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Original Article

Epigenetic regulation of RASGRF1 and its effects on the proliferation and invasion in colorectal cancer cells

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Abstract: Background: As a member of small GTP exchange factor (GEF) family, RASGRF1 activates specific Ras or Rho GTPases. The role of RASGRF1 in cancers seems to be controversial. Aim: In this study, we determined the regulation and role of RASGRF1 in colorectal cancer (CRC). Materials and methods: To analyze the regulation of RASGRF1 in CRC, we firstly detected the methylation status of *RASGRF1* gene promoter by methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP) assays in four colorectal cancer cell line HT29, HCT116, SW480 and SW620. We also detected the relative RASGRF1 mRNA level after 5-Aza-2'-deoxycytidine (5-Aza) treatment. We determined the mRNA and protein level of RASGRF1 in the 4 colorectal cancer lines by RT-PCR and Western blot. To further illustrate the roles of RASGRF1 in CRC tumorigenesis, we overexpressed RASGRF1 in RASGRF1 low-expressing HCT116 cells. The proliferation and apoptosis of these cells were detected by MTT and flow cytometry. We also detected the invasive ability of the cells overexpressing RASGRF1 by wound healing assay and transwell assays. Results: We found that the promoter of *RASGRF1* gene was hypermethylated in CRC cell lines, and restoration of RASGRF1 was induced by 5-Aza-2'-deoxyazacytidine treatment. We also found that overexpression of RASGRF1 inhibits cell proliferation and cell invasion, but does not affect cell apoptosis. Conclusions: These findings strongly suggest that RASGRF1 plays important roles in the development and progression of human CRC.

Keywords: Colorectal cancer, RASGRF1, DNA methylation, cell proliferation, cell invasion

Introduction

Colorectal cancer (CRC) is the third common cancer in males and the second in females and is one leading cause of cancer-related deaths worldwide, with an estimation of around 1,471,808 newly diagnosed patients and 726,028 deaths worldwide in 2015 [1]. Despite the advancement of detection and therapy, the clinical outcome of CRC patients still remains poor.

Aberrant CpG islands methylation in gene promoter is an important epigenetic alteration in the pathogenesis of CRC [2]. Hypermethylation of gene promoter can result in the silencing of tumor suppressor genes or decreased gene expression, consequently contributing to the pathogenesis of CRC [2]. Therefore, identification of potential targets of promoter hypermethyation will provide us new insights into the mechanisms for the initiation and development of CRC, and also provide new approaches for tumor diagnostic and therapeutic improvement [3].

RASGRF1 is a small GTP exchange factor (GEF) which is able to activate specific Ras or Rho GTPases [4]. RASGRF1 is predominantly expressed in the pancreatic islets and in the central nervous system (e.g. hypothalalmus), which explains its role in Ras activation in regulation of the pancreatic mass of insulin-producing cells, insulin secretion, and glucose homeostasis, as well as its involvement in neuronal functions [5]. RASGRF1 promotes the release of GDP from inactive Ras and stabilizes the apoprotein, implying its possible oncogenic function [6]. However, recent studies have demonstrated tumor suppressive roles of RASGRF1. The protein can bind directly to CDC42, another Ras-related GTP binding protein, consequently suppressing CDC42-mediated cellular processes, including tumor cell invasion and transformation [7]. RASGRF1 was also found to be decreased in the gastric cancer, and overex-

pression of RASGRF1 can suppress the proliferation and invasion in gastric cancer cells [8]. Therefore, the role of RASGRF1 in tumorsis not clear and requires further investigation.

In this study, we analyzed the epigenetic regulation of RASGRF1 in CRC cell lines, and the effect of RASGRF1 on tumor cell proliferation and invasion.

Materials and methods

Cell lines and plasmids

Human colorectal cancer cell lines HT29, HCT116, SW480 and SW620 were purchased from Cell Bank at the China Academy of Science (Shanghai, China). All of the human colorectal cancer cell lines were incubated in RPMI medium 1640 (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 mg/ml streptomycin (Life Technologies), and they were cultured at 37°C in a humidified 5% CO_2 incubator. RASGRF1 overexpressing vector or control vector was transfected into HCT116 by using Lipofectamine 2000 (Life Technologies). For hypomethylation, cells were treated with 5-Aza-2'-deoxycytidine (5-Aza) (Sigma-Aldrich, St. Louis, MO, USA).

