Journal of Cellular Physiology

GKN2 Contributes to the Homeostasis of Gastric Mucosa by Inhibiting GKN1 Activity

OLGA KIM,[1] JUNG HWAN YOON,[1] WON SUK CHOI,[1] HASSAN ASHKTORAB,[2] DUANE T. SMOOT,[2] SUK WOO NAM,[1] JUNG YOUNG LEE,[1] AND WON SANG PARK[1]*

[1]*Department of Pathology, College of Medicine, The Catholic University of Korea, Seocho-gu, Seoul, South Korea*

[2]*Department of Medicine, Howard University, Washington, District of Columbia*

Gastrokine 1 (GKN1) plays an important role in maintaining gastric mucosa integrity. Here, we investigated whether gastrokine 2 (GKN2) contributes to the homeostasis of gastric epithelial cells by regulating GKN1 activity. We analyzed cell viability, proliferation, and death in AGS cells transfected with *GKN1*, *GKN2*, *GKN1* plus *GKN2* using MTT, BrdU incorporation, and apoptosis assays, respectively. In addition, the expression levels of the cell cycle- and apoptosis-related proteins, miR-185, DNMT1, and EZH2 were determined. We also compared the expression of GKN1, GKN2, and CagA in 50 non-neoplastic gastric mucosae and measured GKN2 expression in 169 gastric cancers by immunohistochemistry. GKN2 inhibited anti-proliferative and pro-apoptotic activities, miR-185 induction, and anti-epigenetic modifications of GKN1. There was a positive correlation between GKN1 and GKN2 expression ($P = 0.0074$), and the expression of GKN1, but not GKN2, was significantly lower in *Helicobacter pylori* CagA-positive gastric mucosa ($P = 0.0013$). Interestingly, ectopic GKN1 expression in AGS cells increased GKN2 mRNA and protein expression in a time-dependent manner ($P = 0.01$). Loss of GKN2 expression was detected in 126 (74.6%) of 169 gastric cancers by immunohistochemical staining and was closely associated with GKN1 expression and differentiation of gastric cancer cells ($P = 0.0002$ and $P = 0.0114$, respectively). Overall, our data demonstrate that in the presence of GKN2, GKN1 loses its ability to decrease cell proliferation, induce apoptosis, and inhibit epigenetic alterations in gastric cancer cells. Thus, we conclude that GKN2 may contribute to the homeostasis of gastric epithelial cells by inhibiting GKN1 activity.

J. Cell. Physiol. 229: 762–771, 2014. © 2013 Wiley Periodicals, Inc.

Gastric epithelial homeostasis is a dynamic equilibrium. In normal stomach, continuous processes of cell proliferation, differentiation, and self-renewal are counterbalanced by apoptosis. The critical balance between cell proliferation and apoptosis is controlled by a complex network of signaling pathways and transcriptional regulators. Otherwise, homeostatic imbalance can become one of the reasons that lead to many diseases, including gastritis and gastric cancer. However, many of the underlying molecular mechanisms have not been clearly elucidated.

Gastrokine 1 (GKN1) is a 18-kDa stomach-specific protein present in the gastric antrum that plays an important role in maintaining gastric mucosa integrity (Toback et al., 2003). GKN1 protects the antral mucosa and promotes healing by facilitating restitution and proliferation after injury (Toback et al., 2003). Furthermore, it protects the intestinal mucosal barrier by acting on specific tight junction proteins and stabilizing perijunctional actin (Walsh-Reitz et al., 2005). GKN1 is found in mucous secretion granules in gastric surface cells and appears to function as an epithelial cell mitogen (Martin et al., 2003). Several investigations have demonstrated down-regulation of GKN1 in *Helicobacter pylori* (*H. pylori*)-infected gastric mucosal epithelial cells and in most gastric cancers (Shiozaki et al., 2001; Nardone et al., 2007; Yoon et al., 2011). We previously reported that GKN1 has a tumor suppressor activity in gastric carcinogenesis (Yoon et al., 2011). Recently, it was shown that GKN1 induces senescence through p16/Rb pathway activation in gastric cancer cells (Xing et al., 2012) and that its overexpression induces Fas-mediated apoptosis (Rippa et al., 2011), suggesting that, in the absence of GKN1, gastric epithelial cells continuously proliferate without undergoing apoptosis. Thus, the identification of GKN1 inhibitors is essential in order to understand the molecular mechanism of gastric homeostasis.

Gastrokine 2 (GKN2), also known as TFIZ1 (Westley et al., 2005), GDDR (Du et al., 2003), and blottin (Otto et al., 2006), was identified as a binding partner of gastric trefoil factor family-2 peptide (Otto et al., 2006), a mucin-associated protein that participates in the maintenance of homeostasis in the gastrointestinal tract. GKN2 is expressed in superficial gastric foveolar cells (Otto et al., 2006), and its expression is reduced in *H. pylori*-infected gastritis (Resnick et al., 2006) and in gastric cancers (Du et al., 2003). GKN2 has therefore been suggested as a potential biomarker for gastric cancer and

Olga Kim and Jung Hwan Yoon contributed equally to this study.

The authors disclose no potential conflicts of interest.

Author contributions: O.K., J.H.Y., and W.S.P. designed research; O.K, and J.H.Y. performed research and analyzed data; J.H.Y., Y.J.C., W.S.C., S.W.N., and J.Y.L. contributed reagents/materials/analysis tools; O.K. and W.S.P. wrote the paper.

Contract grant sponsor: Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology;
Contract grant number: 2012R1A2A2A01002531.

*Correspondence to: Won Sang Park, Department of Pathology, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Seocho-gu, Seoul 137-701, South Korea.
E-mail: wonsang@catholic.ac.kr

Manuscript Received 27 September 2013
Manuscript Accepted 16 October 2013

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 22 October 2013.
DOI: 10.1002/jcp.24496

© 2013 WILEY PERIODICALS, INC.

10974652, 2014, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/jcp.24496 by University Of Southern Califor, Wiley Online Library on [07/08/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

gastritis. However, the role of GKN2 in gastric homeostasis is still unclear. We hypothesized that GKN2 may play a role in the maintenance of gastric epithelial homeostasis by regulating GKN1.

In the present study, we examined the expression of GKN2 in gastric mucosa and gastric cancer tissues and its effects on cell viability, proliferation, apoptosis, and epigenetic alterations in comparison with GKN1. Our findings suggest that GKN2 contributes to the homeostasis of gastric epithelial cells by inhibiting GKN1 tumor suppressor activity.

Materials and Methods

Cell culture and transfection of *GKN1* and *GKN2*

AGS gastric cancer and HFE-145 non-neoplastic gastric epithelial cell lines were cultured at 37°C in 5% CO_2 in RPMI-1640 medium with 10% heat-inactivated fetal bovine serum (FBS). The complete *GKN1* and *GKN2* cDNAs were cloned into the expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA). AGS cells were transfected in 60-mm diameter dishes with 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

Cell viability was analyzed in AGS gastric cancer cells transfected with *GKN1*, *GKN2*, *GKN1* plus *GKN2* using the MTT [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay, which was performed 24, 48, and 72 h after transient transfection. Absorbance was measured using a spectrophotometer at 540 nm and viability was expressed relative to mock (empty vector + lipofectamin).

To investigate the effect of GKN1 and GKN2 on cell proliferation, a BrdU (bromodeoxyuridine) incorporation assay was performed at 24, 48, and 72 h after transient transfection using the BrdU cell proliferation assay kit (Millipore, Billerica, MA). Absorbance was measured using a spectrophotometer at 450 nm and proliferation was expressed relative to mock.

To measure the proliferative ability of a single cell in vitro, a plate clonogenic assay was performed. Briefly, 1×10^3 AGS cells transfected with each construct were seeded into a six-well plate and 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.

