Human Cancer Biology

GKN1–miR-185–DNMT1 Axis Suppresses Gastric Carcinogenesis through Regulation of Epigenetic Alteration and Cell Cycle

Jung Hwan Yoon¹, Yoo Jin Choi¹, Won Suk Choi¹, Hassan Ashktorab², Duane T. Smoot², Suk Woo Nam¹, Jung Young Lee¹, and Won Sang Park¹

Abstract

Purpose: Gastrokine 1 (GKN1) functions to protect the gastric antral mucosa and promotes healing by facilitating restoration and proliferation after injury. GKN1 is downregulated in Helicobacter pylori–infected gastric epithelial cells and loss of GKN1 expression is closely associated with gastric carcinogenesis, but underlying mechanisms of the tumor-suppressing effects of GKN1 remain largely unknown.

Experimental Design: AGS, MKN1, MKN28 gastric cancer cells and HFE-145 immortalized non-neoplastic gastric mucosal cells were transfected with GKN1 or shGKN1. We conducted molecular and functional studies of GKN1 and miR-185 and investigated the mechanisms of alteration. We also analyzed epigenetic alterations in 80 gastric cancer tissues.

Results: Restoration of GKN1 protein suppressed gastric cancer cell growth by inducing endogenous miR-185 that directly targets epigenetic effectors DNMT1 and EZH2 in gastric cancer cells. In addition, ectopic expression of GKN1 upregulated Tip60 and downregulated HDAC1 in an miR-185–independent manner, thereby inducing cell-cycle arrest by regulating cell-cycle proteins in gastric cancer cells. Notably, GKN1 expression was inversely correlated with DNMT1 and EZH2 expression in a subset of 80 gastric cancer tissues and various gastric cancer cell lines. Interestingly, it was found that GKN1 exerted a synergistic anticancerous effect with 5-fluorouracil on tumor cell growth, which suggests a possible therapeutic intervention method for gastric cancer.

Conclusion: Our results show that GKN1 has an miR-185–dependent and -independent mechanism for chromatic and DNA epigenetic modification, thereby regulating the cell cycle. Thus, the loss of GKN1 function contributes to malignant transformation and proliferation of gastric epithelial cells in gastric carcinogenesis.

Clin Cancer Res; 19(17); 4599–610. ©2013 AACR.

Introduction

Recently, gastrokine 1 (GKN1) was isolated from the gastric mucosa cells of several mammalian species including rats (1). GKN1 is a novel autocrine/paracrine protein that is specifically expressed in gastric mucosa (1, 2). Toback and colleagues reported that GKN1 protects the antral mucosa and promotes healing by facilitating restitution and proliferation after injury (3). Interestingly, GKN1 is downregulated in Helicobacter pylori–infected gastric epithelial cells, and the loss of GKN1 expression is detected in gastric cancer tissues and precancerous lesions such as intestinal metaplasia (4, 5). We also witnessed frequent loss of GKN1 expression in gastric cancers and confirmed tumor suppressor activity of GKN1 through a functional analysis (6). Moreover, GKN1 plays an important role in the epithelial–mesenchymal transition (EMT) and migration of gastric cancer cells (7). Here, we hypothesized that GKN1 plays an important role in cell-cycle progression. This hypothesis is supported by the recent finding that GKN1 inhibits cell growth by inducing G2–M arrest in SGC-7901 cells (8). However, the molecular mechanism through which GKN1 inhibits the cell cycle is still unknown.

Thus, we focused on the effects of GKN1 on cell viability and proliferation, cell cycle, and epigenetic alteration of the cell-cycle–related proteins in AGS, MKN1 and MKN28 gastric cancer, and HFE-145–immortalized non-neoplastic gastric epithelial cells. Overall, we showed that GKN1 plays an important role in the cell-cycle regulation and the epigenetic alterations of tumor-associated genes in gastric cancers.

