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DNA Methylation-Mediated Silencing of Regenerating Protein 1 Alpha (REG1A) Affects Gastric Cancer Prognosis

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Data Collection B
Statistical Analysis C
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Manuscript Preparation E
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Background: Gastric cancer (GC) is one of the most common cause of cancer-related deaths. The clinical trials still lack the effective methods to treat or monitor the disease progression. In this research, the biological function and the underlying molecular mechanism of regenerating protein 1 alpha (REG1A) in GC were investigated.

Material/Methods: Gene expression omnibus (GEO), KMplot datasets and GC tissue microarray (n=164) were used to analyze the expression of REG1A and related patient prognoses in GC. Transwell matrigel assay, flow cytometry analysis and CCK8 cell viability assay were performed to detect the biological functions of REG1A. Western blotting and real-time PCR were used to detect the REG1A expression and PI3K/Akt related signaling.

Results: It was found that the expression of REG1A was significantly downregulated in GC and closely related with clinicopathological findings or patient prognoses. REG1A overexpression could suppress the invasion, cell viability and promote the apoptosis of GC cells. Moreover, we found that the epigenetic methylation suppressed the expression level of REG1A in GC, and REG1A overexpression could suppress the phosphorylation of Akt or GSK3 β signaling.

Conclusions: Taken together, REG1A regulates cell invasion, apoptosis and viability in GC through activating PI3K/Akt-GSK3 β signaling. REG1A may serve as a promising therapeutic strategy for GC.

MeSH Keywords: **Apoptosis • Neoplasm Invasiveness • Stomach Neoplasms**

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Background

Gastric cancer (GC) is the fifth most common malignancy and the third leading cause of cancer-related death worldwide, and half of the world total cases occur at Eastern Asia. GC has 2 distinct morphologic subtypes: gastric intestinal type adenocarcinoma and diffuse gastric adenocarcinoma [1,2]. Gastric intestinal type adenocarcinoma is often associated with intestinal metaplasia and *Helicobacter pylori* infection, and diffuse gastric adenocarcinoma is more often seen in female and young individuals [3]. It has been known that GC included frequent inactivating mutations in cell adhesion and chromatin remodeling genes in addition to TP53 mutations [4,5]. Although some essential factors for resolution were identified in recent years, but the clinical trials still lack the effective methods to treat or monitor the disease progression [6–9].

Regenerating protein 1 alpha (REG1A) is a secreted protein containing 166 amino acids and belongs to the calcium dependent lectins superfamily. In humans, 4 different subtypes of REG genes have been known: REG1A, REG1B, REG3A and REG4. REG1A was first identified by screening a cDNA library of regenerating rat pancreatic islet cells and has been primarily implicated in the regeneration of pancreatic b cells and in the amelioration of diabetes mellitus [10–13]. Previous studies have reported that REG1A play important roles in various cancers, such as: advanced thoracic esophageal squamous cell carcinoma [14], cutaneous melanoma [15], lung cancer [16], hepatocellular carcinoma (HCC) [17], colorectal cancer [18–20], breast cancer [21], bladder cancer [22]. Furthermore, REG1A was also reported to be related with the anti-apoptotic effect and angiogenesis in GC [23–25], but other biological functions, like the invasion and proliferation of GC cells, have not been investigated yet. In this research, we will deeply investigate the biological functions of REG1A and uncover related underlying mechanism.

Here, we found that the expression of REG1A was significantly downregulated in GC. REG1A expression was closely correlated with clinicopathological findings and patient prognoses. DNA methylation-mediated silencing of REG1A could regulate the invasion, apoptosis and viability of GC cells through phosphatidylinositol 3 kinase (PI3K)/Akt-GSK3 β signaling.

Material and Methods

Cell culture

Human GC cell lines, including AGS, BGC-823, HGC-27, MGC-803, MKN-45 and SGC-7901 cells, and human gastric epithelial cell line GES-1 were purchased from Cell Bank of the Chinese Academy of Sciences. RPMI-1640 medium supplemented with 10% (v/v)

fetal calf serum (FCS) and 1% antibiotics was used here. Cells were placed at 37°C in a humidified incubator with 5% CO $_2$.

Clinical samples

A human tissue microarray containing 164 cases of GC samples was obtained from the Department of Bone Tumor, Yantai Mountain Hospital. All human materials were obtained with informed consent, and protocols were approved by the Ethics Review Committee of the World Health Organization Collaborating Center for Research in Human Production.

Immunohistochemical staining

Human tissue microarray was deparaffinized and rehydrated for histopathological evaluation. For immunohistochemical staining, the section was incubated with 0.3% hydrogen peroxide/phosphate-buffered saline for 30 min and blocked with 10% BSA (Sangon). Slides were first incubated using the antibody for REG1A (Abcam) at 4°C overnight with optimal dilution, labeled by HRP second antibody (Abcam) at room temperature for 1 h. Then, the section was treated with DAB substrate liquid (Thermo) and counterstained by hematoxylin. The section was observed and photographed with a microscope (Carl Zeiss). The final expression of REG1A was designated as low or high expression group as follows: 0–35% positive staining was low expression and more than 36% positive staining was high expression.