DNA isolation and bisulfite modification

Total genomic DNA was isolated from colorectal cancer cell lines by using the Universal Genomic DNA Extraction Kit Ver.3.0 (Takara, Dalian, Liaoning, China) as recommended by the manufacturer. Bisulfite modification of DNA was carried out by using Epitect Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Methylation-specific PCR

DNA sequences of RASGRF1 promoter were analyzed with the MethPrimer program to design methylation-specific PCR primers. Bisulfite-modified DNA was amplified with primers specific for methylated or unmethylated sequences. The methylated DNA was amplified using M primers: 5'-TATTTTCGTTGTAAAGCGTTC-3' (sense) and 5'-TTACACGACATTTATCCGAA-3' (antisense), and unmethylated DNA was amplified using U primers: 5'-ATTTATTTTTGTTGTAAAGTGTTT-3' (sense), and 5'-TTACACAACATTTATCCAAAAAC-3' (antisense). Methylation-specific PCR for *RASGRF1* gene promoter was conducted in a total PCR volume of 20 μl, and the PCR products were analyzed by 1% agarose gel electrophoresis.

Bisulfite sequencing PCR

Genomic DNA from HT29, HCT116, SW480 and SW620 cell lines was included in BSP analysis. The bisulfite-treated RASGRF1 promoter containing 37 CpG sites was amplified with the primers 5'-AGATGGTGTTTTTTTTTTATAGGG-3' (sense) and 5'-AAATTTTCTACAAATACCCCCC-3' (antisense). The amplified PCR products were cloned into a pGEM T-Easy vector (Promega, Madison, WI, USA). 5-10 clones for each sample were sequenced. The percentage of methylated CpG dinucleotides was calculated to evaluate the methylation level of RASGRF1.

RNA isolation and reverse-transcriptase polymerase chain reaction

The total RNA was isolated from cells with Trizol Reagent (Applied Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. A total of 1 μg RNA was used to synthesize first strand cDNA using the Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA)). RASGRF1 mRNA levels were amplified in Eppendorf system by using Ex Taq (Takara, Dalian, China). The sequences of the RASGRF1 primers were as follows: forward 5'-GCCACCAATCGTGTCTTGAA-3' and reverse 5'-CAAAGTCCTGAGAGTGCTTGGA-3'. GAPDH was used as an internal control. The PCR products were analyzed by 1% agarose gel electrophoresis. For real-time PCR assays, PCR reaction was conducted with 2 × SYBR Green PCR Master Mix (Takara) based on the manufacturer's protocol. ABI 7900 sequence detection system was used to amplification. All the experiments were repeated for at least three times.

Protein extraction and Western blotting

Total protein extracted from four CRC cell lines and transfected cells (HCT116) were obtained by using SDS lysis buffer (Sigma). Protein concentrations were measured using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Equal amounts (40 μg) of proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford,