Authors' Affiliations: ¹Department of Pathology, College of Medicine, The Catholic University of Korea, Seoul, South Korea; and ²Department of Medicine, Howard University, Washington, District of Columbia

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

Corresponding Author: Won Sang Park, Department of Pathology, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Seicho-gu, Seoul 137-701, South Korea. Phone: 82-2-590-1192; Fax: 82-2-537-6586; E-mail: wonsang@catholic.ac.kr

doi: 10.1158/1078-0432.CCR-12-3675

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Translational Relevance

In cancer cells, treatment with inhibitors of epigenetic modification can reactivate epigenetically silenced genes and restore normal gene function. Our extensive investigation provides evidence that gastrokine 1 (GKN1) acts as a tumor suppressor by downregulating epigenetic modifications and cell-cycle progression, and that it has a synergistic cytotoxic effect with 5-fluorouracil. We also show that GKN1 plays a key role in epigenetic modification and cell proliferation in an miR-185-dependent and -independent manner. Thus, there is potential for GKN1 and miR-185 to be novel therapeutic agents for gastric cancer therapy.

Materials and Methods

Cell culture and transfection of GKN1 and shGKN1

AGS, MKN1 and MKN28 gastric cancer cells, and HFE-145-immortalized non-neoplastic gastric epithelial cells were obtained from the American Type Culture Collection and Dr. Hassan Ashktorab (Howard University, Washington, DC). GKN1 cDNA and shGKN1 were cloned into the pcDNA3.1 expression vector and pSilencer 3.1 H1-neo (Invitrogen). AGS, MKN1, MKN28, and HFE-145 cells were transfected in 60-mm-diameter dishes with the expression plasmids (2 μg total DNA) using Lipofectamine Plus transfection reagent (Invitrogen) according to the manufacturer’s recommendations.

Measurement of cell viability, proliferation, and colony formation

For cell viability analysis, MTT assay was conducted at 24, 48, and 72 hours after transfection of each construct, as described previously. To further examine whether GKN1 contributes to the chemosensitivity of 5-fluorouracil (5-FU), MTT assay was conducted on AGS and HFE-145 cells at 24 and 48 hours after simultaneous treatment with GKN1 and in HFE-145 cells after transfection with shGKN1, as described previously. After blocking, the membrane was subsequently probed with antibodies against G0–G1 phase proteins and G2–M phase proteins. Protein bands were detected using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

Expression of cell-cycle regulators

Expression of G0–G1 phase proteins, including p53, p21, p16, p15, CDK4, cyclin D1, E2F, and G2–M phase proteins, including cdc2, cyclin B, cdc25A, cdc25C, aurora A, and Plk1, was examined in AGS cells 24 and 48 hours after transfection with GKN1 and in HFE-145 cells after transfection with shGKN1, as described previously. After blocking, the membrane was subsequently probed with antibodies against G0–G1 phase proteins and G2–M phase proteins. Protein bands were detected using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

Expression of epigenetic regulators

We also analyzed the expression levels of EZH2, DNMT1, HDAC1, and Tip60, which are involved in the epigenetic process, in AGS, MKN1, and MKN28 cells 24 and 48 hours after transfection with GKN1 and in HFE-145 cells transfected with shGKN1. Expression of DNMT1 and EZH2 was also examined in 80 frozen advanced gastric cancer and corresponding non-cancerous gastric tissues, HFE-145 cells, and 10 gastric cancer cell lines by Western blot analysis. After blocking, the membrane was subsequently probed with anti-DNMT1, anti-EZH2, anti-methyl histone H3, and anti-HDAC1 monoclonal antibody (BD bioscience). Approval was obtained from the Institutional Review Board of The Catholic University of Korea, College of Medicine (CUMC09U089).

Measurement of DNMT1, EZH2, CDKN2A and miRNA-185 expression

Real-time reverse transcription (RT)-PCR was carried out using SYBR Green Q-PCR Master Mix (Stratagene) according to the manufacturer’s instructions. DNMT1, EZH2, and CDKN2A mRNAs were quantified by SYBR Green quantitative PCR (qPCR) and normalized to mRNA of the housekeeping gene GAPDH. To investigate whether GKN1 directly regulates miR-185 expression, expression of primary and precursor miR-185 was also quantified by qPCR and normalized to human U6 snRNA. Data are reported as relative quantities according to an internal calibrator using the 2−ΔΔCT method. Sequences of the primers are described in Supplementary Table S1.