Measurement of apoptosis

Apoptosis was measured using an Annexin V binding assay according to the manufacturer's instructions (BD Biosciences, San Jose, CA). Annexin V binds to cells that display phosphatidylserine on the cell membrane, whereas propidium iodide (PI) stains cellular DNA with a compromised cell membrane. Transfected AGS cells were washed twice in cold PBS and resuspended in 100 μl binding buffer. In each case, 100 μl supernatant was mixed with 400 μl blocking solution, 5 μl of annexin V-FITC (1 μg/ml) and 5 μl of PI (2 μg/ml) were added, and the mixture was incubated in the dark for 15 min. Cells were analyzed using a fluorescence-activated cell sorter (BD Biosciences). Annexin V-positive cells without PI staining were counted as apoptotic cells.

We examined caspase-3 and -7 activity with the Apo-One Homogeneous Caspase 3/7 assay kit (Promega Corporation, Madison, WI), as described previously (Arimura et al., 2012). Expression of apoptosis-related proteins was also examined by western blot analysis in AGS cells transfected with *GKN1* or *GKN2*. Briefly, cell lysates were separated on a 10% polyacrylamide gel and transferred onto a Hybond PVDF membrane (Amersham Pharmacia Biotech, Piscataway, NJ). After blocking, the membrane was probed with antibodies against the following apoptosis-related proteins: anti-caspase 8 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-caspase 3, anti-cleaved caspase 3, anti-PARP, anti-cleaved PARP (Cell Signaling, Danvers, MA); anti-GAPDH (Abcam, Cambridge, UK); and anti-mouse IgG and anti-rabbit IgG (Sigma, St. Louis, MO). The enhanced chemiluminescence (ECL) plus western blotting detection system (Amersham Pharmacia Biotech) was used to detect bound antibodies. The intensity of bands was quantified using LAS 3000 (Fuji Film, Japan).

Cell cycle analysis by flow cytometry

AGS cells from each experimental group were collected and stained with PI for 45 min in the dark prior to analysis. The percentage of cells in different phases of the cell cycle was determined using a FACSCalibur Flow Cytometer with CellQuest 3.0 software (BD Biosciences). Experiments were performed in triplicate.

Expression of cell cycle regulators

To determine whether GKN1 and GKN2 are involved in regulation of the cell cycle, we examined the expression of G0/G1 phase proteins (including p53, p21, p16, CDK4, and cyclin D1) and G2/M phase proteins (including cdc2, cdc25c, cyclin B, and cyclin E) in AGS cells 24 and 48 h after transfection with *GKN1*, *GKN2*, *GKN1* plus *GKN2*. Cell lysates were separated on a 10% polyacrylamide gel and transferred to a Hybond PVDF membrane (Amersham Pharmacia Biotech). After blocking, the membrane was probed with antibodies against p53 and p21 (Santa Cruz Biotechnology), and p16, CDK4, cyclin D1, cdc2, cdc25c, cyclin B, and cyclin E (Cell Signaling Technology). Protein bands were detected using ECL (Amersham Pharmacia Biotech).

Measurement of miRNA-185, DNMT1, and EZH2 expression

To investigate whether GKN1 and GKN2 regulate miR-185 expression, we quantified the expression of miR-185 in AGS cells transfected with *GKN1*, *GKN2*, *GKN1* plus *GKN2* by quantitative (Q)-PCR and normalized expression to that of human U6 snRNA. Data are reported as relative quantities according to an internal calibrator based on the $2^{-\Delta\Delta C_t}$ method (Pfaffl, 2001). Sequences of the primers are described in Supplementary Table S1. In addition, to study the effect of GKN2 on epigenetic alterations, the expression of DNMT1 and EZH2 was measured in AGS cells transfected with *GKN1, GKN2*, *GKN1* plus *GKN2* by western blot analysis. After blocking, the membrane was subsequently probed with anti-DNMT1 and anti-EZH2 antibodies (BD Biosciences). Protein bands were detected using ECL reagents (Amersham Pharmacia Biotech).

GKN1, GKN2, and CagA expression in non-cancerous gastric mucosa

Expression of GKN1, GKN2, and *H. pylori* CagA was examined in 50 non-neoplastic gastric mucosal tissues by western blot analysis. Tissue samples were ground to a very fine powder in liquid nitrogen using a pestle and mortar and resuspended in ice-cold Nonidet P-40 lysis buffer supplemented with a 1× protease inhibitor mix (Roche). Cell lysates were separated on 12% polyacrylamide gel and blotted onto a Hybond-PVDF transfer membrane (Amersham), which was subsequently probed with anti-GKN1 monoclonal antibody (Sigma–Aldrich), anti-GKN2 monoclonal antibody (Abcam), anti-CagA antibody (Santa Cruz Biotechnology), and anti-GAPDH antibody (Abcam), and then incubated with anti-mouse, anti-rabbit and anti-mouse IgG conjugated with horseradish peroxidase, respectively. Protein bands were detected using ECL western blotting detection reagents (Amersham, Pharmacia Biotech). Approval was obtained

from the Institutional Review Board of The Catholic University of Korea, College of Medicine (CUMC09U089). There was no evidence of familial cancer in any of the patients.

After quantification of mRNA extracted from 50 frozen non-neoplastic gastric mucosa samples, cDNA was synthesized using the reverse transcription kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. For Q-PCR, 50 ng cDNA was amplified using Fullvelocity SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) and 20 pM/μl each of forward and reverse primers on the Stratagene Mx 3000P QPCR system using previously published techniques (Walker et al., 2006). The specific oligonucleotide primers for mRNA were synthesized according to published information on the *GKN1*, *GKN2*, and *GAPDH* genes. The primer sequences are described in Supplementary Table S1. To ensure the fidelity of DNA and mRNA extraction and reverse transcription, all samples were subjected to PCR amplification with oligonucleotide primers specific to the constitutively expressed GAPDH gene and expression was normalized to that of GAPDH. Primers for SYBR Green analysis were designed based on gene-specific non-homologous DNA sequences. The standard curve method was used for quantification of the relative amount of gene expression products. This method provides unit-less normalized expression values that can be used for direct comparison of the relative amounts of target DNA and mRNA in different samples. All samples were tested in duplicate and average values were used for quantification.

Measurement of GKN2 expression

The effect of GKN1 on GKN2 expression in AGS cells was examined by real-time RT-PCR and western blot analysis 24, 48, and 72 h after transient transfection. Previously, it was reported by Baus-Loncar et al. (2007) that *GKN2* gene expression is significantly downregulated in AGS and KATO-III gastric adenocarcinoma cell lines by the p50 and p65 subunits of NF-κB and upregulated by co-transfection with the NF-κB specific inhibitor (IkB). To examine whether GKN1 is involved in the regulation of GKN2 expression through inactivation of the NF-κB pathway, we compared the effects of TNF-α and GKN1 on p-p65, p65, and IκB expression and then analyzed their influence on GKN2 in HFE-145 and AGS cells using real-time RT-PCR and western blot analyses. HFE-145 and AGS cells were transfected with *GKN1* or *shGKN1* and treated with TNF-α. Real-time RT-PCR was performed using SYBR Green Q-PCR Master Mix (Stratagene, La Jolla, CA) according to the manufacturer's instructions. *GKN2* mRNA expression was quantified by SYBR Green Q-PCR and normalized to mRNA expression of the housekeeping gene *GAPDH*. The primer sequences are described in Supplementary Table S1. For Western blot analysis, the following antibodies were used: p-p65 (Cell Signaling Technology), p65 and IκB (Santa Cruz Biotechnology).

Immunohistochemistry

Tissue microarray recipient blocks containing 169 gastric cancer tissues were constructed from formalin-fixed paraffin embedded specimens. Three tissue cores (2 mm in diameter) were transferred to a new recipient paraffin block using a microarray instrument (Beecher Instruments, Micro-Array Technologies, Silver Spring, MD), according to established methods (Kononen et al., 1998). One cylinder of normal gastric mucosa adjacent to each tumor was also transferred to the recipient block. Sections (2 μm thick) were cut the day before use and stained according to standard protocol.