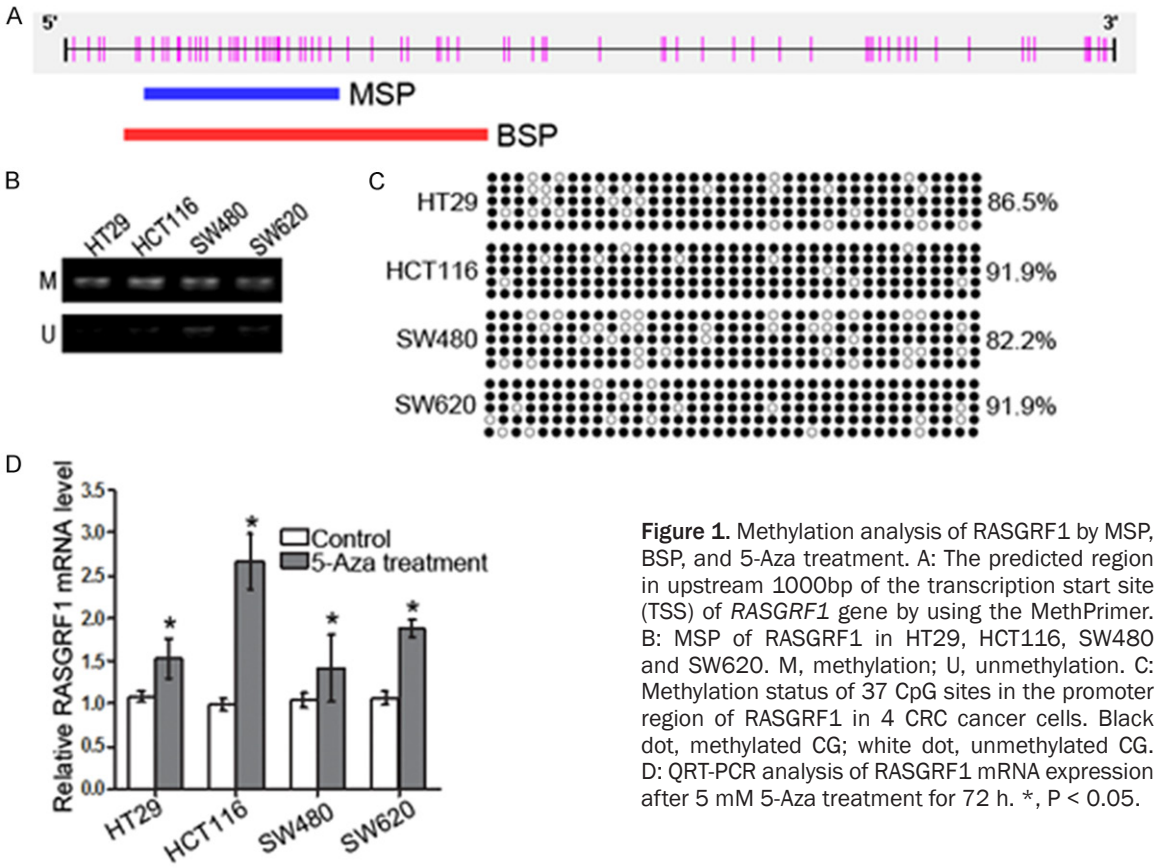

Figure 1. Methylation analysis of RASGRF1 by MSP, BSP, and 5-Aza treatment. A: The predicted region in upstream 1000bp of the transcription start site (TSS) of *RASGRF1* gene by using the MethPrimer. B: MSP of RASGRF1 in HT29, HCT116, SW480 and SW620. M, methylation; U, unmethylation. C: Methylation status of 37 CpG sites in the promoter region of RASGRF1 in 4 CRC cancer cells. Black dot, methylated CG; white dot, unmethylated CG. D: QRT-PCR analysis of RASGRF1 mRNA expression after 5 mM 5-Aza treatment for 72 h. *, $P < 0.05$.

MA, USA). The protein were incubated with primary antibodies at 4°C overnight followed by incubation with secondary peroxidase labeled antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The primary antibodies RASGRF1, E-Cadherin, Vimentin and Fibronectin were all obtained from Santa Cruz, and GAPDH was purchased from Millipore.

Cell proliferation assay

After transfection, cells were plated in 96-well plate at a density of 2×10^3 cells/well, and cell viability was measured at 24, 48, 72, and 96 h using the methylthiazoletetrazolium (MTT) assay kit (Promega, Madison, Wisconsin, USA). Absorbance was measured on a microplate reader (Thermo, USA) at a wave length of 570 nm. This assay was repeated at least three times.

Flow cytometry

After transfection, cells were harvested and double stained with PI and FITC-conjugated Annexin-V provided by the apoptosis detection kit (Biotyme, China) according to the manufacturer's instruction. Cells were analyzed by BD Fluorescence-activated cell sorting (FACS) Caliber (BD Biosciences). Cells that stain positive for PI and negative for FITC-Annexin V undergoing apoptosis. Cells that stain positive for both FITC-Annexin V and PI are in the end-stage of apoptosis. This assay was repeated at least three times.

Wound healing assay

Cells were seeded into 6-well plate and cultured overnight before transfection with RASGRF1 overexpressing vector or control vector. After 48 h, the cell monolayers were gently scratched with a pipette tip across the diameter of the well and rinsed with media to remove cellular debris. The surface distance of the scratch was quantified immediately after wounding and then measured at 24 through a microscope. This assay was repeated at least three times.