Measurement of DNMT1 activity

The DNMT1 activity was analyzed using the DNMT1 Activity Assay Kit (Abcam) according to manufacturer’s instructions. Briefly, the cell pellet was lysed in lysis buffer (10 mmol/L Tris-Hcl pH 7.5, 10 mmol/L NaCl, and 2 mmol/L MgCl2) containing protease inhibitor mixture (Complete; Roche Molecular Biochemicals). Then, 6 μL of 20% NP-40 was added, and the mixture was incubated for 10 minutes at 4°C and centrifuged for 5 minutes. The supernatant was collected and the pellet

Flow cytometric analysis of the cell cycle

For cell-cycle analysis, AGS cells from each experimental group were collected and stained with propidium iodide (PI) for 45 minutes in the dark before analysis.

The percentages of cells in different phases of the cell cycle were determined using a FACSCalibur Flow Cytometer with CellQuest 3.0 software (BD Biosciences).
containing the nuclei was resuspended in 50 μL of extraction buffer (20 mmol/L HEPES, pH 7.9, 420 mmol/L NaCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, and 10% glycerol) followed by incubation for 30 minutes at 4°C and collection of the nuclear extract by centrifugation.

**Bisulfite genomic sequencing**

Methylation status of the promoter region of the p16 and E-cadherin genes in AGS cells was determined using sodium bisulfite treatment of the DNA followed by bisulfite genome sequencing (BGS), as described previously (6). Bisulfite-treated DNA was amplified using primers for BGS (Supplementary Table S1), and sequencing of the PCR products was carried out using the Cyclic Sequencing Kit (Perkin-Elmer), according to the manufacturer’s recommendations.

**Statistical analysis**

The Student t test was used to analyze the effect of GKN1 on cell viability and proliferation. All experiments were carried out in triplicate to verify the reproducibility of the findings. Data are expressed as means ± SD from at least 3 independent experiments. Association between GKN1, miR-185, DNMT1, and EZH2 expression and the tumor histologic type was tested using χ² and Spearman correlation tests. P < 0.05 was considered the limit of statistical significance.

**Results**

**GKN1 reduces cell viability, proliferation, and colony formation**

When we transfected AGS, MKN1, and MKN28 cells with GKN1, we witnessed a significant time-dependently induced decrease in cell growth and proliferation (Fig. 1A and B and Supplementary Fig. S1). In contrast, when we silenced GKN1 with shGKN1 in HFE-145 cells with GKN1 expression, we found a time-dependent increase in cell growth and proliferation (Fig. 1A and B). In the colony formation assay, GKN1 dramatically reduced the number and size of surviving colonies in the AGS gastric cancer cell lines compared with the empty vector–transfected control cells (mock; Fig. 1C). In addition, treatment with 5-FU induced a decrease in time- and dose-dependent cell viability in both cell lines (Fig. 1D and E). AGS cells treated with GKN1 and recombinant GKN1 protein showed synergistic inhibition of cell viability with 5-FU in a dose- and time-dependent manner (Fig. 1F). In flow cytometric analysis, treatment with 5-FU alone had no effect on the cell cycle, whereas AGS cells treated with 5-FU and GKN1 or recombinant GKN1 protein showed increased numbers of sub-G₀-G₁ cells and indicated a modest effect on the cell-cycle G₀-G₁ and G₂-M phase progression (Fig. 1G). Furthermore, the exogenous GKN1 expression synergistically induced increased expression of p53 and p21 and decreased expression of cyclin B and D in AGS cells (Fig. 1H).

**GKN1 induced G₀-G₁ and G₂-M arrest**

Ectopic expression of GKN1 resulted in increased numbers of sub-G₀ cells and showed a modest effect on the cell-cycle G₀-G₁ and G₂-M phase progression (Fig. 2A).

For G₀-G₁ arrest in AGS cells, GKN1 induced re-expression of p16 and downregulated expression of Cdk4, cyclin D, E2F1, and cyclin A (Fig. 2B). As CDKN2A is hypermethylated in gastric cancers (10), we investigated methylation status of the promoter region of the CDKN2A gene in AGS cells after treatment of 5-aza-dC. Expectedly, 5-aza-dC induced demethylation of the CDKN2A promoter region and re-expression of the p16 protein (Supplementary Fig. S2). In shGKN1-transfected HFE-145 cells, GKN1 silencing downregulated expression of p16 and p21, but upregulated expression of positive cell-cycle regulators including CDK4 and cyclin A (Fig. 2B). For G₂-M arrest, GKN1 downregulated expression of p-cdc2, PLK1, CDK2, cdc25a, cdc25c, and cyclin B in AGS cells, whereas GKN1 silencing in HFE-145 cells upregulated expression of p-cdc2, PLK1, and cdc2 (Fig. 2C).