To enhance the signal, we used antigen retrieval in citrate buffer and signal amplification with biotinylated tyramide (Park et al., 2000). The sections were incubated overnight at 4°C with GKN2 antibodies (1/100; Abcam, Cambridge, UK). Detection was carried out using biotinylated goat anti-rabbit antibodies (Sigma), followed by incubation in a peroxidase-linked avidin-biotin complex. Diaminobenzidine was used as a chromogen and the slides were counterstained with Mayer's hematoxylin. Staining for GKN2 antigen was considered positive when >30% of the cytoplasm stained positively. The results were reviewed independently by two pathologists. For negative controls, primary antibodies were replaced with non-immune serum. We compared GKN2 expression patterns with our previous GKN1 expression data (Yoon et al., 2011).

Statistical analysis

A Student's *t*-test was used to analyze the effects of GKN1 and GKN2 on cell viability, proliferation, and apoptosis. Data were expressed as means ± SD from at least three independent experiments. The relationship between GKN1, GKN2, and CagA expression levels and the clinicopathologic parameters of gastric cancers was examined using Spearman correlation and Chi-square tests. $P < 0.05$ was considered the limit of statistical significance.

Results

GKN2 inhibits anti-proliferative and pro-apoptotic activities of GKN1

Cell viability, proliferation, and colony formation assays were performed in AGS gastric cancer cells transfected with *GKN1*, *GKN2*, *GKN1* plus *GKN2*. Transfection with *GKN1* resulted in a significant reduction in cell viability and proliferation in a time-dependent manner ($P < 0.0001$ and $P < 0.001$, respectively), whereas transfection with *GKN2* did not have any effect on cell viability and proliferation. Interestingly, in AGS cells transfected with *GKN1* plus *GKN2*, GKN2 significantly inhibited the effect of GKN1 on cell viability and proliferation (Fig. 1A,B). In the colony formation assay, ectopic expression of GKN1 dramatically reduced the number and size of surviving colonies compared with the mock or *GKN2*–transfected cells, and GKN2 markedly rescued the effect of GKN1 on colony formation (Fig. 1C).

Next, we assayed cell death by staining cells with FITC-labeled annexin V. As expected, the percentage of apoptotic cells and the level of caspase 3/7 activity were significantly increased in cells transfected with *GKN1* ($P < 0.05$). However, no induction of apoptosis and caspase 3/7 activity was observed in AGS cells transfected with *GKN2* and *GKN1* plus *GKN2* (Fig. 2A,B). In addition, cleaved forms of caspase 8, caspase 3, and PARP were present in *GKN1*-transfected cells, but there was no change in the expression of these proteins in *GKN2* and *GKN1* plus *GKN2*-transfected AGS cells (Fig. 2C). These results indicate that GKN2 inhibit the effects of GKN1 on cell viability, proliferation, and apoptosis.

GKN2 inhibits GKN1-induced G2/M arrest

As shown in Figure 3, GKN1 dramatically increased the number of cells in G2/M phase, whereas GKN2 alone showed no effect on the cell cycle. Furthermore, when we co-transfected *GKN1* and *GKN2* into AGS cells, GKN2 completely inhibited the GKN1-induced G2/M arrest.

To determine whether GKN1 and GKN2 are involved in regulation of the cell cycle, we examined the expression of G0/G1 phase proteins (p53, p21, p16, CDK4, and cyclin D1) and G2/M phase proteins (cdc2, cdc25c, cyclin A, cyclin B, and cyclin E) 24 and 48 h after transfection of AGS cells with *GKN1*, *GKN2*, *GKN1* plus *GKN2*. GKN1 downregulated the expression of CDK4, p-cdc2, cdc25c, cyclin A, and cyclin B and upregulated the expression of p53, p21, and p16. But GKN2 markedly inhibited the effect of GKN1 on the expression of the above cell cycle regulators (Fig. 3B). Thus, although GKN2 alone had no direct influence on the regulation of cell cycle progression

10974652, 2014, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/jcp.24496 by University Of Southern Califor, Wiley Online Library on [07/08/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

10974652, 2014, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/jcp.24496 by University Of Southern Califor, Wiley Online Library on [07/08/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

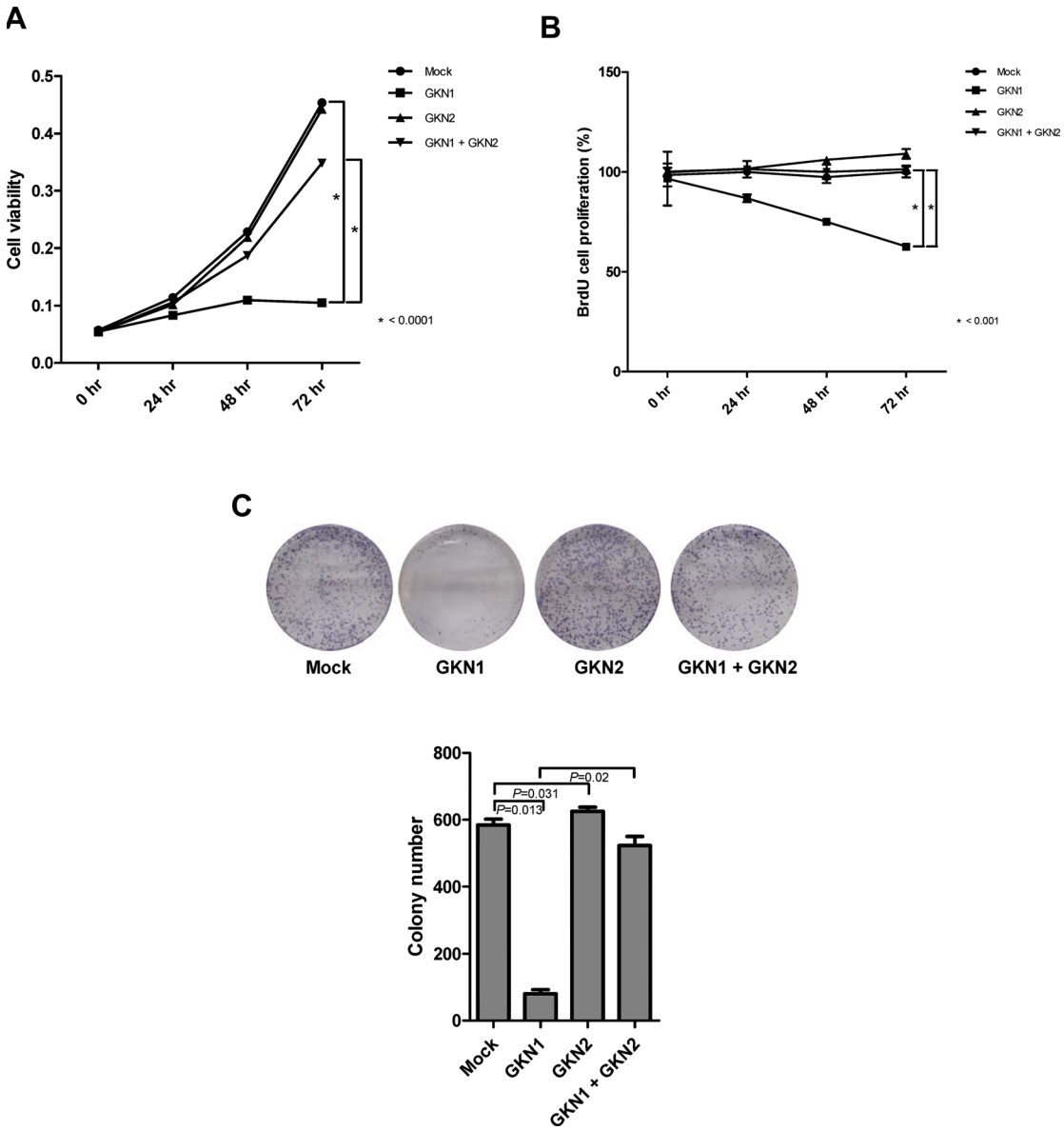

Fig. 1. GKN2 inhibits the anti-proliferative activity of GKN1. A: In the MTT assay, GKN1-transfected AGS cells demonstrated a significant reduction in cell viability in a time-dependent manner, whereas transfection with *GKN2* had no effect on cell viability. Transfection of AGS cells with *GKN1* plus *GKN2* abrogated the effect of GKN1 on cell viability. B: In the BrdU incorporation assay, *GKN1*-transfected AGS cells showed a time-dependent inhibition of cell proliferation, whereas transfection with *GKN2* cells had no effect on cell proliferation. Transfection of AGS cells with *GKN1* plus *GKN2* demonstrated that GKN2 inhibited the anti-proliferative effect of GKN1 in gastric cancer cells. C: In the colony formation assay, ectopic expression of GKN1 dramatically reduced the number and size of surviving colonies in the AGS cells compared with mock or *GKN2*-transfected cells, whereas GKN2 markedly reversed the effect of GKN1 on colony formation.

and cell death, it inhibited the anti-proliferative and pro-apoptotic activities of GKN1 in gastric cancer cells.