Quantitative real-time PCR

Total RNA was extracted by Trizol (Takara), and reverse transcribed by PrimeScript RT-PCR kit (Perfect Real-Time). Quantitative real-time PCR analyses were performed with SYBR Premix Ex Taq (Takara) on a 7500 real-time PCR system (Applied Biosystems).

Western blotting

Cells were lysed in lysis buffer and proteins were separated by SDS-PAGE under reducing condition. The membrane was blocked in phosphate-buffered saline/Tween-20 containing 5% BSA. Then, the antibodies for REG1A (Abcam), phospho-Akt (Cell Signaling), total-Akt (Cell Signaling), phospho-GSK3 β (Cell Signaling), total-GSK3 β (Cell Signaling), GAPDH (Sigma), and species-specific secondary antibodies were used to incubate the membrane separately. The secondary antibodies were detected using the Odyssey imaging system (LI-COR).

Lentivirus production and cell transduction

We cotransfected 293T cells with pEZ-lv105 vector (GeneCopoeia) by using Lipofectamine 2000 (Invitrogen) for virus packaging. Viruses were harvested at 24, 48, and 72 h

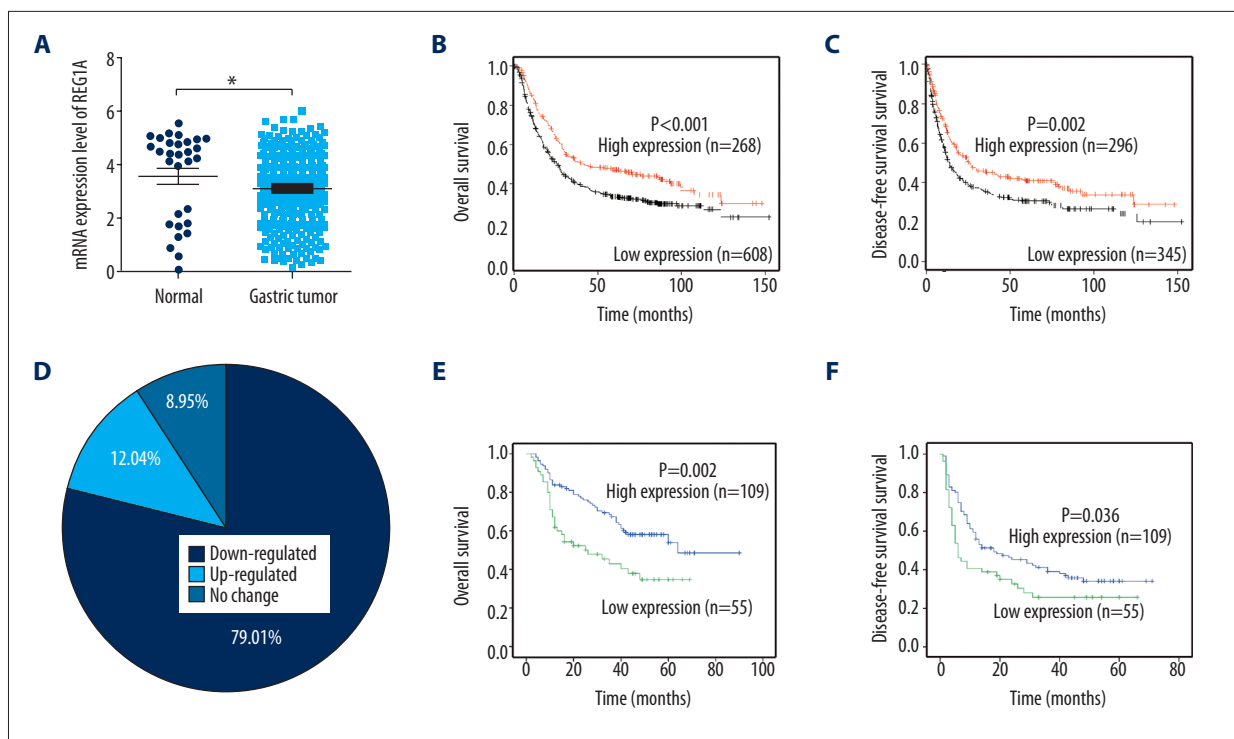


Figure 1. The expression of REG1A is downregulated in gastric cancer (GC) tissues and closely related with patient prognoses. (A) REG1A mRNA expression level in GC and normal gastric tissues. We obtained this dataset from TCGA. ** $P < 0.01$. (B, C) KMplot analysis of overall survival (OS) (B, $P < 0.001$) and disease-free survival (DFS) (C, $P = 0.002$) for the expression of REG1A. (D) The expression of REG1A was downregulated in 79.01% of GC tissues. (E, F) Kaplan-Meier analysis of OS (E, $P = 0.002$) and DFS (F, $P = 0.036$) for the expression of REG1A in 164 cases GC tissue microarray.

after transfection. After determining virus titers, 1×10^5 cells were infected with 1×10^6 recombinant lentivirus-transducing units by using 6 $\mu\text{g/ml}$ polybrene (Sigma).