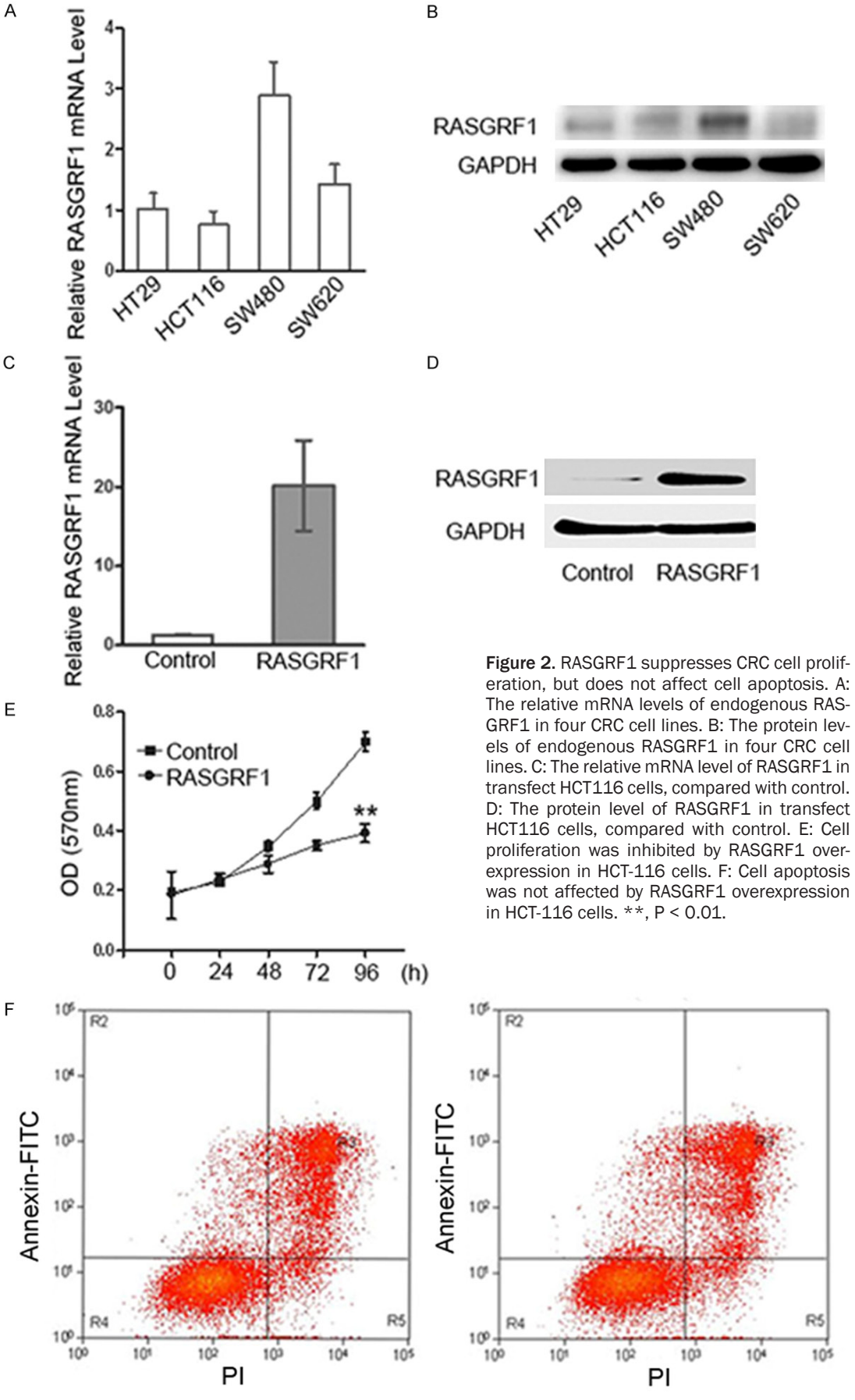

Figure 2. RASGRF1 suppresses CRC cell proliferation, but does not affect cell apoptosis. A: The relative mRNA levels of endogenous RASGRF1 in four CRC cell lines. B: The protein levels of endogenous RASGRF1 in four CRC cell lines. C: The relative mRNA level of RASGRF1 in transfect HCT116 cells, compared with control. D: The protein level of RASGRF1 in transfect HCT116 cells, compared with control. E: Cell proliferation was inhibited by RASGRF1 overexpression in HCT-116 cells. F: Cell apoptosis was not affected by RASGRF1 overexpression in HCT-116 cells. **, $P < 0.01$.