GKN2 inhibits GKN1-induced miR-185 expression and suppression of epigenetic regulators

We also examined whether GKN2 inhibits GKN1-induced expression of miR-185, a microRNA that regulates epigenetic alterations (Yoon et al., 2013a). A significantly increased level of miR-185 was detected in AGS cells transfected with *GKN1* ($P < 0.05$), but ectopic expression of GKN2 did not affect miR-185 expression (Fig. 4A). However, in cells transfected with *GKN1* plus *GKN2*, GKN2 completely abrogated the GKN1-induced miR-185 expression (Fig. 4A). Previously, we reported that GKN1 upregulates expression of miR-185 by binding to and down-regulating c-Myc (Yoon et al., 2013a). In this study, we found that GKN2 has no effect on c-Myc expression, although it inhibits the suppression of c-Myc by GKN1 in cells transfected with *GKN1* plus *GKN2* (Fig. 4B). Additionally, when we explored the effect of GKN2 on epigenetic alterations, we observed upregulation of DNMT1 and EZH2 in *GKN2*-transfected AGS cells, whereas GKN1 significantly downregulated the expression of these genes. Interestingly, transfection of AGS cells with *GKN1* plus *GKN2* showed that

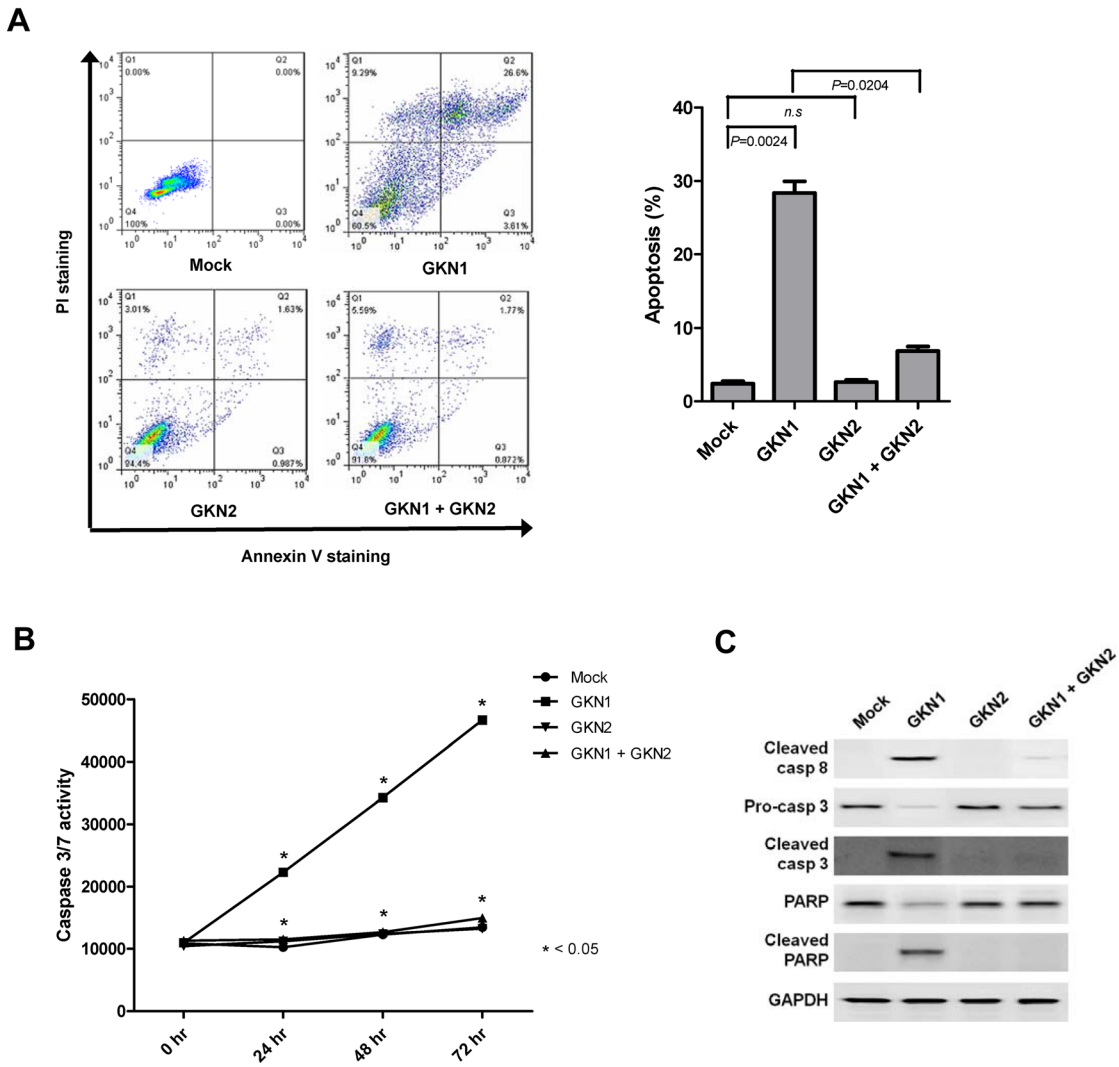

Fig. 2. GKN2 inhibits the pro-apoptotic effect of GKN1. A: An Annexin V-binding assay showed a significant increase in the percentage of apoptotic cells in wild-type *GKN1*-transfected cells. GKN2 inhibited the pro-apoptotic effect of GKN1 in AGS cells transfected with *GKN2* and *GKN1* plus *GKN2*. B: GKN1 significantly increased the activity of caspase-3 and -7, however, no induction of caspase-3 and -7 activity was observed in cells transfected with *GKN2* and *GKN1* plus *GKN2*. C: Western blot analysis revealed the presence of cleaved forms of caspase 8, caspase 3, and PARP in *GKN1*-transfected cells, but no change in the expression of these proteins in cells transfected with *GKN2* and *GKN1* plus *GKN2*.

GKN2 inhibited the effect of GKN1 on DNMT1 and EZH2 (Fig. 4B).

GKN1, GKN2, and CagA expression in normal gastric mucosa

As shown in Figure 5, expression of GKN1 and GKN2 was detected in 47 (94%) and 48 (96%) out of 50 non-neoplastic gastric mucosa tissues, respectively. Positivity for *H. pylori* CagA was found in 25 (50%) gastric mucosa tissues, and 24 of 48 gastric mucosa tissues (50%) with GKN2 expression showed CagA positivity. Statistically, there was a close correlation between GKN1 and GKN2 expression ($P = 0.0074$). GKN1 protein expression was significantly lower in *H. pylori* CagA-positive gastric mucosa than in CagA-negative mucosa ($P = 0.0013$). However, GKN2 mRNA and protein expression was not associated with CagA positivity ($P = 0.3136$ and $P = 0.4668$, respectively) (Fig. 5B). These results suggest that *H. pylori* CagA may be involved in regulating the expression of GKN1, but not GKN2.

GKN1 increases GKN2 expression through inactivation of the NF-κB pathway and activation of IκB

As shown in Figure 6A,B, ectopic GKN1 expression increased *GKN2* mRNA and protein expression in a time-dependent manner ($P = 0.01$). To define how GKN1 regulates GKN2 expression, we first compared the effects of TNF-α and GKN1 on p-p65, p65, and IκB expression in AGS cells. As expected, TNF-α increased p-p65 and p65 expression and decreased IκB expression, whereas GKN1 downregulated p-p65 and p65 expression and upregulated IκB expression (Fig. 6C). Furthermore, repression of GKN1 expression in HFE-145 cells transfected with *shGKN1* resulted in increased p-p65 and p65 expression and decreased IkB expression (Fig. 6C).

