GKN1-miR-185-DNMT1 axis suppresses gastric carcinogenesis through regulation of epigenetic alteration and cell cycle

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Abstract (word count: 250)

Purpose: Gastrokine 1 (GKN1) functions to protect the gastric antral mucosa and promotes healing by facilitating restoration and proliferation after injury. GKN1 is down-regulated 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 performed 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 up-regulated Tip60 and down-regulated HDAC1 in a 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 anti-cancerous effect with 5-FU on tumor cell growth, which suggests a possible therapeutic intervention method for gastric cancer.

Conclusion: Our results demonstrate that GKN1 has a 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.

**Keywords:** GKN1, miR-185, Cell cycle, Epigenetic modification, 5-FU
Translational Relevance

In cancer cells, treatment with inhibitors of epigenetic modification can reactivate epigenetically silenced genes and restore normal gene function. Our extensive investigation provide evidence that gastrokine 1 (GKN1) acts as a tumor suppressor by down-regulating epigenetic modifications and cell cycle progression, and that it has a synergistic cytotoxic effect with 5-FU. We also show that GKN1 plays a key role in epigenetic modification and cell proliferation in a miR-185-dependent and -independent manner. Thus, there is the potential for GKN1 and miR-185 to be novel therapeutic agents for gastric cancer therapy.
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 et al. reported that GKN1 protects the antral mucosa and promotes healing by facilitating restitution and proliferation after injury (3). Interestingly, GKN1 is down-regulated 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, MKN28 gastric cancer and HFE-145 immortalized non-neoplastic gastric epithelial cells. Overall, we demonstrated that GKN1 plays an important role in the cell cycle regulation and the epigenetic alterations of tumor-associated genes in gastric cancers.
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 (Manassas, VA, USA) and Dr. Hassan (Washington, DC, USA). GKN1 cDNA and shGKN1 were cloned into the pcDNA3.1 expression vector and pSilencer 3.1 H1-neo (Invitrogen, Carlsbad, CA, USA). 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 performed at 24, 48 and 72 hrs after transfection of each construct, as described previously (6). To further examine whether GKN1 contributes to the chemosensitivity of 5-fluorouracil (5-FU), MTT assay was performed on AGS and HFE-145 cells at 24 and 48 hrs after simultaneous treatment with GKN1, recombinant GKN1 protein and 5-FU.

For cell proliferation assay, a BrdU incorporation assay was performed at 24, 48 and 72 hrs after transient transfection of each construct using the BrdU cell proliferation assay kit (Millipore, Billerica, MA, USA), according to the manufacturer’s instructions.

To measure the proliferative ability of a single cell in vitro, a plate clonogenic assay was performed. Briefly, AGS cells transfected with each construct were cultured in RPMI 1640 for 2 weeks to allow colony formation. Colonies were fixed in 1%
formaldehyde, stained with 0.5% crystal violet solution, and counted using the colono-
count program.

**Flow-cytometry 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 min 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, Heidelberg, Germany).

**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 hrs after transfection with GKN1 and in HFE-145 cells after transfection with shGKN1, as described previously (6). 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, Piscataway, NJ, USA).

**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 hrs 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 RT-PCR was performed using SYBR Green Q-PCR Master Mix (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions. *DNMT1*, *EZH2*, and *CDKN2A* mRNAs were quantified by SYBR Green Q–PCR 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 Q–PCR and normalized to human U6 snRNA. Data are reported as relative quantities according to an internal calibrator using the $2^{-\Delta\Delta CT}$ method (12). 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 mM Tris-Hcl pH 7.5, 10 mM NaCl and 2 mM MgCl$_2$) containing protease inhibitor mixture (Complete; Roche Molecular Biochemicals). Then, 6 μl of 20% NP-40 was added and the mixture was incubated for 10 min at 4°C and centrifuged
for 5 min. The supernatant was collected and the pellet containing the nuclei was resuspended in 50 μl of extraction buffer (20 mM Hepes pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA and 10 % glycerol) followed by incubation for 30 min at 4°C and collection of the nuclear extract by centrifugation.

**Bisulfite genomic sequencing (BGS)**

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 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, Foster City, CA), according to the manufacturer’s recommendations.

