

RGS4 Regulates Proliferation And Apoptosis Of NSCLC Cells Via microRNA-16 And Brain-Derived Neurotrophic Factor

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Purpose: Regulator of G-protein signaling (RGS) proteins are GTPase-activating proteins that target the α -subunit of heterotrimeric G proteins. Many studies have shown that RGS proteins contribute to tumorigenesis and metastasis. However, the mechanism in which RGS proteins, especially RGS4, affect the development of non-small cell lung cancer (NSCLC) remains unclear. The aim of this study was to characterize the role of RGS4 in NSCLC.

Methods: *RGS4* expression in NSCLC tissues was assessed using an immunohistochemistry tissue microarray. Additionally, *RGS4* was knocked down using short-hairpin RNA to assess the regulatory function of RGS4 in the biological behaviors of human NSCLC cell lines. A xenograft lung cancer model in nude BALB/c mice was established to study whether *RGS4* knockdown inhibits cancer cell proliferation in vivo.

Results: We observed an increase in RGS4 protein levels in NSCLC samples. *RGS4* knockdown inhibited cell proliferation and induced apoptosis in H1299 and PC9 cell lines, but did not affect cell migration. Moreover, we found that *RGS4* negatively regulated the expression of microRNA-16 (miR-16), a tumor suppressor. The inhibition of miR-16 resulted in upregulated *RGS4* expression. We also found that RGS4 regulated the expression of brain-derived neurotrophic factor (*BDNF*) and activated the BDNF-tropomyosin receptor kinase B signaling pathway.

Conclusion: This study revealed that *RGS4* overexpression positively correlated with the development of NSCLC. Downstream RGS4 targets (eg, miR-16 and BDNF) might be involved in the development of NSCLC and may serve as potential therapeutic targets for its treatment.

Keywords: NSCLC, regulator of G-protein signaling 4, microRNA-16, BDNF, proliferation

Introduction

Among malignancies, lung cancer is the leading cause of death worldwide.¹ According to the GLOBOCAN database, there were 1.825 million cases of lung cancer worldwide in 2012.² It was estimated that there were 0.7 million new cases of lung cancer and 0.6 million deaths due to lung cancer in 2015 in China.³ Non-small cell lung cancer (NSCLC) accounts for 80% of all lung carcinomas,⁴ and some NSCLCs have resistance to cytotoxic agents.⁵ Thus, developing novel biomarkers and therapeutic targets for NSCLC treatment is a critical.

G-protein-coupled receptors (GPCRs) are the main extracellular surface receptors involved in signal transduction. Growing evidence suggests that GPCR expression and activation of encoded proteins are associated with the development of many types of tumors. Furthermore, several studies have demonstrated excessive activation of GPCR

expression in NSCLC. Unfortunately, there are few drugs that can directly inhibit GPCR expression.⁶⁻¹¹

Regulator of G-protein signaling (RGS) proteins are GPCR-mediated response regulators in cells.^{12,13} To date, more than 20 RGS family proteins have been identified. RGS proteins function as guanosine triphosphatase (GTPase)-activating proteins (GAPs) that accelerate GTP hydrolysis. Many studies have indicated that mutations to RGS genes are associated with several disease states, including schizophrenia¹⁴ and tumorigenesis.¹³⁻²⁰ For example, RGS2 expression is significantly downregulated in androgen-independent prostate cancer,²¹ while RGS5 expression is upregulated in hepatocellular carcinoma²² and in the vasculature of renal cell carcinoma.²³ Moreover, single-nucleotide polymorphisms (SNPs) in RGS6 are linked to a significant decrease in the risk of bladder cancer.²⁴