10974652, 2014, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/jcp.24496 by University Of Southern Califor, Wiley Online Library on [07/08/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

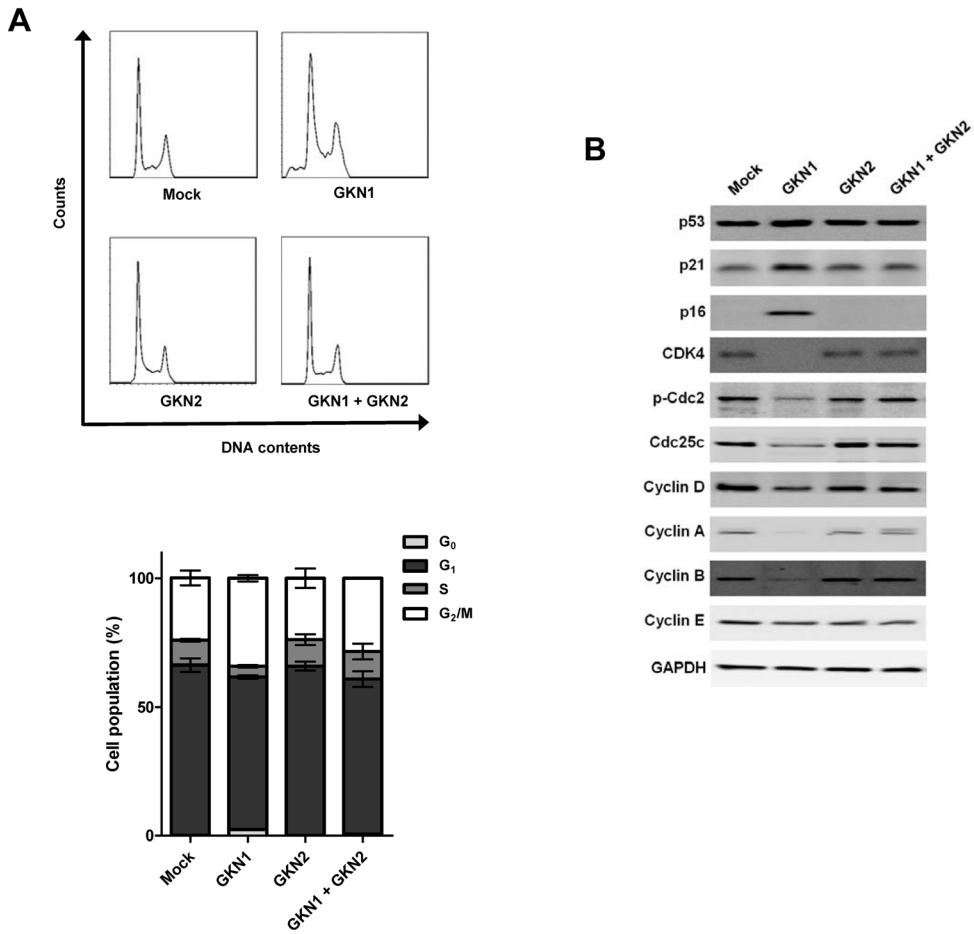

Fig. 3. GKN2 inhibits the GKN1-induced G2/M arrest. A: Cell cycle arrest at G2/M phase was detected in AGS cells transfected with GKN1. But GKN2 completely inhibited GKN1-induced G2/M arrest in *GKN2* and *GKN1* plus *GKN2*-transfected AGS cells. B: GKN1 down-regulated the expression of CDK4, p-cdc2, cdc25c, cyclin A, and cyclin B and up-regulated the expression of p53, p21, and p16 in AGS cells. GKN2 markedly inhibited the effect of GKN1 on the expression of these G1/S- and G2/M-related cell cycle regulators.

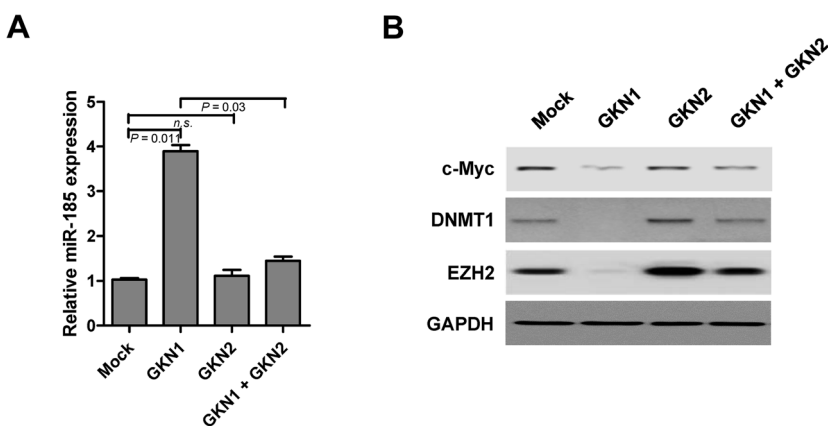

Fig. 4. GKN2 inhibits GKN1-induced miR-185 expression and GKN1 suppressive effect on epigenetic regulators. A: GKN2 completely abrogated the induction of miR-185 expression by GKN1 in AGS cells transfected with *GKN1* plus *GKN2*. B: GKN1 negatively regulated the expression of DNMT1, EZH2, and c-Myc. GKN2 inhibited the suppressive effect of GKN1 on epigenetic regulators and c-Myc in AGS cells transfected with *GKN2* and *GKN1* plus *GKN2*.

10974652, 2014, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/jcp.24496 by University Of Southern Califor, Wiley Online Library on [07/08/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

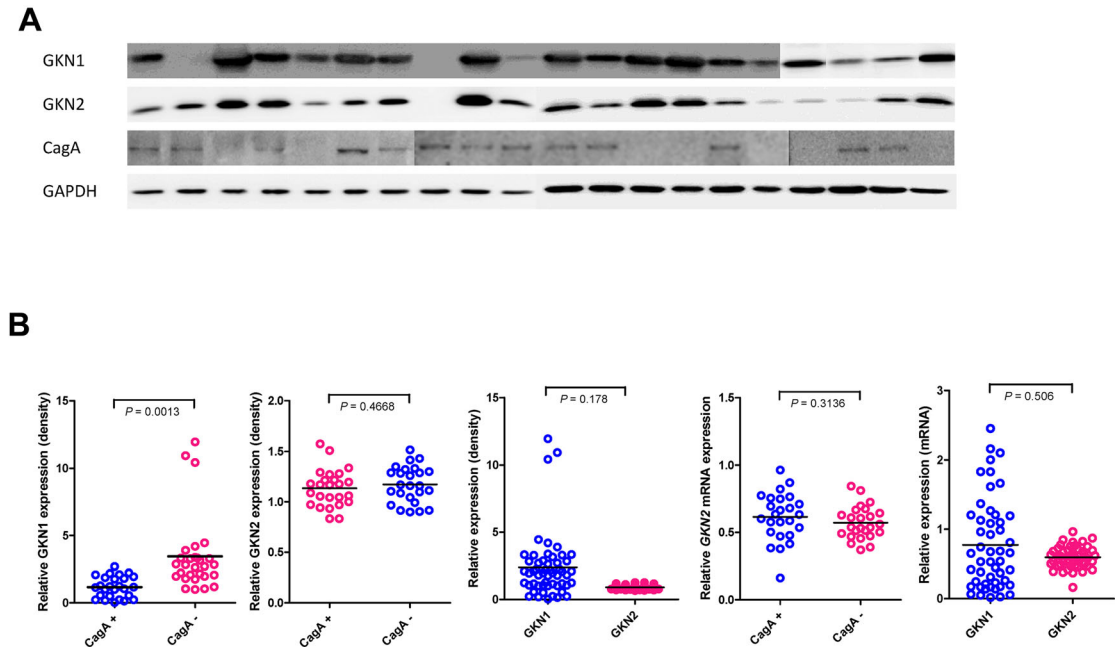

Fig. 5. GKN1, GKN2, and CagA expression in non-neoplastic gastric mucosa. A: Expression of GKN1 and GKN2 was detected in 47 (94%) and 48 (96%) out of 50 non-neoplastic gastric mucosa tissues, respectively. Positivity for *H. pylori* CagA was present in 25 gastric mucosa tissues (50%). Statistically, there was a close correlation between the expression of GKN1 and GKN2 ($P = 0.0074$). B: GKN1 protein expression was significantly lower in *H. pylori* CagA-positive gastric mucosa compared with CagA-negative mucosa ($P = 0.0013$). GKN2 mRNA and protein expression was not associated with CagA positivity ($P = 0.3136$ and $P = 0.4668$, respectively).