**Statistical analysis**

Student’s *t*-test was used to analyze the effect of GKN1 on cell viability and proliferation. All experiments were performed in triplicate to verify the reproducibility of the findings. Data are expressed as means ± SD from at least three independent experiments. Association between GKN1, miR-185, DNMT1 and EZH2 expression and the tumor histologic type was tested using Chi-Square and Spearman correlation tests. A *P*-value less than 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-dependent inhibition 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-cytometry 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 demonstrated increased numbers of sub-G0 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 cyclin 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 demonstrated a modest effect on the cell cycle G0/G1 and G2/M phase progression (Fig.
For G0/G1 arrest in AGS cells, GKN1 induced re-expression of p16 and down-regulated expression of Cdk4, cyclin D, E2F1 and cyclin A (Fig. 2B). Since CDKN2A is hypermethylated in gastric cancers (13), 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 de-methylation of the CDKN2A promoter region and re-expression of the p16 protein (Supplementary Fig. S2). In shGKN1-transfected HFE-145 cells, GKN1 silencing down-regulated expression of p16 and p21, but up-regulated expression of positive cell cycle regulators including CDK4 and cyclin A (Fig. 2B). For G2/M arrest, GKN1 down-regulated expression of p-cdc2, PLK1, CDK2, cdc25a, cdc25c and cyclin B in AGS cells, whereas GKN1 silencing in HFE-145 cells up-regulated expression of p-cdc2, PLK1 and cdk2 (Fig. 2C).

**GKN1 functions as an epigenetic regulator**

As shown in Figure 3A, GKN1 induced Tip60 expression, which acetylated DNMT1 (14), 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 up-regulated 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 C).

In human gastric cancer tissues, GKN1-negative cancers showed overexpression of DNMT1 and EZH2, whereas GKN1-positive cancers demonstrated underexpression of these proteins (Fig. 3D). Sixty-nine (86%) out of 80 gastric cancers showed decreased expression of GKN1 and increased expression of DNMT1 (79%) and EZH2 (84%) proteins, compared to 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 expression from the large cohorts of gastric cancer patients 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 down-regulated, while $DNMT1$ and $EZH2$ gene expression was significantly up-regulated, in gastric cancer cohorts (Fig. 3G).

**GKN1 inhibits DNMT1 expression by up-regulating miR-185 expression**

We also examined whether GKN1 induces expression of miR-185, which directly targets DNMT1 (15, 16). 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 Fig. S5B). Interestingly, ectopic expression of c-Myc, which binds to a promoter of the miR-185 (http://genome.ucsc.edu/), inhibited primary, precursor and mature miR-185 expression, but GKN1 up-regulated expression of primary, precursor and mature miR-185 by binding to and down-regulating c-Myc (Supplementary Fig. S5C and S5D). In addition, miR-185 decreased cell viability in AGS, MKN1 and MKN28 cells, but increased cell viability in HFE-145 cells (Fig. 4B and Supplementary Fig. S6A). miR-185 increased the sub-G0 cell population and induced cell cycle arrest, especially in G2/M phase (Fig. 4C). In particular, miR-185 inhibited DNMT1 and EZH2 activities by binding to a plausible recognition site in the 3’-UTR region of the DNMT1 and EZH2 (Fig. 4D). Over-expression of miR-185 reduced the expression of DNMT1 and EZH2 in AGS, MKN1 and MKN28 cells, as well as the expression of the cell cycle-related proteins including cyclin D, cyclin E and cyclin B in AGS cells (Fig. 4E and Supplementary Fig. S6B). In HFE-145 cells, silencing of miR-185 induced up-regulation of DNMT1, EZH2 and the positive cell cycle regulators, but not HDAC1 and Tip60 (Fig. 4E). Moreover, overexpression of miR-185 in AGS cells resulted in decreased DNMT1 activity, DNMT1 and EZH2 mRNA expression, and increased CDKN2A mRNA expression (Fig. 4F and G). In MKN1 and MKN28 cells, we also found that miR-185 reduced DNMT1 and EZH2 mRNA expression (Supplementary Fig. S6C). However, HFE-145 cells with anti-miR-185 treatment showed expression or activity patterns exactly opposite to miR-185 in AGS cells (Fig. 4F and G). In human gastric cancer tissues, there was decreased expression of GKN1 and miR-185 in 69 (86.3%) out of 80 gastric cancer tissues and increased DNMT1 and EZH2 expression in 69 (86%) cancer tissues. Statistically, we found a close relationship between GKN1 and
miR-185 expression and an inverse relationship between miR-185 and DNMT1 and EZH2 in gastric cancer tissues (Fig. 4H). Furthermore, we recapitulated miR-185 expression from the large cohorts from the NCBI GEO database (accession numbers GSE23739) and found that miR-185 expression was significantly down-regulated in gastric cancer cohorts (Fig. 4I). In addition, decreased expression of miR-185 and GKN1, and increased expression of DNMT1 and EZH2 were found in 10 gastric cancer cell lines (Fig. 4J).