Transwell assays for cell migration and invasion

A total of 1×10^5 cells for each well were resuspended in 100 μl RPMI medium 1640, and added to the top chambers with 8-μm pore sized filter inserts (Corning Costar, Tewksbury, MA, USA). For cell invasion assay, before seeding cells, the top surface of the filter membrane was coated with 30% of BD Matrigel matrix (BD Biosciences, San Jose, CA, USA). For cell migration assay, Matrigel coating was not performed. The bottom chambers were soaked by complete medium. After overnight incubation, cells on the top surface of the filter membranes were removed, then the migrated or invaded cells were fixed by methanol and stained with crystal violet before counted. This assay was repeated at least three times.

Statistical analysis

The analysis was conducted using SPSS version 15.0. All experimental data are presented as mean ± SD. All statistical analyses were performed using Student's t-test. *$P < 0.05$ was considered significant.

Results

RASGRF1 expression is downregulated by promoter hypermethylation in CRC cells

We first predicted the CpG islands in upstream 1000bp of the transcription start site (TSS) of *RASGRF1* gene by using the MethPrimer, as shown in **Figure 1A**. We performed MSP to detect *RASGRF1* promoter methylation status in four CRC cell lines, including HT29, HCT116, SW480 and SW620, and the location of MSP products was indicated in **Figure 1A**. We found that there was a clear band in the four cell lines amplified with methylated primers, whereas no band was found with unmethylated primers in HT29, HCT116 and SW620 (**Figure 1B**). For SW480, there was a not-clear band found with unmethylated primers (**Figure 1B**). MSP results were further validated by BSP assays in HT29, HCT116, SW480 and SW620. The promoter region including 37CpG sites was amplified by PCR and then sequenced (**Figure 1A**). Tumor cell lines were significantly hypermethylated; and DNA methylation levels were 86.5%, 91.9%, 82.2% and 91.9%, respectively (**Figure 1C**). These results indicated hypermethylation of RASGRF1 promoter in CRC cells. To demonstrate the hypothesis that downregulation of RASGRF1 caused by hypermethylation of the promoter region, CRC cell lines were treated with the demethylating agent 5-Aza. The mRNA expression of RASGRF1 was increased in the 5-Aza treated cells compared with that in the control cells. These results strongly indicated that hypermethylation is responsible for down-expression of RASGRF1 in CRC cells (**Figure 1D**).

Restoration of RASGRF1 expression inhibits cell proliferation, doesn't affect cell apoptosis

After detection of RASGRF1 mRNA expression and protein level in four CRC cells (**Figure 2A** and **2B**), we decided to selected HCT116 cells, which had a lower level of endogenous RASGRF1. For further RASGRF1 overexpression analysis, we transfected RASGRF1 overexpressing vector into HCT116 cells. Empty vector was used as a control. The mRNA and protein expressions of RASGRF1 in the cells were evaluated by qRT-PCR and Western blotting analysis (**Figure 2C** and **2D**). Then, the effects of RASGRF1 overexpression on tumor cell proliferation and apoptosis were determined by MTT assays and flow cytometry. The results showed that cell proliferation was impaired in HCT116 cells overxpressing RASGRF1 compared with control cells (**Figure 2E**). However, the apoptosis analysis by flow cytometry showed that RASGRF1 overexpression had no effect on cell apoptosis (**Figure 2F**).