Next, we examined the effects of GKN1 and TNF-α on *GKN2* mRNA and protein expression. We found that TNF-α decreased GKN2 expression in both HFE-145 and AGS cells and GKN1 conversely increased GKN2 expression ($P < 0.05$; Fig. 6D). However, silencing of GKN1 in HFE-145 cells decreased the expression of GKN2 (Fig. 6E). In addition, TNF-α treatment of *GKN1*-transfected AGS cells rescued GKN2 expression. These results suggest that GKN1 may increase GKN2 expression through inactivation of the NF-κB pathway and activation of IκB in gastric cancer cells. Nevertheless, further studies are needed to clarify the exact mechanisms regulating GKN1-dependent expression of GKN2.

Expression of GKN1 and GKN2 in gastric cancers by immunohistochemistry

Immunohistochemical staining of gastric mucosal cells revealed moderate-to-strong immunopositivity for GKN2, mainly in the cytoplasm (Fig. 6F). A reduction or loss of GKN2 expression was detected in 12 (75%) and 114 (74.5%) of upper and lower tumor locations, respectively. According to Lauren's classification, GKN2 was expressed in 11 (15.5%) of 71 intestinal-type and 32 (32.7%) of 98 diffuse-type gastric cancers (Table I). Statistically, there was no significant relationship between altered expression of GKN2 protein and clinicopathologic parameters, including tumor location, tumor size, and lymph node metastasis (χ^2 test, $P > 0.05$, Table I). However, loss of GKN2 expression was more frequent in intestinal-type gastric cancers ($P = 0.0114$). Previously, we found reduction or loss of GKN1 expression in 151 (89.3%) of 169 gastric cancers (Yoon et al., 2011). When we compared GKN2 and GKN1 expression in these cases, 119 (70.4%) showed immunonegativity for both GKN1 and GKN2 proteins and there was a close correlation between GKN1 and GKN2 expression ($P = 0.0002$; Table I).

Discussion

In response to damage, protective and reparative mechanisms rapidly restore gastric mucosal integrity by stimulating the migration of epithelial cells over denuded areas ('restitution'), increasing mucus production, and re-establishing epithelial proliferation and differentiation programmes (Baus-Loncar et al., 2007). GKN1 performs a mucosal barrier role to prevent gastric mucosa from systemic exposure to foreign antigens, bacteria, and gastric acid (Shiozaki et al., 2001; Oien et al., 2004). It is possible that inactivation of GKN1 renders the gastric mucosa vulnerable to carcinogens or gastric injury, and eventually evokes genetic alterations of cancer-related genes. Interestingly, GKN2 expression is reduced in *H. pylori* infected gastritis (Resnick et al., 2006) and gastric cancers (Du et al., 2003), prompting recognition of GKN2 as a putative stomach-specific tumor suppressor gene and implying its potential utility in clinical prediction/diagnosis (Menheniott et al., 2013). However, the precise role of GKN2 and the molecular mechanisms that maintain gastric homeostasis remain to be elucidated.

Previously, we reported that GKN1 has a tumor suppressor function in HFE-145 and AGS cells, inhibiting cell proliferation and inducing apoptosis (Yoon et al., 2011; Yoon et al., 2013a). In this study, we examined whether GKN2 maintains gastric homeostasis by inhibiting GKN1. Although GKN2 alone did not have any effect on cell viability, proliferation, and apoptosis, it inhibited the effects of GKN1 on these cellular processes (Figs. 1 and 2). In cell cycle analysis, GKN1 dramatically increased the number of cells in G2/M phase (Fig. 3). However, GKN2 completely inhibited GKN1-induced G2/M arrest and the effect of GKN1 on the expression of G0/G1- and G2/M-related cell cycle regulators (Fig. 3A,B). These findings are consistent with the results of GKN1 silencing by *shGKN1* and transfection with anti-miR-185 (Yoon et al., 2013a) and

10974652, 2014, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/jcp.24496 by University Of Southern Califor, Wiley Online Library on [07/08/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