**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%) out 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, D and Supplementary Fig. S8A, B). Thus, re-expression of *E-cadherin* and *CDKN2A* was induced by de-methylation due to the suppression of *EZH2* and *DNMT1* through up-regulation 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 the cell viability, proliferation and colony formation of AGS cells (Fig. 1). Treatment of GKN1 and 5-FU synergistically inhibited cell viability and proliferation by up-regulating p53 and p21 (Fig. 1). The activation of p53 and p21 by 5-FU was previously reported in mouse L-TK and AGS cells (17, 18). 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 the key modulators that suppress cyclin D1/CDK4, 6 or cyclin E/CDK2 complex in the G1/S transition (19). 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 cdc25, 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 if GKN1 inhibits the expression of epigenetic regulators (20). 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 (21). EZH2, a histone methyltransferase, is involved in epigenetic silencing of a large number of genes involved in differentiation and proliferation (22). EZH2 expression is also regulated at transcriptional, post-transcriptional and post-translational levels in human cancer (23) and its overexpression is considered to be an important positive regulator of cancer cell growth in multiple human malignancies (24). Moreover, EZH2 promotes EMT by interacting with Snail and suppressing expression of E-cadherin (25). Snail induces DNA methylation of E-cadherin promoter by recruiting HDAC1 and DNMT1 (26). In particular, EZH2 containing Polycomb Repressor Complex 2 transcriptionally represses cell cycle suppressor INK-ARF to drive cell cycle progression (22) and acts as a major enzyme that methylates lysine-27 of histone H3 (H3-K27) (24). 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 gastric cancer patients treated with cisplatin/5FU-based neoadjuvant chemotherapy revealed that the concordant methylation of multiple genes suggested an association with worse response to therapy (27). Since 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 and regulate DNA methylation (15, 16). Interestingly, GKN1 up-regulated miR-185 expression in gastric epithelial cells (Fig. 4A) and miR-185 inhibited cell growth by inducing cell cycle arrest, mainly at G2/M phase, with inactivation of DNMT1 and EZH2 and increase of CDKN2A mRNA expression (Fig. 4). Unexpectedly, GKN1 down-regulated HDAC1 and Tip60 in a miR-185 independent manner (Fig. 4E). Before these experiments, we confirmed miR-185 expression levels after miR-185 and anti-miR-185 transfection in AGS and HFE-145 cells, respectively (Supplementary Fig. S5). Previously, the binding site of the miR-185 in the 3'-UTR region of the DNMT1 has been reported (15). Here, we found that miR-185 inhibits EZH2 activity by binding to a plausible recognition site in the 3'-UTR region of the EZH2 (Fig. 4D). In gastric cancer tissues and cell lines, the GKN1 expression level was positively associated with miR-185 expression and inversely correlated with DNMT1 and EZH2 expression (Fig. 4H and J). Furthermore, miR-185 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 down-regulating epigenetic regulators and positive cell cycle components in a 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 up-regulated expression of both primary and precursor miR-185 by binding to and down-regulating c-Myc (Supplementary Fig. S5C and S5D). However, further studies are strongly necessary to clarify the molecular mechanism of c-Myc down-regulation by the GKN1.
Since DNMT1 is primarily involved in the maintenance of methylation during DNA replication (28), 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.

Based on 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 down-regulates DNMT1 by inhibition of HDAC1 and induction of Tip60 in a miR-185-independent manner. Therefore, GKN1 leads to de-methylation of *E-cadherin* and the *CDKN2A* promoter region and re-expression of E-cadherin and CDKN2A. Thus, the data here suggest that GKN1 has a 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.
Reference


Figure Legends

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 BrdU incorporation assay, GKN1-transfected AGS cells showed time-dependent inhibition of cell proliferation, but shGKN1-transfected HFE-145 cells induced cell proliferation. C. In colony formation assay in AGS cells, GKN1 significantly inhibited colony formation. D, E. 5-FU treatment of AGS (D) and HFE-145 cells (E) induced a time- and dose-dependent inhibition of cell viability. F. GKN1 and recombinant GKN1 protein treatment showed synergistic inhibition of cell viability with 5-FU. G. Treatment of GKN1 and recombinant GKN1 protein showed a synergistic effect on G0/G1 and G2/M cell cycle arrest with 5-FU. H. Western blot analysis following GKN1 transfection and recombinant GKN1 protein treatment with 5-FU in AGS cells showed a synergistic increased expression of p53 and p21, but decreased expression of cyclin B and D.

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 down-regulation of the expression of CDK4, cyclin D, E2F1, and cyclin A. Knock-down of GKN1 in HFE-145 cells down-regulated the expression of p16 and p21, but up-regulated the expression of cell cycle positive regulators including CDK4 and cyclin A. C. For G2/M phase proteins, GKN1 down-regulated 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.