**GKN1 functions as an epigenetic regulator**

As shown in Fig. 3A, GKN1 induced Tip60 expression, which acetylated DNMT1 (11), and reduced the expression of DNMT1, EZH2, methyl histone H3, and HDAC1 in AGS cells (Fig. 3A). In MKN1 and MKN28 cells, GKN1 also reduced expression of DNMT1 and EZH2 (Supplementary Fig. S3). However, GKN1 silencing in HFE-145 cells upregulated expression of these proteins. When we examined the DNMT1 activity in GKN1-transfected AGS cells and in shGKN1-transfected HFE-145 cells, decreased and increased DNMT1 activity was observed, respectively (Fig. 3B). Moreover, GKN1 inhibited DNMT1 and EZH2 mRNA expression and increased CDKN2A mRNA expression in AGS cells (Fig. 3C). In addition, DNMT1, EZH2, and HDAC1 silencing in AGS cells by shDNMT1, shEZH2, and shHDAC1 also significantly reduced cell growth (Supplementary Fig. S4A), increased CDKN2A mRNA expression, and induced G₀-G₁ and G₂-M cell-cycle arrest (Supplementary Fig. S4B and S4C).

In human gastric cancer tissues, GKN1-negative cancers showed overexpression of DNMT1 and EZH2, whereas GKN1-positive cancers showed underexpression of these proteins (Fig. 3D). Sixty-nine (86%) of 80 gastric cancers showed decreased expression of GKN1 and increased expression of DNMT1 (79%) and EZH2 (84%) proteins, compared with samples from the corresponding normal gastric mucosa (Fig. 3E). Histologically, reduced GKN1 expression and overexpression of DNMT1 and EZH2 were closely associated with diffuse type and poorly differentiated gastric cancer (P < 0.05, Supplementary Table S2). Moreover, HFE-145 cells showed strong GKN1 protein expression, but 10 gastric cancer cell lines did not express GKN1. HFE-145 cells did not express DNMT1 and EZH2, whereas there was strong expression of both proteins in 10 gastric cancer cell lines (Fig. 3F). To further confirm our results, we recapitulated GKN1, DNMT1, and EZH2 gene
Figure 1. Tumor-suppressing and anti-cancer effects of GKN1. A, in MTT assay, GKN1-transfected AGS cells showed time-dependent inhibition of cell viability, whereas shGKN1-transfected HFE-145 cells showed an increase in cell viability. B, in BrdUrd incorporation assay, GKN1-transfected AGS cells
expression from the large cohorts of patients with gastric cancer that are available from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (accession numbers GSE27342). GKN1 gene expression was significantly and consistently downregulated, whereas DNMT1 and EZH2 gene expression was significantly upregulated, in gastric cancer cohorts (Fig. 3G).

We also examined whether GKN1 induces expression of miR-185, which directly targets DNMT1 (12, 13). A significantly increased level of miR-185 was detected in the AGS, MKN1, and MKN28 cells transfected with GKN1, but GKN1 silencing in HFE-145 cells significantly decreased miR-185 expression (Fig. 4A and Supplementary DNA counts).

Figure 2. GKN1 negatively regulates cell-cycle progression. A, cell-cycle arrest at G0–G1 and G2–M phases was found in AGS cells transfected with GKN1. B, ectopic GKN1 expression in AGS cells showed the re-expression of p16 and downregulation of the expression of CDK4, cyclin D, E2F1, and cyclin A. Knockdown of GKN1 in HFE-145 cells downregulated the expression of p16 and p21 but upregulated the expression of cell-cycle–positive regulators including CDK4 and cyclin A. C, for G2–M phase proteins, GKN1 downregulated expression of p-cdc2, PLK1, CDK2, cdc25a, cdc25c, and cyclin B in AGS cells, whereas GKN1 silencing in HFE-145 cells upregulated expression of p-cdc2, PLK1, and CDK2.

