C. Wang, C.-W. Chen, Y.-S. Shan
Development of methodology: C.-W. Chen, C.-L. Yang, I.-M. Tsai, Y.-C. Hou, Y.-S. Shan
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.-W. Chen, Y.-S. Shan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H.-C. Wang, C.-W. Chen, C.-L. Yang, I.-M. Tsai, Y.-C. Hou, C.-J. Chen, Y.-S. Shan
Writing, review, and/or revision of the manuscript: H.-C. Wang, C.-W. Chen, C.-L. Yang, C.-J. Chen, Y.-S. Shan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.-C. Wang, C.-W. Chen, Y.-S. Shan
Study supervision: C.-W. Chen, Y.-S. Shan

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