RGS4 is a negative regulator of GPCR that can block the transmission of related signaling factors by accelerating proteolysis of G-protein. Studies have shown that RGS4 can form complex signaling molecule transduction complexes with receptors, effectors, scaffold proteins and other signaling molecules, affecting the intracellular localization, activity and stability of signaling, and thereby regulating cell function. Under physiological conditions, RGS4 is expressed in certain populations of differentiating neurons in the central nervous system, including the cerebral cortex. Four common SNPs in RGS4 are reported to be linked to schizophrenia,²⁵ and upregulated RGS4 expression has been detected in colorectal cancers.²⁶ Furthermore, RGS4 is associated with increased cell viability, invasion, and/or motility in thyroid cancer,²⁷ glioma,^{18,20} ovarian cancer, ascites,²⁸ and triple-negative breast cancer.¹³ However, the role of RGS4 remains poorly understood in lung cancer. Cheng et al (2016) reported that RGS4 expression is generally downregulated in NSCLC specimens, and that overexpression of RGS4 in NSCLC cells inhibits invasion and migration.¹⁶ However, the underlying mechanism has not been elucidated. The aim of this study was to determine whether RGS4 regulates NSCLC development, and to elucidate the mechanisms involved. The data obtained from our study enriched our understanding of NSCLC development and provided a new target for the clinical treatment of NSCLC.

Materials And Methods

Reagents

Antibodies against RGS4, brain-derived neurotrophic factor (BDNF), Bax, and Bcl-2 were purchased from Proteintech

(Chicago, IL). Rabbit anti-cleaved caspase-3 and rabbit anti-phosphorylated-Akt S473 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. We purchased the tissue microarray with NSCLC tissue samples from Shanghai Zhuoli Biotechnology Co., Ltd. (Shanghai, China).

Immunohistochemistry

The immunohistochemistry tissue microarray involved anti-RGS4 antibodies. Tissue samples were deparaffinized and rehydrated using standard techniques. Sections were first immersed in 3% H₂O₂ for 10 min to quench endogenous peroxidase activity, then treated with a goat serum blocking reagent prior to incubation with anti-RGS4 antibodies overnight at 4°C. An immunohistochemistry kit (Fuzhou Maixin Biotech Co., Ltd., Beijing, China) was used to visualize any bound antibodies. For NCs, the primary antibody was eliminated.

Cell Culture And Transfection

The human lung adenocarcinoma cell lines H1299 and PC9 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO₂. Transient transfections were performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. The shRNAs against RGS4 (5'-AAC ATG CTA GAG CCT ACA ATA-3') and the NC sequence (5'-ATA ACA TCC GAG ATC GTA CAA-3') were incorporated into the pSUPER vector.

RNA Isolation And Quantitative Real-Time PCR

Total RNA was extracted using the Ultrapure RNA Kit (CWBio, Beijing, China), and the extracted RNA was then used as the template for a reverse transcription with the First Strand cDNA Synthesis Kit (CWBio). The relative RGS4 expression level was determined in a qRT-PCR assay using the UltraSYBR Mixture (CWBio). Relative mRNA levels were calculated according to the $2^{-\Delta\Delta Ct}$ method and were normalized to GAPDH levels. The following primers were used for qRT-PCR: RGS4-F (5'-GCC AAG AGG AAG TCA AGA AA-3'), RGS4-R (5'-CTT

CAC AGC TGA TCC AGA AG-3'), and GAPDH-F (5'-CGG AGT CAA CGG ATT TGG TCG TAT-3') and GAPDH-R (5'-AGC CTT CTC CAT GGT GA A GAC-3').

CCK-8 Assay

The CCK-8 assay was performed following the manufacturer's protocol (Solarbio, Beijing, China). Cells were added to 96-well plates at a density of 1×10^4 cells/well, with three replicate wells per group. The cells were cultured for 3 days, then 10 μ l of CCK-8 solution was added to each well. Plates were incubated for an additional 2 h at 37 °C. The OD450 value was used to estimate cell proliferation in different groups. The experiment was independently performed in triplicate.

Colony Formation Assay

H1299 and PC9 cells were trypsinized, counted, and added to a 6-well plate at a density of 100 cells/well. The plate was incubated at 37 °C for 14 days, and the culture medium was replaced every 2 days. Colonies were fixed with 4% paraformaldehyde for 10 min and stained with crystal violet for 20 min. Colonies were counted under a light microscope and photographed with a digital camera. The experiment was performed independently three times.

Assessment Of Apoptosis

Apoptosis was detected in situ by the TUNEL technique using the In Situ Cell Death Detection Kit (Roche, Basel, Switzerland). Cells were seeded onto sterile glass coverslips in a 6-well plate and subsequently examined according to the manufacturer's instructions.