GKN1 inhibits DNMT1 expression by upregulating miR-185 expression

We also examined whether GKN1 induces expression of miR-185, which directly targets DNMT1 (12, 13). A significantly increased level of miR-185 was detected in the AGS, MKN1, and MKN28 cells transfected with GKN1, but GKN1 silencing in HFE-145 cells significantly decreased miR-185 expression (Fig. 4A and Supplementary DNA counts).
Figure 3. GKN1 negatively regulates expression of epigenetic regulators. A, GKN1 upregulated Tip60 and downregulated expression of DNMT1, EZH2, methyl histone H3, and HDAC1. B, GKN1 directly inhibited DNMT1 activity. C, GKN1 significantly downregulated DNMT1 and EZH2 and upregulated CDKN2A mRNA expression. D–F, an inverse association of GKN1 expression with DNMT1 and EZH2 in gastric cancer tissue (D and E) and cell lines (F) was observed. G, recapitulated GKN1, DNMT1, and EZH2 gene expression levels in the large cohort of patients with gastric cancer (NCBI GEO database, accession number GSE27342). The relative expression levels of GKN1, DNMT1, and EZH2 mRNA in noncancerous (Normal) and gastric cancer (Tumor) tissues are illustrated by scatter plot. The median expression level of each group is indicated by horizontal lines. Gene expression levels are shown on the ordinate (log2 intensity). The differential GKN1, DNMT1, and EZH2 expression for these 2 categories was determined by the unpaired t test ($P < 0.0001$; 2-tailed), respectively.
miR-185 is required for GKN1 tumor suppressor activity

Next, we investigated whether GKN1 activity is dependent on miR-185 expression. Treatment with anti-miR-185 in AGS, MKN1, and MKN28 cells transfected with GKN1 showed a moderate ablation of GKN1-induced growth-inhibitory activity (Fig. 5A and Supplementary Fig. S7) and cell-cycle arrest (Fig. 5B). Silencing of miR-185 with anti-miR-185 recovered expression of DNMT1, EZH2, and positive cell-cycle regulators and decreased expression of p16 (Fig. 5C). Also, miR-185 silencing in AGS cells transfected with GKN1 increased the number and size of surviving colonies, DNMT1 activity, and DNMT1 and EZH2 mRNA expression (Fig. 5D–F) and decreased CDKN2A mRNA expression (Fig. 6F).

GKN1 regulates methylation status of CDKN2A and E-cadherin

To determine whether GKN1 functions as a hypomethylating agent, we sequenced 218- and 417-bp fragments, containing 17 and 34 CpG dinucleotides, respectively, in the promoter and around the translation start sites of the E-cadherin (Bis-E-cadherin) and in the promoter of CDKN2A (Bis-CDKN2A; Fig. 6A and C). For the E-cadherin gene, 7 methylated CpG sites were completely unmethylated in GKN1-transfected cells, but treatment of GKN1 with anti-miR-185 reverted this hypomethylation. For the CDKN2A gene, 30 (88.24%) of 34 CpG islands were methylated in AGS cells. Ectopic GKN1 expression turned 26 methylated CpG sites into unmethylated CpG sites, whereas anti-miR-185 treatment inhibited the hypomethylating activity of GKN1 (Fig. 6B and D and Supplementary Fig. S8A and S8B). Thus, re-expression of E-cadherin and CDKN2A was induced by demethylation due to the suppression of EZH2 and DNMT1 through upregulation of Tip60 and decreased HDAC1 activity following GKN1 expression (Fig. 6E).

Discussion

In this study, we addressed the molecular mechanism underlying the tumor suppressor activity of GKN1 in gastric cancer. GKN1 significantly reduced cell viability, proliferation, and colony formation of AGS cells (Fig. 1). Treatment of GKN1 and 5-FU synergistically inhibited cell viability and proliferation by upregulating p53 and p21 (Fig. 1). The activation of p53 and p21 by 5-FU was previously reported in mouse L-TK and AGS cells (14, 15). The fact that GKN1 suppressed cell proliferation implies that GKN1 can modulate cell-cycle-regulating components. Negative cell-cycle regulators such as p15 and p21 are key modulators that suppress cyclin D1/CDK4, 6 or cyclin E/CDK2 complex in the G1–S transition (16). Here, we found a concomitant increase of G0–G1 phase and G2–M phase in GKN1-transfected cells (Fig. 2A). GKN1 selectively induced p16 re-expression and elicited concomitant suppression of CDK4, cyclin D1, and E2F expression in the G1–S transition (Fig. 2B). GKN1 also inactivated G2–M-phase progression in AGS cells by regulating the expression of cdck5, PLK1, and cyclin B (Fig. 2C). These findings are consistent with previous reports (2, 8) and strongly suggest that GKN1 functions as a gastric tumor suppressor by regulating a set of proteins involved in cell-cycle control and has synergic effects with 5-FU.