Invasion assays

Cells were detached and resuspended in serum-free RPMI-1640 medium. We placed 2×10^4 cells in 0.1 ml in matrigel (BD)-coated inserts (Millipore) seated on the 24-well plate, then we added 5% FBS RPMI-1640 medium to the bottom chamber. Cells were incubated at 37°C . After 48 h, filters were fixed and stained with 0.1% (w/v) Crystal Violet. Non-invading cells were removed, and invading cells were counted under a microscope at $400\times$. Three grids per field were counted and the experiments were repeated twice.

Apoptosis assays

We placed 5×10^5 cells/well in 12-well plates and serum starved for 48 h at 37°C . After 48 h, adherent cells were detached harvested in complete RPMI-1640 medium. After centrifuging at 1000 rpm for 5 min, the cells were washed with $1 \times \text{PBS}$ and stained with 100 μl binding buffer containing 3 μl Annexin V and 3 μl propidium iodide (PI). After incubating at room temperature for 15 min, the cells were analyzed by flow cytometry (BD).

DAC and TSA treatment

Cells were treatment with 5 or 10 μM of 5-aza-2'-deoxycytidine (DAC, Sigma-Aldrich) or 300 or 600 nM Trichostatin A (TSA, Selleckchem) for 3 days and drug in medium were replaced every 24 h. Control cells were incubated with the same volume of DMSO.

Statistical analysis

Data are presented as means \pm standard error of the mean (SEM). Survival time was analyzed by Kaplan-Meier method. The Pearson's χ^2 test and t test were used for comparisons between groups.

Results

REG1A is downregulated in GC tissues and is closely related with vascular embolism, tumor size, and patient prognoses

To investigate the expression of REG1A in GC, we first analyzed the microarray data from the Gene expression omnibus (GEO). The serial number of the GEO dataset used in this research was GSE 62944. We downloaded the raw data of GSE62944 from

The Cancer Genome Atlas (TCGA). It was shown that the infection of *H. pylori* virus was not found in these patients. The dataset showed that the expression level of REG1A was significantly downregulated in GC tissues compared with normal gastric tissues (Figure 1A). By analyzing data from KMplot, we found that REG1A expression was closely related with patient prognoses. High REG1A expression was associated with improved overall survival (OS) ($P < 0.001$) and disease-free survival (DFS) ($P = 0.002$) (Figure 1B, 1C).

To further investigate the clinical significance of REG1A in GC, we used a GC tissue microarray containing 164 samples. We performed immunohistochemistry to detect the positive staining of REG1A in 164 cases GC tissue microarray. We found that the expression of REG1A was significantly downregulated in 79.01%, up-regulated in 12.04%, and no change in 8.95% of GC patients, compared with normal gastric tissues (Figure 1D). REG1A expression was closely related with tumor size, vascular embolism, differentiation, patient smoking history, and TNM stage (Table 1). We also found that high REG1A expression was associated with improved OS ($P = 0.002$) and DFS ($P = 0.036$) of these patients (Figure 1E, 1F).

Overexpression of REG1A suppresses the cell viability of GC cells

To investigate the biological functions of REG1A in GC, we detected the relative mRNA expression level of REG1A in 6 GC cell lines and human gastric epithelial cell line GES-1. As shown in Figure 2A, we found that the expression level of REG1A in MGC-803 and BGC-823 cells was obviously lower than in other GC cells. Then, we established stable cell lines transduced by the lentivirus carrying the REG1A gene, named as Lenti-REG1A, in MGC-803 and BGC-823 cells. By real-time PCR and Western blotting analysis, we found that REG1A was overexpressed in MGC-803 (Figure 2B, 2D) and BGC-823 cells (Figure 2C, 2E).

By CCK8 cell viability assay, we investigated the cell viability of MGC-803 and BGC-823 cells after REG1A overexpression. We found that the cell viability of MGC-803 and BGC-823 cells was significantly suppressed by REG1A overexpression at 24, 48, and 72 h (Figure 2F, 2G).

Overexpression of REG1A suppresses the invasion and promotes the apoptosis of GC cells

We further investigated the invasion and apoptosis of MGC-803 and BGC-823 cells after REG1A overexpression. By transwell matrigel invasion assay, we found that REG1A overexpression suppressed the invasiveness of MGC-803 and BGC-823 cells after 48 h (Figure 3A, 3B). After the cells were serum starved for 48 h, we found that REG1A overexpression significantly promoted the apoptosis of GC cells by flow cytometry analysis.

The results showed that the apoptosis rate was obviously increased by REG1A overexpression in MGC-803 and BGC-823 cells (Figure 3C, 3D).