RASGRF1 overexpression inhibits cell migration and invasion in HCT116 cells

To study the effects of RASGRF1 on cell migration and invasion in colorectal cancer cells, we employed wound healing assay and Transwell assays. **Figure 3A** exhibited representative photographs of wound healing assays, and the percentage of wound healing distance was 63.0% less in RASGRF1 overexpressed HCT116 cells than the control (**Figure 3A**, lower panel). Consistently, the number of migrated cells per field was 1.86-fold less in RASGRF1 overexpressing cells compared with the control utilizing Transwell assay (**Figure 3B**). The in vitro invasion of HCT116 cells was measured by a Matrigel Transwell assay, and RASGRF1 overex-

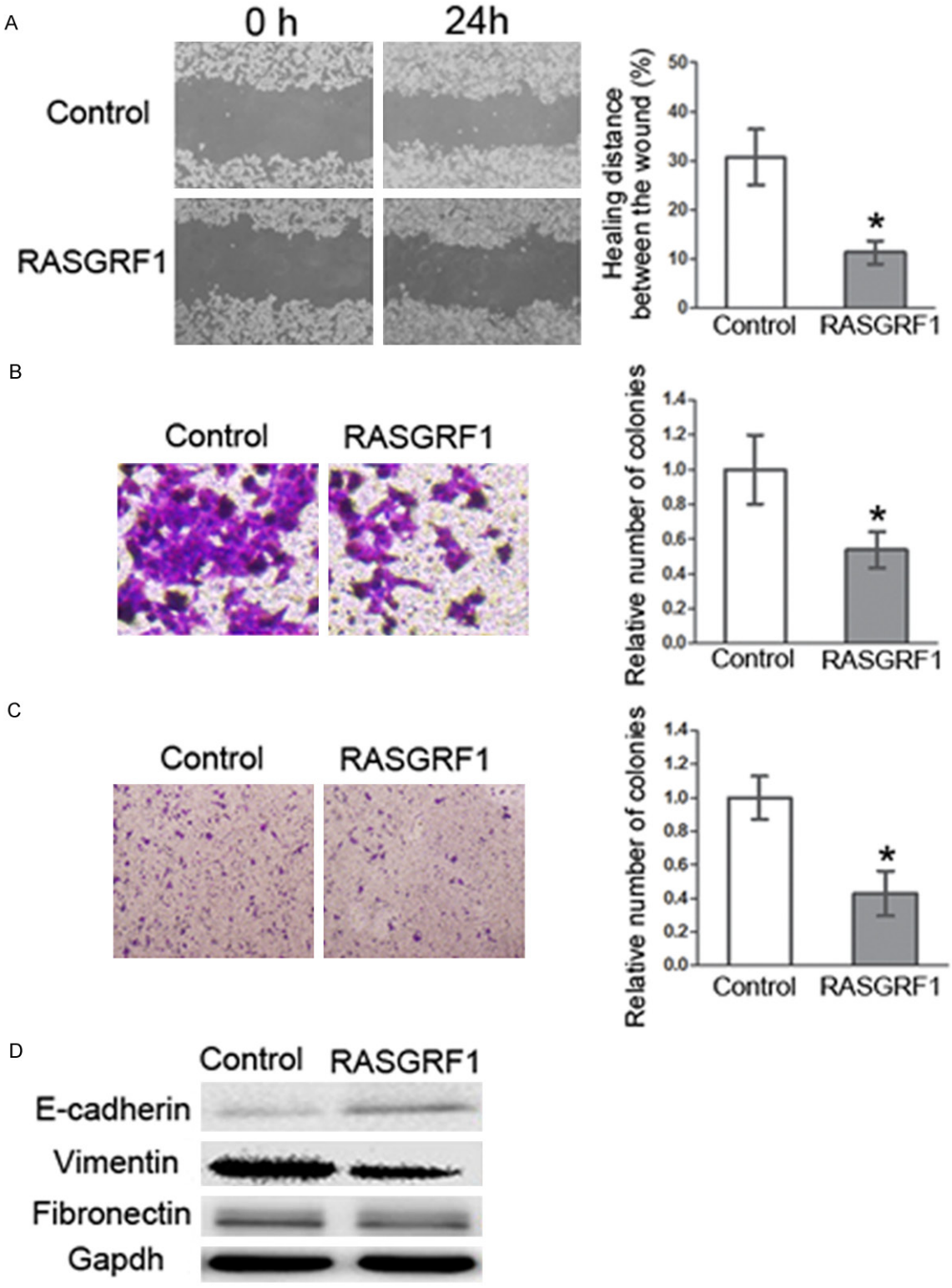

Figure 3. RASGRF1 inhibits tumor cell migration and invasion. A: Determination of cell migration ability by wound healing assay in RASGRF1 overexpressing HCT116 cells. Cells were seeded into 6-well plate and transfected with RASGRF1 overexpressing vector or control vector, incubated for 2 days prior to starvation by serum-free medium, and then subjected to wound healing assay. B: Effect of RASGRF1 overexpressionon cell migration determined by Transwell assay. C: Influence of RASGRF1 overexpression on cell invasion assessed by Matrigel Transwell assay. Invasive cells were plotted as the average number of cells per field of view from three different experiments. D: Related molecules in EMT process by Western blotting in RASGRF1 overexpressing HCT116 cells. *, $P < 0.05$.

pression caused a 2.34-fold decrease in the number of invaded cells per field (**Figure 3C**). To sum up, these observations indicated that the upregulation of RASGRF1 in colorectal cancer cells resulted in the inhibition of cell migration and invasion. We further evaluated the molecules involved in EMT process by Western blotting, and found a stronger band of E-Cadherin and weaker bands of Vimentin compared with controls (**Figure 3D**).

Discussion

RASGRF1 is a small GTP exchange factor (GEF) which can promote the release of GDP from inactive Ras and stabilize the apo-protein, so it seems to exert oncogenic effect [6]. However, recent studies have identified its tumor suppressive function. The protein has been demonstrated to bind directly to CDC42, another Ras-related GTP binding protein, thereby blocking CDC42-mediated cellular processes, including tumor cell invasion and transformation [7]. RASGRF1 was also found to be reduced in the gastric cancer, and it suppresses cell proliferation and invasion in gastric cancer [8].