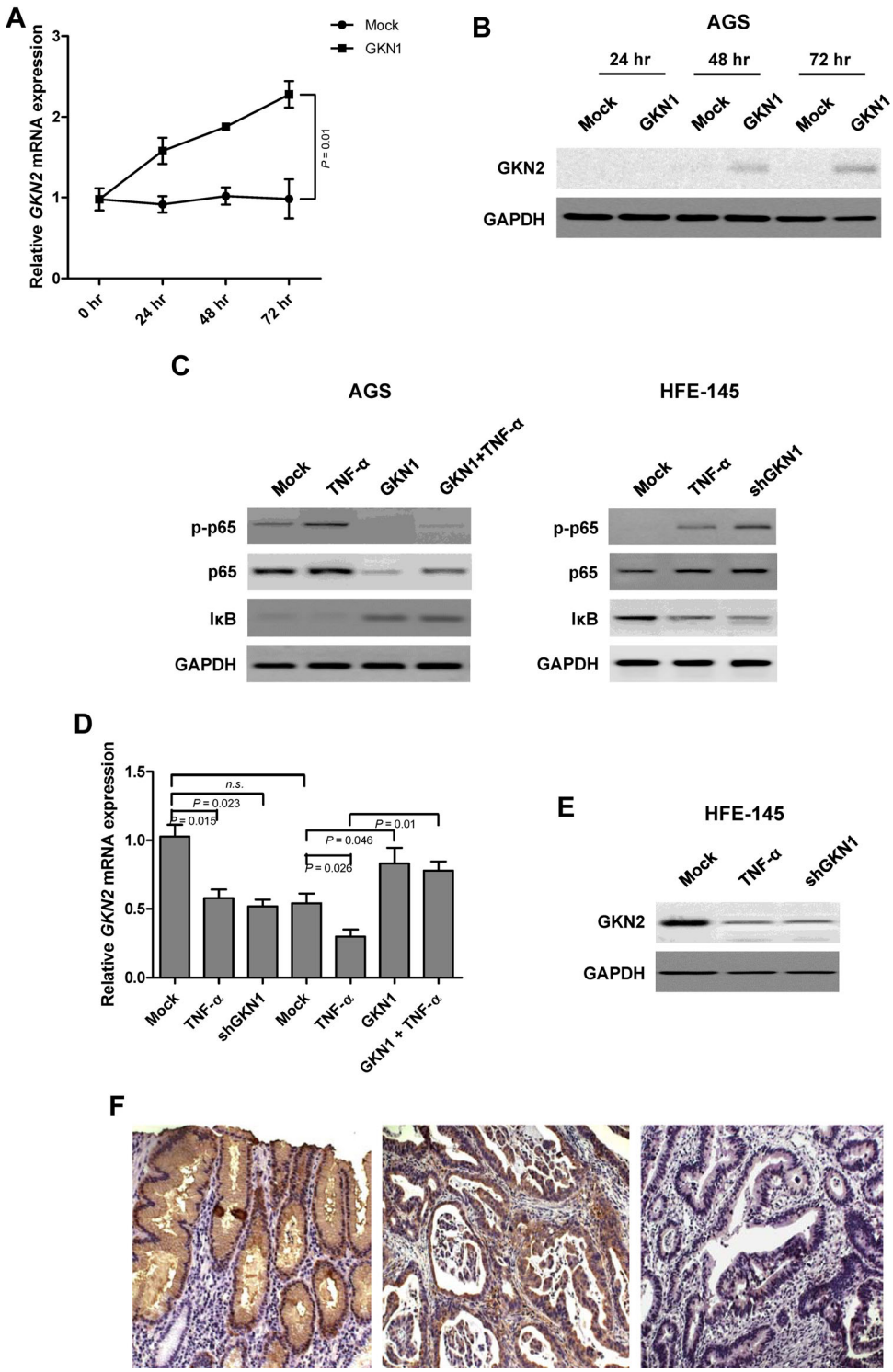

Fig. 6. GKN1 regulates GKN2 expression through inactivation of the NF-κB pathway and activation of IκB. Ectopic expression of GKN1 increased GKN2 mRNA (A) and protein (B) expression in a time-dependent manner. C: TNF-α increased p-p65 and p65 expression and decreased IκB expression, whereas GKN1 down-regulated NF-κB expression and up-regulated IκB expression. Repression of GKN1 expression in HFE-145 cells transfected with shGKN1 resulted in increased p-p65 and p65 expression and decreased IκB expression. D: Real-time RT-PCR showed that TNF-α decreased GKN2 expression in both HFE-145 and AGS cells. Conversely, GKN1 increased GKN2 expression in AGS cells. TNF-α treatment of *GKN1*-transfected AGS cells rescued GKN2 expression. E: Silencing of GKN1 in HFE-145 cells decreased the expression of GKN2. F: GKN2 immunostaining of gastric cancers showed intense GKN2 immunoreactivity in gastric mucosal epithelial cells (left part), strong immunoreactivity in intestinal-type gastric cancers (middle), and negative GKN2 staining in intestinal-type gastric cancers (right part).

10974652, 2014, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/jcp.24496 by University Of Southern Califor, Wiley Online Library on [07/08/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE I. Association between GKN2 and GKN1 expression and clinicopathologic parameters in gastric cancers

Parameters	GKN2 protein expression		
	+	−	*P*-value
Tumor location			0.9643
Upper	4	12	
Lower	39	114	
Size			0.6827
<6.5 cm	21	57	
>6.5 cm	22	69	
No. of metastatic L/N[a]			0.6643
N0 (=0)	0	4	
N1 (≥1 and ≤6)	15	44	
N2 (≥7 and ≤15)	26	74	
N3 (>15)	2	4	
Differentiation[b]			0.0114
Intestinal	11	60	
Diffuse	32	66	
GKN1 expression			0.0002
+	11	7	
−	32	119	

[a]L/N, lymph node.
[b]Lauren's classification.

strongly suggest that GKN2 plays an important role in maintaining the homeostasis of gastric mucosal epithelial cells by regulating GKN1 tumor suppressor activities.

To further examine the effect of GKN2 on epigenetic alterations that are regulated by GKN1 (Yoon et al., 2013a), we investigated the involvement of GKN2 in the regulation of miR-185, DNMT1, and EZH2. As expected, GKN2 completely downregulated GKN1-induced miR-185 expression (Fig. 4A) and inhibited the suppressive effect of GKN1 on DNMT1 and EZH2 expression (Fig. 4B). Furthermore, because GKN1 upregulates expression of primary, precursor, and mature miR-185 by binding to and downregulating c-Myc (Yoon et al., 2013a), we examined the effect of GKN2 on c-Myc. We found that GKN2 had no direct effect on c-Myc expression, but inhibited the suppressive activity of GKN1 on c-Myc (Fig. 4B). These results suggest that GKN2 may inhibit GKN1-induced miR-185-dependent tumor suppressor activity. However, our results are not consistent with a previous report that GKN2 inhibits gastric cancer growth in a TFF1-dependent manner (Chu et al., 2012). Thus, it is likely that in the presence of TFF1, GKN2 forms a heterodimer that is important for the tumor suppressor activity of TFF1, whereas GKN2 monomer inhibits anti-proliferative and pro-apoptotic activities of GKN1. Further studies to elucidate the exact functions of GKN2 might provide new insights into the mechanisms of gastric mucosal homeostasis.

To verify the association between GKN2 and GKN1 expression, we also analyzed mRNA and protein expression of GKN1 and GKN2 in 50 non-neoplastic gastric mucosal tissues. Interestingly, there was a close correlation between the expression of GKN1 and GKN2 ($P = 0.0074$). In addition, GKN1 protein expression was significantly lower in *H. pylori* CagA-positive gastric mucosa than in CagA-negative mucosa ($P = 0.0013$), whereas GKN2 mRNA and protein expression were not associated with CagA positivity ($P = 0.3136$ and $P = 0.4668$, respectively; Fig. 5B). These results suggest that GKN2 expression may be positively correlated with that of GKN1, and *H. pylori* CagA may be involved in regulation of GKN1 expression, but not GKN2 expression.

GKN1 and GKN2 are specifically expressed by surface mucous cells of the antrum and fundus (Toback et al., 2003). Like GKN1, GKN2 is downregulated during gastric cancer development. GKN2 was initially identified through the profound expression loss revealed by subtractive hybridization between gastric tumors and disease-free tissues (Du et al., 2003). Ubiquitous loss of GKN2 (TFIZ1) expression has been reported in gastric cancers and is significantly associated with lymph node metastasis in combination with high TFF1 expression (May et al., 2009). A large cohort study by Moss et al. showed that GKN2 loss correlated with shorter survival time in intestinal-type gastric cancer and that loss of GKN1 expression was positively correlated with loss of GKN2 (Moss et al., 2008). In the present study, reduction or loss of GKN2 expression was detected in 134 (79.3%) of 169 gastric cancers and was closely associated with differentiation of cancer cells and GKN1 expression (Table I). To define whether GKN1 affects GKN2 expression, we analyzed GKN2 mRNA and protein expression in *GKN1*-transfected AGS cells. Ectopic GKN1 expression increased both mRNA and protein expression of GKN2 in a time-dependent manner ($P = 0.01$; Fig. 6A,B). Conversely, silencing of GKN1 in HFE-145 cells decreased the expression of GKN2 (Fig. 6E).

Previously, Baus-Loncar et al. (2007) reported that GKN2 gene expression is significantly reduced in AGS and KATO-III gastric adenocarcinoma cell lines by the p50 and p65 subunits of NF-κB. Interestingly, *H. pylori* induces NF-κB stimulation and release of IL-8 and TNF-α in a *cag*PAI-dependent manner (Kumar Pachathundikandi et al., 2011) and co-transfection with NF-κB specific inhibitor (IkB) results in further upregulation of the *GKN2* gene (Baus-Loncar et al., 2007). In addition, Resnick et al. (2006) found that *GKN2* mRNA was the most strongly upregulated in the gastric transcriptome after *H. pylori* eradication, and GKN1 was similarly upregulated. In our previous study, we reported that GKN1 inhibits the NF-κB signaling pathway and cytokine expression (Yoon et al., 2013b). Therefore, we hypothesized that GKN1 might be involved in the regulation of GKN2 expression through inactivation of the NF-κB signaling pathway. As expected, while comparing the effects of TNF-α and GKN1 on p-p65, p65, and IκB expression in AGS cells, we observed that GKN1 suppressed the expression of p-p65 and p65 and increased the expression of IκB, whereas TNF-α upregulated expression of p-p65 and p65, and downregulated expression of IκB (Fig. 6C). However, repression of GKN1 expression in HFE-145 cells by transfection with *shGKN1* increased the expression p-p65 and p65 and decreased expression of IkB (Fig. 6C). Finally, we found that TNF-α decreased GKN2 mRNA and protein expression in both HFE-145 and AGS cells, whereas GKN1 conversely increased GKN2 expression in AGS cells ($P < 0.05$; Fig. 6D,E). Moreover, TNF-α treatment of *GKN1*-transfected AGS cells rescued GKN2 expression (Fig. 6D). Taken together, our data suggest that GKN2 expression might be regulated in a GKN1-dependent manner by inactivating the NF-κB signaling pathway and activating IκB, and negative feedback inhibition might occur in gastric epithelial cells to maintain homeostasis. However, GKN1 expression was positively correlated with that of GKN2 and *H. pylori* CagA, whereas there was no association between GKN2 and *H. pylori* CagA expression. Thus, we cannot completely rule out the possibility that another signaling pathway, independent of GKN1, is involved in the regulation of GKN2 expression. Thus, further studies are needed to identify the molecular mechanisms regulating transcription of the GKN1 and GKN2 genes.