REG1A overexpression has no effect on gastric epithelial cells, but REG1A knockdown increases the proliferation of gastric epithelial cells

We used a non-cancer cell line-gastric epithelial cell line (GES-1 cell) to further investigate the biological functions of REG1A. We overexpressed the REG1A gene in GES-1 cells (Figure 4A). By performing transwell matrigel invasion assay and flow cytometry analysis, we found that REG1A overexpression had no effects on the invasion and apoptosis of GES-1 cells (Figure 4C). Additionally, by CCK8 cell viability assay, we found that the cell viability of GES-1 cells was not affected by REG1A overexpression (Figure 4D).

Furthermore, we knocked down REG1A in GES-1 cells by siRNA (Figure 4B). We found that REG1A knockdown increased the proliferation of GES-1 cells at 48 and 72 h, but it had no effects on the invasion and apoptosis of GES-1 cells (Figure 4E, 4F).

DNA methylation silences REG1A expression in GC, and REG1A overexpression suppresses the phosphorylation of Akt and GSK3 β signaling

We further investigated why REG1A was downregulated in GC. Using 5-aza-2'-deoxycytidine (DAC), a specific methyltransferase inhibitor, and Trichostatin A (TSA), a histone deacetylase inhibitor, we found that methylation occurred in all of the 4 tested GC cell lines, and histone acetylation existed in 1 of them. These data indicate that epigenetic methylation suppresses the expression level of REG1A in GC (Figure 5A–5D).

To uncover the underlying mechanism of REG1A in GC, we detected PI3K/Akt-related signaling pathway in REG1A-overexpressed MGC-803 cells and control cells by Western blotting. Interestingly, we found that the phosphorylation of Akt and GSK3 β was significantly suppressed by the overexpression of REG1A (Figure 5E, 5F), suggesting that the regulation of REG1A in GC cell invasion, apoptosis and viability are mainly dependent on PI3K/Akt-GSK3 β signaling.

Discussion

In previous studies, REG1A was shown to act as a factor to reduce epithelial apoptosis in inflammation [26]. In recent years, it was found that REG1A plays important roles in the tumorigenesis of various types of human cancer [16–22]. But some biological functions and related mechanism of REG1A in GC remain unclear. In our research, the exact role of REG1A in GC

Table 1. Correlation of the clinicopathological findings with REG1A expression. Pearson's χ^2 test was used. The bolding stands for P values with significant differences.

Variable	REG1A (n)		P
	High	Low	
Age	≤50 years	39	0.102
	>50 years	52	
Gender	Female	11	0.912
	Male	80	
Smoking history	Yes	21	<0.001
	No	70	
Lauren subtype	Diffuse	26	0.713
	Intestine	65	
Location	Upper	12	0.682
	Middle	15	
	Lower	21	
	Remnant stomach	43	
Tumor size	≤5 cm	68	<0.001
	>5 cm	23	
Differentiation	well	36	<0.001
	moderate	44	
	poor	11	
Vascular embolism	Yes	16	<0.001
	No	75	
Alcohol habits	Yes	13	0.121
	No	78	
TNM stage	I	65	<0.001
	II	17	
	III	9	

Pearson's χ^2 test was used. The bolding stands for the P-values with significant differences.

was investigated. By analyzing GEO and KMplot datasets, we found that the expression of REG1A was obviously downregulated in GC. High REG1A expression was associated with improved OS and DFS of GC patients. We further confirmed that REG1A expression was closely related with tumor size, vascular embolism, differentiation, patient smoking history, TNM stage, and prognoses of patients by using GC tissue microarray, showing that REG1A may play an important role in the development of GC.

Further, it has been reported that REG1A plays an important role in the anti-apoptotic effect and angiogenesis in GC [23–25]. In the present study, we revealed that REG1A also affected other

biological functions of GC cells. We found that the invasion and viability of GC cells were suppressed, and the apoptosis of GC cells was supported by REG1A overexpression. REG1A overexpression was proved to have no effects on the invasion, apoptosis or cell viability of non-cancer cell line-gastric epithelial cells. These data indicate that REG1A regulates the invasion, apoptosis, and viability of GC cells, meaning it is involved in the progression of GC.

It is generally considered that, during carcinogenesis processes, epigenetic modifications of the genome, including DNA methylation and histone modification, are established as a consequence of interactions between cellular lineage and