Accumulating evidence demonstrates that promoter hypermethylation of tumor-related gene plays important roles in the tumorgenesis of CRC, and also can be used as a sensitive marker for early diagnosis, prognosis prediction, and therapeutic target of CRC [2, 3]. In this study, we demonstrate that promoter methylation may play an important role in the regulation of RASGRF1 expression. First of all, methylation status of RASGRF1 was examined by MSP in 4 CRC cell lines. RASGRF1 was fully methylated in three CRC cells, including HT29, HCT116 and SW620, while partially methylated in SW480 cells. Subsequently, BSP results confirmed that RASGRF1 promoter hypermethylation was found in CRC cells, indicating a high prevalence of RASGRF1 methylation in CRC. Considering epigenetic silencing of tumor-related genes has been shown to be reversible, we measured RASGRF1 expression in CRC cells cultured in the presence of the hypomethylation agent 5-Aza. The mRNA levels of RASGRF1 in CRC cells were significantly upregulated after 5-Aza treatment. In general, these results revealed that the promoter methylation of RASGRF11 has a critical role in its transcriptional down-regulation, which may be involved in CRC development.

We are interest in the function of RASGRF1 in tumor cell proliferation. We transfected RASGRF1 overexpressing vector into HCT116 cells, which had lower level of endogenous RASGRF1, and found an inhibitory effect on cell proliferation. We also tested the effect of RASGRF1 on tumor cell apoptosis, but the result showed that it did not affect cell apoptosis. We evaluated roles of RASGRF1 in colorectal cancer cell migration and invasion. We found that overexpression of RASGRF1 in HCT116 cells not only prevents tumor cell migration, but also hampers cell invasion. Epithelial-mesenchymal transition endows the properties of migration and invasion in cancer cells. During EMT process, some molecular repertoire of the cell undergodramatic changes. We observed a decrease in Vimentin and an increasein E-Cadherin in RASGRF1 overexpressing cells as compared with control. Therefore, these data revealed that RASGRF1 could suppress the invasive ability of colorectal cancer cells.

Conclusions

In conclusion, this study describes RASGRF1 as a novel epigenetic silencing gene in CRC, thereby providing a potential marker for early diagnosis. Moreover, our findings indicate that promoter hypermethylation contributes to lower RASGRF1 expression, which may be involved in CRC tumor growth and metastasis.

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Disclosure of conflict of interest

None.

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