Because GKN1 induced p16 re-expression in AGS cells (Fig. 2), we set out to determine whether GKN1 inhibits the expression of epigenetic regulators (17). Interestingly, we observed a near-complete inactivation of DNMT1, EZH2, methyl histone H3, and HDAC1 in GKN1-transfected cells (Fig. 3). In addition, DNMT1, EZH2, and HDAC1 silencing in AGS cells suppressed cell growth by inducing G0–G1 and G2–M cell-cycle arrest (Supplementary Fig. S4).
Furthermore, GKN1 expression was inversely correlated with DNMT1 and EZH2 expression in gastric cancer tissues and cell lines (Fig. 3D–F). DNMT1 is primarily involved in the maintenance of methylation during DNA replication phase, and the overexpression of DNMT1 has been reported in gastric cancer (18). EZH2, a histone methyltransferase, is involved in epigenetic silencing of a large number of genes involved in differentiation and proliferation (19). EZH2 expression is also regulated at transcriptional, posttranscriptional, and posttranslational levels in human cancer (20), and its overexpression is considered to be an important positive regulator of cancer cell growth in multiple human malignancies (21). Moreover, EZH2 promotes EMT by interacting with Snail and suppressing expression of E-cadherin (22). Snail induces DNA methylation of E-cadherin promoter by recruiting HDAC1 and DNMT1 (23). In particular, EZH2 containing polycomb repressor complex 2 transcriptionally represses cell-cycle suppressor INK-ARF to drive cell-cycle progression (19) and acts as a major enzyme that methylates lysine-27 of histone H3 (H3-K27; ref. 21). Thus, these results suggest that GKN1 inhibits the development and progression of gastric cancer by regulating the expression of epigenetic regulatory components and EMT-related proteins. Interestingly, a previous study that involved a group of patients with gastric cancer treated with cisplatin/5-FU–based neoadjuvant chemotherapy revealed that the concordant methylation of multiple genes suggested an association with worse response to therapy (24). As GKN1 synergistically enhanced the effect of cell growth inhibition induced by 5-FU (Fig. 1), all of these data suggest that GKN1 functions as a tumor suppressor by regulating abnormal epigenetic modification associated with gastric tumorigenesis, such as DNA methylation, DNA acetylation, and histone modification.

Next, to identify a potential molecular pathway involved in the regulation of DNMT1 and EZH2 expression by GKN1, we analyzed miR-185, which is known to directly target DNMT1 activity. Therefore, GKN1 leads to demethylation of multiple genes involved in epigenetic silencing suppressed the inhibitory effects of GKN1 on cell proliferation, cell-cycle, tumorigenicity, expression of DNMT1 and EZH2, and DNMT1 activity (Fig. 5). All of these data indicate that GKN1 suppresses gastric cancer cell growth by downregulating epigenetic regulators and positive cell-cycle components in an miR-185–dependent and -independent manners.

To define how GKN1 regulates the expression of miR-185, we analyzed expression of primary and precursor miR-185 and c-Myc in AGS and HFE-145 cells and found that GKN1 upregulated expression of both primary and precursor miR-185 by binding to and downregulating c-Myc (Supplementary Fig. S5C and S5D). However, further studies are necessary to clarify the molecular mechanism of c-Myc downregulation by the GKN1.

As DNMT1 is primarily involved in the maintenance of methylation during DNA replication (25), we examined the methylation status of the CDKN2A and E-cadherin genes and found that GKN1-induced miR-185 converted hypermethylated CDKN2A and E-cadherin to the unmethylated form. Detailed methylation analysis of 32 and 17 CpG sites at the CDKN2A and E-cadherin CpG islands, respectively, confirmed the demethylated status (Fig. 6). Thus, these results strongly suggest that GKN1 functions as a hypomethylating agent and inhibit gastric carcinogenesis by regulating epigenetic alterations in cell-cycle regulatory components.