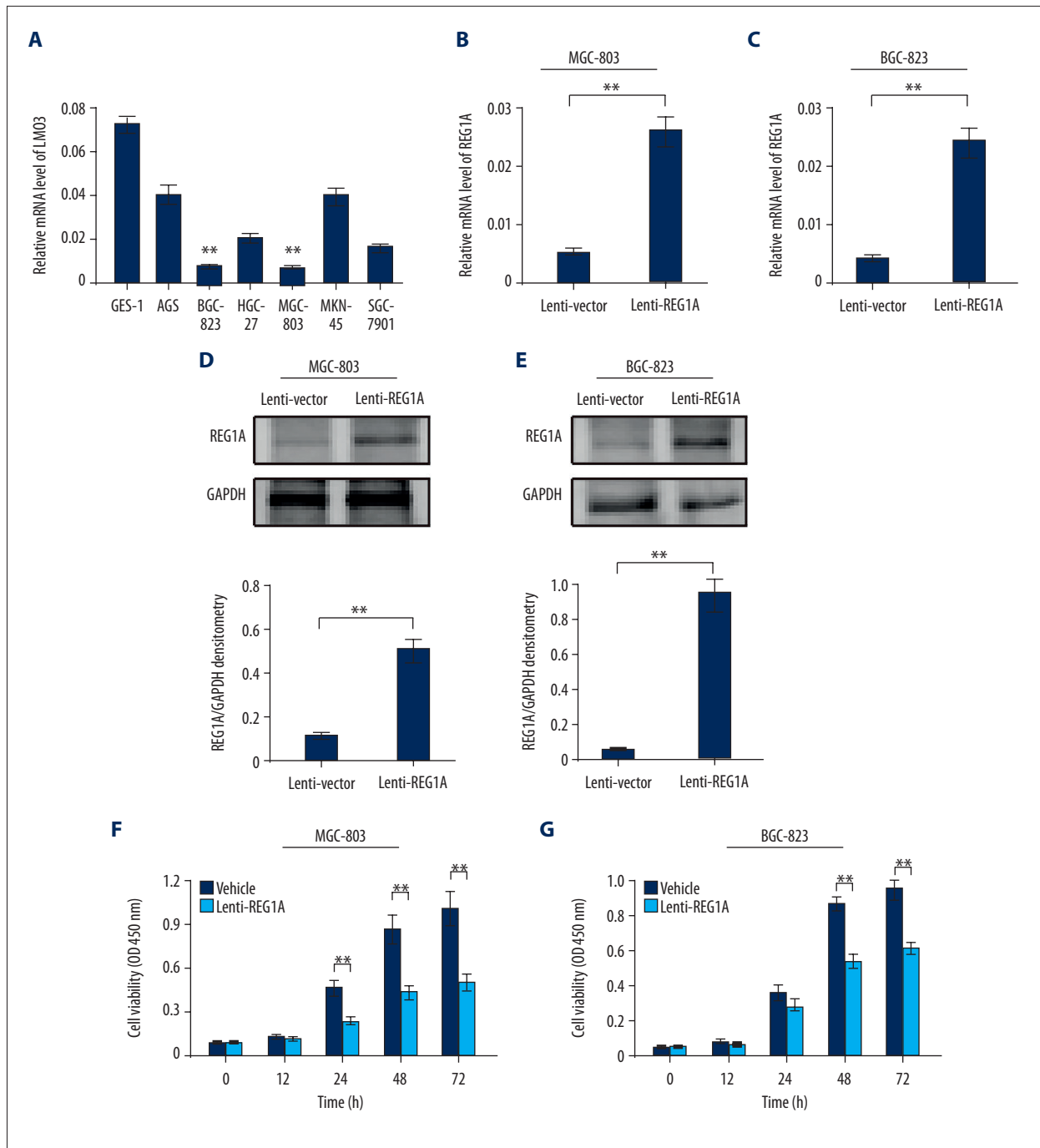


Figure 2. Overexpression of REG1A reduces the cell viability of GC cells. **(A)** The mRNA expression level of REG1A in 6 GC cell lines and human gastric epithelial cell line GES-1. **(B, C)** REG1A mRNA expression level in MGC-803 **(B)** and BGC-823 **(C)** GC cells, which were infected with lenti-vector or lenti-REG1A. **(D, E)** REG1A protein expression level in MGC-803 **(D)** and BGC-823 **(E)** GC cells, which were infected with lenti-vector or lenti-REG1A. Statistical analysis of REG1A expression in the 2 groups is shown below. **(F)** CCK8 assay analysis of MGC-803 cells infected with lenti-vector or lenti-REG1A at 0, 12, 24, 48, and 72 h. **(G)** CCK8 assay analysis of BGC-823 cells infected with lenti-vector or lenti-REG1A at 0, 12, 24, 48, and 72 h. ** $P < 0.01$.