In conclusion, in this study we describe a novel function of GKN2: inhibition of the effects of GKN1 on cell viability, proliferation, and apoptosis in the maintenance of gastric mucosal homeostasis. Interestingly, ectopic GKN2 expression significantly suppressed GKN1-induced anti-growth signaling by inhibiting miR-185 expression and inducing epigenetic modification. Furthermore, GKN2 expression was regulated in a GKN1-dependent manner by inactivation of the NF-κB signaling pathway. Finally, our results suggest that GKN2 has a determinative role in gastric mucosal homeostasis by regulating GKN1 activities. Additional and translational studies of GKN1

10974652, 2014, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/jcp.24496 by University Of Southern Califor, Wiley Online Library on [07/08/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

and GKN2 will broaden our understanding of gastric mucosal homeostasis and the pathogenesis of gastritis and gastric cancer.

Literature Cited

Arimura Y, Yano T, Hirano M, Sakamoto Y, Egashira N, Oishi R. 2012. Mitochondrial superoxide production contributes to vancomycin-induced renal tubular cell apoptosis. Free Radic Biol Med 52:1865–1873.

Baus-Loncar M, Lubka M, Pusch CM, Otto WR, Poulsom R, Blin N. 2007. Cytokine regulation of the trefoil factor family binding protein GKN2 (GDDR/TFIZ1/blottin) in human gastrointestinal epithelial cells. Cell Physiol Biochem 20:193–204.

Chu G, Qi S, Yang G, Dou K, Du J, Lu Z. 2012. Gastrointestinal tract specific gene GDDR inhibits the progression of gastric cancer in a TFF1 dependent manner. Mol Cell Biochem 359:369–374.

Du JJ, Dou KF, Peng SY, Wang WZ, Wang ZH, Xiao HS, Guan WX, Liu YB, Gao ZQ. 2003. Down-regulated full length novel gene GDDR and its effect on gastric cancer. Zhonghua Yi Xue Za Zhi 83:1166–1168.

Kononen J, Bubendorf L, Kallioniemi A, Bärlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP. 1998. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 4:844–847.

Kumar Pachathundikandi S, Brandt S, Madassery J, Backert S. 2011. Induction of TLR-2 and TLR-5 expression by Helicobacter pylori switches cagPAI-dependent signalling leading to the secretion of IL-8 and TNF-α. PLoS ONE 6:e19614.

Martin TE, Powell CT, Wang Z, Bhattacharyya S, Walsh-Reitz MM, Agarwal K, Toback FG. 2003. A novel mitogenic protein that is highly expressed in cells of the gastric antrum mucosa. Am J Physiol Gastrointest Liver Physiol 285:G332–G343.

May FE, Griffin SM, Westley BR. 2009. The trefoil factor interacting protein TFIZ1 binds the trefoil protein TFF1 preferentially in normal gastric mucosal cells but the co-expression of these proteins is deregulated in gastric cancer. Int J Biochem Cell Biol 41:632–640.

Menheniott TR, Kurklu B, Giraud AS. 2013. Gastrokines: Stomach-specific proteins with putative homeostatic and tumor suppressor roles. Am J Physiol Gastrointest Liver Physiol 304:G109–G121.

Moss SF, Lee JW, Sabo E, Rubin AK, Rommel J, Westley BR, May FE, Gao J, Meitner PA, Tavares R, Resnick MB. 2008. Decreased expression of gastrokine 1 and the trefoil factor interacting protein TFIZ1/GKN2 in gastric cancer: Influence of tumor histology and relationship to prognosis. Clin Cancer Res 14:4161–4167.

Nardone G, Rippa E, Martin G, Rocco A, Siciliano RA, Fiengo A, Cacace G, Malorni A, Budillon G, Arcari P. 2007. Gastrokine 1 expression in patients with and without Helicobacter pylori infection. Dig Liver Dis 39:122–129.

Oien KA, McGregor F, Butler S, Ferrier RK, Downie I, Bryce S, Burns S, Keith WN. 2004. Gastrokine 1 is abundantly and specifically expressed in superficial gastric epithelium, down-regulated in gastric carcinoma, and shows high evolutionary conservation. J Pathol 203:789–797.

Otto WR, Patel K, McKinnell I, Evans MD, Lee CY, Frith D, Hanrahan S, Blight K, Blin N, Kayademir T, Poulsom R, Jeffery R, Hunt T, Wright NA, McGregor F, Oien KA. 2006. Identification of blottin: A novel gastric trefoil factor family-2 binding protein. Proteomics 6:4235–4245.

Park WS, Oh RR, Park JY, Lee JH, Shin MS, Kim HS, Lee HK, Kim YS, Kim SY, Lee SH, Yoo NJ, Lee JY. 2000. Somatic mutations of the trefoil factor family 1 gene in gastric cancer. Gastroenterology 119:691–698.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45.

Resnick MB, Sabo E, Meitner PA, Kim SS, Cho Y, Kim HK, Tavares R, Moss SF. 2006. Global analysis of the human gastric epithelial transcriptome altered by Helicobacter pylori eradication in vivo. Gut 55:1717–1724.

Rippa E, La Monica G, Allocca R, Romano MF, De Palma M, Arcari P. 2011. Overexpression of gastrokine 1 in gastric cancer cells induces Fas-mediated apoptosis. J Cell Physiol 226:2571–2578.

Shiozaki K, Nakamori S, Tsujie M, Okami J, Yamamoto H, Nagano H, Dono K, Umeshita K, Sakon M, Furukawa H, Hiratsuka M, Kasugai T, Ishiguro S, Monden M. 2001. Human stomach-specific gene, CA11, is down-regulated in gastric cancer. Int J Oncol 19:701–707.

Toback FG, Walsh-Reitz MM, Musch MW, Chang EB, Del Valle J, Ren H, Huang E, Martin TE. 2003. Peptide fragments of AMP-18, a novel secreted gastric antrum mucosal protein, are mitogenic and motogenic. Am J Physiol Gastrointest Liver Physiol 285:G344–G353.

Walker DG, Link J, Lue LF, Dalsing-Hernandez JE, Boyes BE. 2006. Gene expression changes by amyloid β-peptide-stimulated human postmortem brain microglia identify activation of multiple inflammatory processes. J Leukoc Biol 79:596–610.

Walsh-Reitz MM, Huang EF, Musch MW, Chang EB, Martin TE, Kartha S, Toback FG. 2005. AMP-18 protects barrier function of colonic epithelial cells: Role of tight junction proteins. Am J Physiol Gastrointest Liver Physiol 289:G163–G171.

Westley BR, Griffin SM, May FE. 2005. Interaction between TFF1, a gastric tumor suppressor trefoil protein, and TFIZ1, a brichos domain-containing protein with homology to SP-C. Biochemistry 44:7967–7975.

Xing R, Li W, Cui J, Zhang J, Kang B, Wang Y, Wang Z, Liu S, Lu Y. 2012. Gastrokine 1 induces senescence through p16/Rb pathway activation in gastric cancer cells. Gut 61:43–52.

Yoon JH, Song JH, Zhang C, Jin M, Kang YH, Nam SW, Lee JY, Park WS. 2011. Inactivation of the Gastrokine 1 gene in gastric adenomas and carcinomas. J Pathol 223:618–625.

Yoon JH, Choi YJ, Choi WS, Ashktorab H, Smoot DT, Nam SW, Lee JY, Park WS. 2013a. GKN1-miR-185-DNMT1 axis suppresses gastric carcinogenesis through regulation of epigenetic alteration and cell cycle. Clin Cancer Res 19:4599–4610.

Yoon JH, Cho ML, Choi YJ, Back JY, Park MK, Lee SW, Choi BJ, Ashktorab H, Smoot DT, Nam SW, Lee JY, Park WS. 2013b. Gastrokine 1 regulates NF-κB signaling pathway and cytokine expression in gastric cancers. J Cell Biochem 114:1800–1809.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

10974652, 2014, 6, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/jcp.24496 by University Of Southern Califor, Wiley Online Library on [07/08/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License