On the basis of our data, we propose the following model describing the role of GKN1 in cell cycle and epigenetic alterations. GKN1-induced miR-185 inhibits EZH2 and DNMT1 activity. Also, GKN1 downregulates DNMT1 by inhibition of HDAC1 and induction of Tip60 in an miR-185–independent manner. Therefore, GKN1 leads to demethylation of E-cadherin and the Cdkn2a promoter region and re-expression of E-cadherin and Cdkn2a. Thus,
**Figure 1.**

A. Cell growth (absorbance 540 nm) over time.

B. Cell-cycle phase (%).


D. Colony formation assay: Mock, GKN1, GKN1 + anti-miR-185.

E. DNMT1 activity (OD/h/mg).

F. Relative mRNA expression:
   - DNMT1
   - EZH2
   - CDKN2A

**Legend:**
- *Mock*
- *GKN1*
- *GKN1 + 100 nmol anti-miR-185*

**Significance:**
- *P < 0.05*
- *P < 0.01*
- *P < 0.001*

**Note:**
- The data represents a study comparing the effects of GKN1 and anti-miR-185 on cell growth, cell-cycle phase, and gene expression in cancer cells.
the data here suggest that GKN1 has an miR-185–dependent and -independent mechanism for chromatic and DNA epigenetic modification and cell-cycle regulation (Fig. 6E). It is plausible that modulating GKN1-induced miR-185 activity and stimulating its anti-cancerous effect could significantly impact the development of novel cancer treatments, which will ultimately achieve the goal of gastric cancer prevention and remission.

Figure 6. Ectopic expression of GKN1 induces DNA hypomethylation in AGS cells. A, region of BGS in Bis-E-cadherin, which spans the region from -183 to +35 with respect to the Exon 1. Bis-E-cadherin contains 17 CpG dinucleotides underlined. B, methylation pattern of the bis-E-cadherin region of the E-cadherin 5’ CpG island in mock, GKN1, and GKN1 with anti-miR-185–transfected AGS cells. C, region of BGS in Bis-CDKN2A, which spans the region from -832 to -415 with respect to the exon 1. Bis-CDKN2A contains 34 CpG dinucleotides underlined. D, methylation pattern of the bis-CDKN2A region of the CDKN2A 5’ CpG island in mock, GKN1, and GKN1 with anti-miR-185–transfected AGS cells. Each circle in the figure represents a single CpG site; *, the unmethylated CpG; ●, methylated CpG residues. E, schematic model depicting how GKN1 regulates the cell cycle and epigenetic regulators. GKN1 regulated the cell cycle throughout the demethylation of E-cadherin and CDKN2A gene promoters in miR-185–dependent and -independent manners.

Figure 5. GKN1-induced miR-185 negatively regulates expression of epigenetic regulators and the cell cycle. A, GKN1-transfected AGS cells showed time-dependent inhibition of cell growth but cotransfection of GKN1 with anti-miR-185 resulted in recovery of cell growth, *P < 0.05 compared with mock by Student t test. B, GKN1-transfected AGS cells showed G0–G1 and G2–M cell-cycle arrest but cotransfection of GKN1 with anti-miR-185 brought about recovery of the cell cycle. C, ectopic GKN1 expression downregulated expression of epigenetic regulators and positive cell-cycle regulators and upregulated p16 expression but cotransfection of GKN1 with anti-miR-185 completely suppressed the effect of GKN1. D, GKN1 significantly inhibited colony formation, but anti-miR-185 treatment reversed GKN1 effects on colony formation. E, GKN1-transfected AGS cells showed inhibition of DNMT1 activity but cotransfection of GKN1 with anti-miR-185 induced recovery of DNMT1 activity. F, ectopic expression of GKN1 downregulated DNMT1 and EZH2 mRNA expression and upregulated CDKN2A mRNA expression. However, anti-miR-185 treatment with GKN1-transfected AGS cells induced recovery of DNMT1 and EZH2 mRNA expression and decreased CDKN2A mRNA expression.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: J.H. Yoon, S.W. Nam, J.Y. Lee, W.S. Park
Development of methodology: J.H. Yoon, S.W. Nam, W.S. Park
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.H. Yoon, Y.J. Choi, W.S. Choi, D. Smoot, W.S. Park
Analysis and interpretation of data (e.g., statistical analysis, bio-statistics, computational analysis): J.H. Yoon, Y.J. Choi, W.S. Choi, W.S. Park
Writing, review, and/or revision of the manuscript: J.H. Yoon, W.S. Park

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doi:10.1158/1078-0432.CCR-12-3675

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