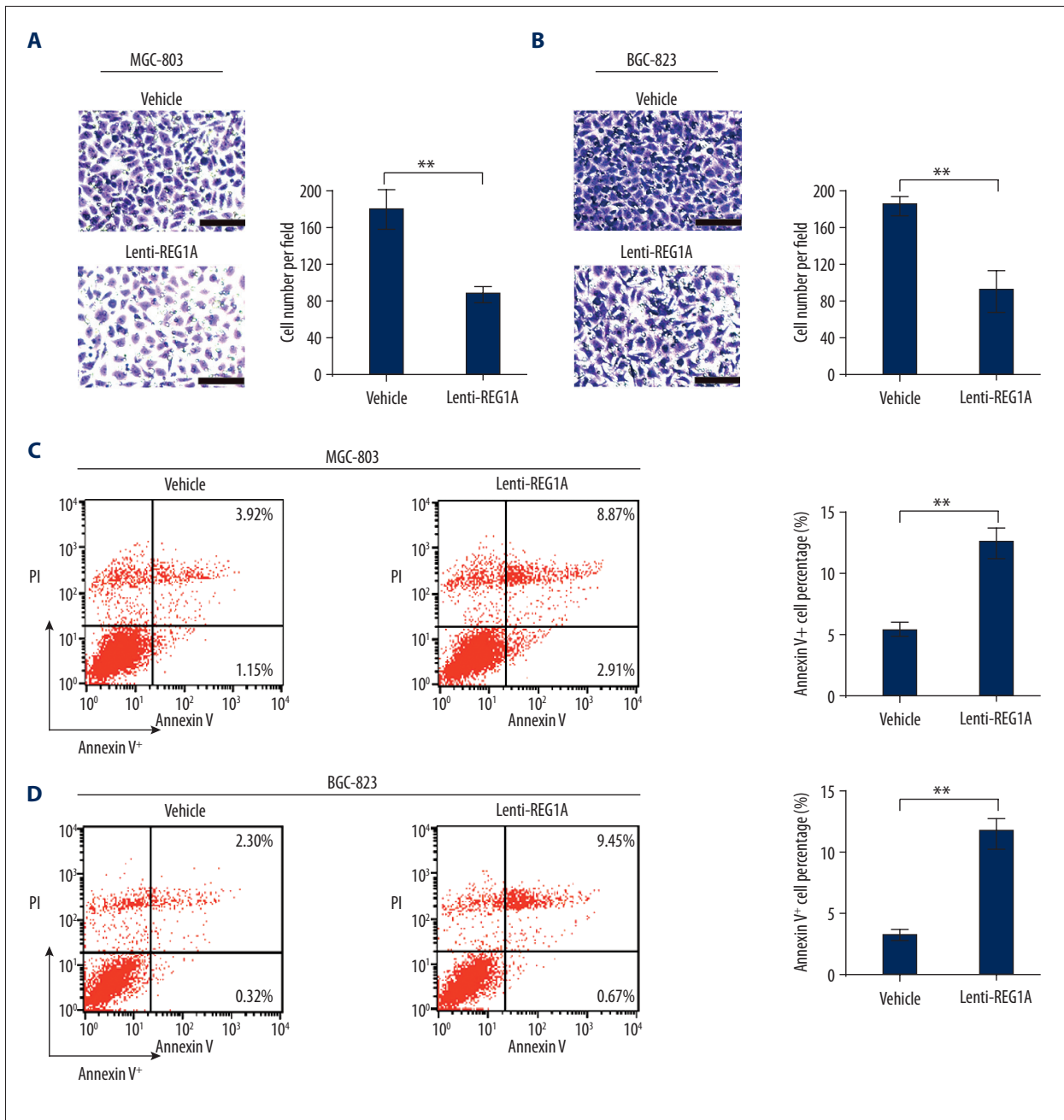


Figure 3. REG1A overexpression suppresses the invasion and supports the apoptosis of GC cells. **(A)** Representative photos of invaded MGC-803 cells infected with lenti-vector or lenti-REG1A. Statistical analysis of invaded MGC-803 cells in the 2 groups is shown right. Scale bars: 10 μ m. **(B)** Representative photos of invaded BGC-823 cells infected with lenti-vector or lenti-REG1A. Statistical analysis of invaded BGC-823 cells in the 2 groups is shown right. Scale bars: 10 μ m. **(C)** Flow cytometry analysis of apoptotic MGC-803 cells infected with lenti-vector or lenti-REG1A. Statistical analysis of apoptotic MGC-803 cells in the 2 groups is shown right. **(D)** Flow cytometry analysis of apoptotic BGC-823 cells infected with lenti-vector or lenti-REG1A. Statistical analysis of apoptotic BGC-823 cells in the 2 groups is shown at right. ** $P < 0.01$.