**Figure 3. GKN1 negatively regulates expression of epigenetic regulators.** A. GKN1 up-regulated Tip60 and down-regulated expression of DNMT1, EZH2, methyl histone H3 and HDAC1. B. GKN1 directly inhibited DNMT1 activity. C. GKN1 significantly down-regulated DNMT1 and EZH2, and up-regulated CDKN2A mRNA expression. D-F. An inverse association of GKN1 expression with DNMT1 and EZH2 in gastric cancer tissue (D, E) and cell lines (F) was observed. G. Recapitulated GKN1, DNMT1 and EZH2 gene expression levels in the large cohort of gastric cancer patients (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 scatterplot. 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 two categories was determined by the unpaired t-test ($P < 0.0001$; two-tailed), respectively.

**Figure 4. GKN1-induced miR-185 has tumor suppressor activity.** A. GKN1 transfected AGS cells and GKN1 knock-downed HFE-145 cells induced increased and decreased miR-185 expression, respectively. B. In MTT assay, miR-185 inhibited cell growth of AGS cells in a time-dependent manner, whereas anti-miR-185 transfected HFE-145 cells showed increased cell growth. *$P < 0.05$ compared to control miRNA by student t-test. C. miR-185 transfected AGS cells showed G0/G1 and G2/M cell cycle arrest. D. Prediction schema of binding sites between miR-185 and the 3’-UTRs for the
DNMT1 and EZH2. **E.** miR-185 down-regulated expression of epigenetic regulators (DNMT1 and EZH2) and positive cell cycle regulators, but not HDAC1 and Tip60. **F.** miR-185 negatively regulated DNMT1 activity. **G.** miR-185 down-regulated expression of DNMT1 and EZH2, and up-regulated CDKN2A mRNA expression. **H.** Positive correlation of GKN1 mRNA with miR-185 expression and inverse relationship of DNMT1 and EZH2 mRNA expression with GKN1 and miR-185 were found in 80 gastric cancer tissues. The differential GKN1, DNMT1, EZH2 and miR-185 expression for these two categories was determined by the unpaired t-test ($P < 0.0001, P < 0.0001, P < 0.0001, P < 0.0001$; two-tailed). **I.** Recapitulated miR-185 expression levels of the large cohort of gastric cancer patients (NCBI GEO database, accession number GSE23739). The relative expression of miR-185 in noncancerous (Normal) and gastric cancer (Tumor) tissues is illustrated as described in Fig. 3. The differential miR-185 expression for these two categories was determined by the unpaired t-test ($P = 0.0043$; two-tailed). **J.** In 10 gastric cancer cell lines, there was also positive correlation of GKN1 with miR-185 and an inverse relationship of DNMT1 and EZH2 with GKN1 and miR-185 expression.

**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 co-transfection of GKN1 with anti-miR-185 resulted in recovery of cell growth. *$P < 0.05$ compared to mock by student t-test. **B.** GKN1 transfected AGS cells showed G0/G1 and G2/M cell cycle arrest, but co-transfection of GKN1 with anti-miR-185 brought about recovery of the cell cycle. **C.** Ectopic GKN1 expression down-regulated expression of epigenetic regulators and positive cell cycle
regulators and up-regulated p16 expression, but co-transfection 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 co-transfection of GKN1 with anti-miR-185 induced recovery of DNMT1 activity. F. Ectopic expression of GKN1 down-regulated DNMT1 and EZH2 mRNA expression and up-regulated 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.

Figure 6. The ectopic expression of GKN1 induces DNA hypomethylation in AGS cells. A. Region of bisulfite genomic sequencing 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 bisulfite genomic sequencing 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 de-methylation of E-cadherin and CDKN2A gene promoters in miR-185 dependent and independent manners.
Figure 2

A

Graphs showing DNA content analysis over 24 and 48 hours for Mock and wtGKN1 treatments in AGS and HFE-145 cells. The histograms display the proportion of cells in sub-G0, G0/G1, S, G2, and M phases.

B

Table comparing G1/S phase protein expression levels for Mock and wtGKN1 treatments in AGS and HFE-145 cells after 24 and 48 hours. Proteins analyzed include GKN1, p53, p16, p21, CDK4, Cyclin A, Cyclin D, E2F1, Cyclin B, and GAPDH.

C

Table comparing G2/M phase protein expression levels for Mock and wtGKN1 treatments in AGS and HFE-145 cells after 24 and 48 hours. Proteins analyzed include GKN1, p-CDC2, PLK1, CDK2, Cdc25c, Cdc25a, Cyclin B, and GAPDH.
Figure 6

A

B

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D

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GK1

Figure 6

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Figure 6

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GK1
GKN1-miR-185-DNMT1 axis suppresses gastric carcinogenesis through regulation of epigenetic alteration and cell cycle

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