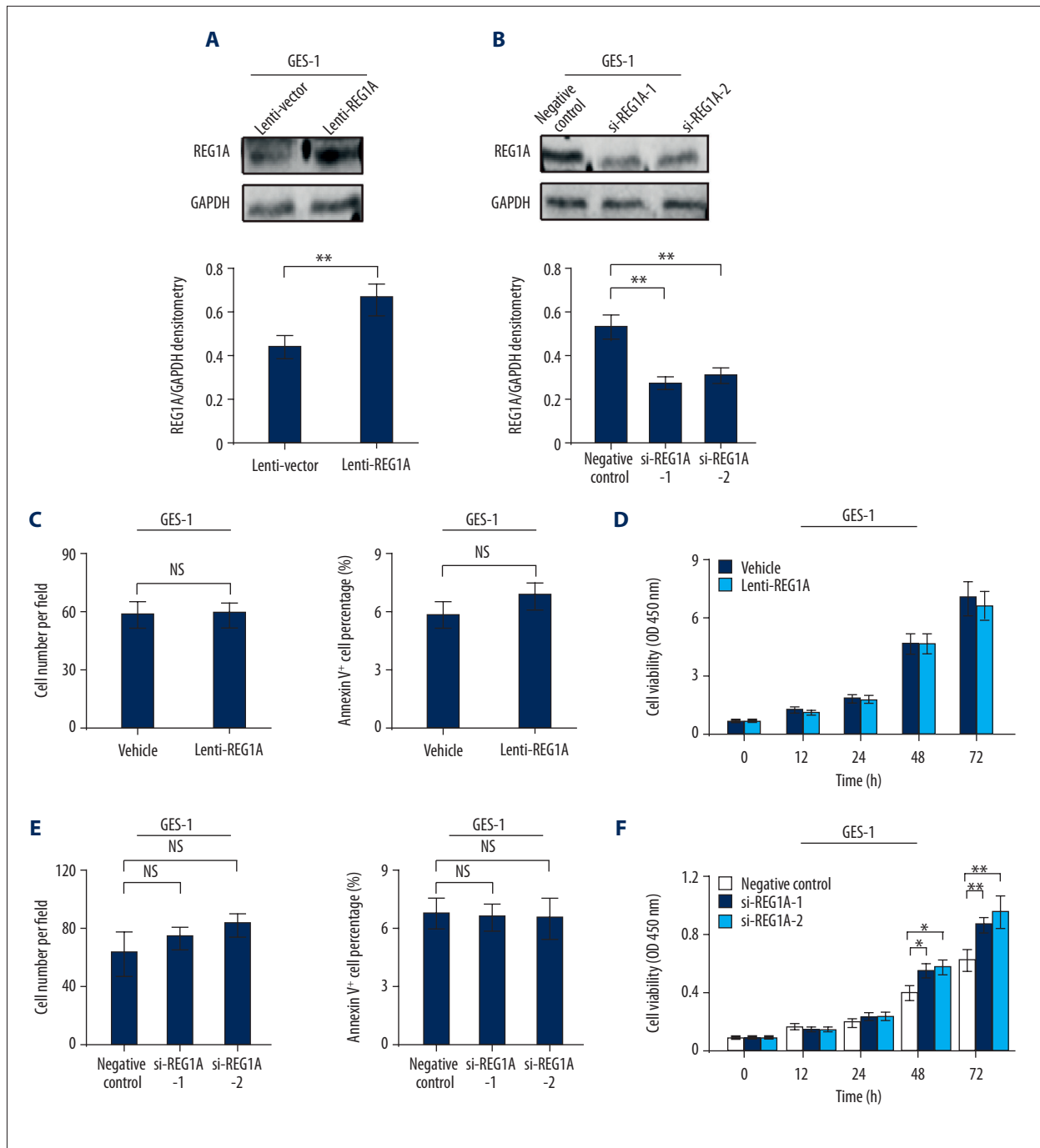


Figure 4. REG1A overexpression has no effect on gastric epithelial cells, while REG1A knockdown promotes the proliferation of gastric epithelial cells. (A) REG1A protein expression level in GES-1 cells, which were infected with lenti-vector or lenti-REG1A. Statistical analysis of REG1A expression in the 2 groups is shown below. (B) REG1A protein expression level in GES-1 cells, which were infected with siRNA of REG1A. Statistical analysis of REG1A expression is shown below. (C) Statistical analysis of invaded or apoptotic GES-1 cells infected with lenti-vector or lenti-REG1A. (D) CCK8 assay analysis of GES-1 cells infected with lenti-vector or lenti-REG1A at 0, 12, 24, 48, and 72 h. (E) Statistical analysis of invaded or apoptotic GES-1 cells infected with siRNA of REG1A. (F) CCK8 assay analysis of GES-1 cells infected with siRNA of REG1A at 0, 12, 24, 48, and 72 h. * $P < 0.05$, ** $P < 0.01$.

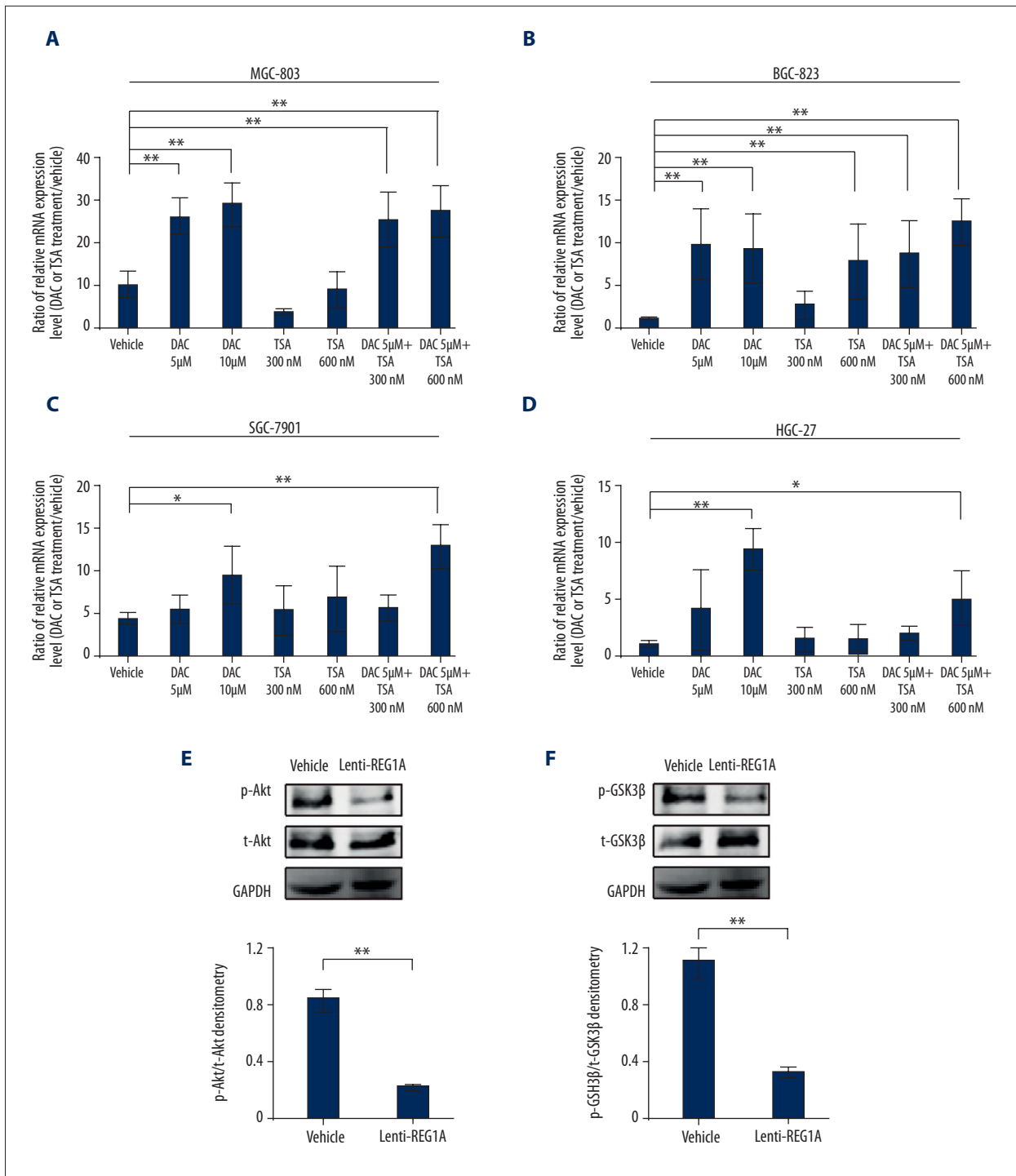


Figure 5. Epigenetic methylation suppresses REG1A expression in GC, and overexpression of REG1A suppresses Akt and GSK3β phosphorylation. (A–D) Relative mRNA expression levels of REG1A in MGC-803 (A), BGC-823 (B), SGC-7901 (C), and HGC-27 (D) cells after treatment with DAC and TSA. (E) Western blotting analysis of phospho-Akt and total-Akt in REG1A overexpressed and control MGC-803 cells. Statistical analysis of phospho-Akt/total-Akt densitometry is shown below. (F) Western blotting analysis of phospho-GSK3β and total-GSK3β in REG1A overexpressed and control MGC-803 cells. Statistical analysis of phospho-GSK3β/total-GSK3β densitometry is shown below. ** $P < 0.01$.

environmental input. However, increasing evidence indicates that epigenetic changes are responsible for initiation, proliferation, invasion, and metastasis of tumor cells by affecting the tumor microenvironment [27,28]. In the present study, by treatment of DAC and TSA in GC cell lines, we proved that epigenetic DNA methylation suppresses the expression level of REG1A in GC. Additionally, we conclude that REG1A was epigenetically silenced by the aberrant hypermethylation of CpG islands in promoter in GC. The epigenetic regulation needs further in-depth study.

PI3K/Akt is a very important signaling pathway involved in various cancers, including GC. GSK3 β is a multi-substrate target protein kinase and also an important downstream regulator of the PI3K/Akt pathway. The overexpression of REG1A is correlated with decreased p-AKT and p-GSK3 β expression, resulting in the suppression of GC cells invasion and viability

and increasing apoptosis. Our results show that the PI3K/Akt-GSK3 β signaling pathway participates in the regulatory effects of REG1A on GC cells.

Conclusions

We found that REG1A overexpression suppressed GC cells invasion, viability, and promoted apoptosis. Epigenetic methylation suppressed the expression level of REG1A in GC. REG1A exerts its functional roles in GC mainly through the PI3K/Akt and GSK3 β signaling pathways. REG1A may be used as a potential therapeutic strategy for GC in future.

Conflicts of interest

None.

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