Immunofluorescence Assay

Tissue samples on slides were deparaffinized and rehydrated using standard techniques. Sections were immersed in 3% H₂O₂ for 10 min to quench endogenous peroxidase activity. They were then treated with goat serum blocking reagent and incubated with anti-RGS4 antibodies for 1 h at room temperature. Samples were rinsed four times with phosphate-buffered saline (PBS) and then incubated with Alexa Fluor 488-labeled secondary antibodies (1:1000 dilution in 5% normal goat serum) for 1 h at room temperature. Samples were rinsed twice with PBS, and then glass coverslips were mounted on the slides using VECTASHIELD with DAPI (Vector Laboratories, Burlingame, CA). The tissue samples were analyzed and images were captured using a Nikon confocal microscopy system (Nikon, Tokyo, Japan).

Wound Healing Assay

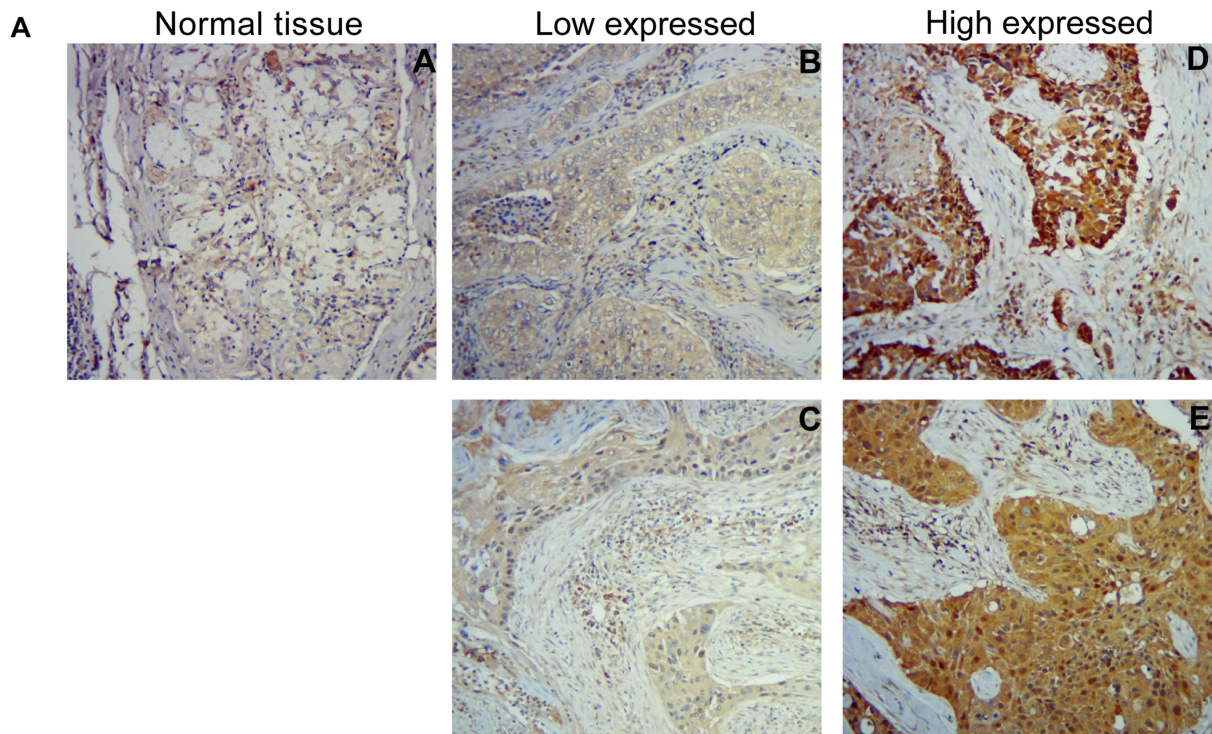
A wound healing assay was completed to detect and compare the migration of H1299 and PC9 cells among experimental groups. Specifically, 2×10^5 cells/well were added to 6-well plates. A sterile 200- μ L pipette tip was used to scratch a straight line through the cell layer in each well. Fresh medium was added to the wells, then the plates were incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO₂. Images were captured to estimate the cell migration changes in different groups. The assays were repeated three times.

Transwell Assay

A 200- μ l cell suspension (2×10^5 cells/mL) was added to the upper chamber of a 24-well transwell containing polycarbonate filters coated with Matrigel (8 μ m pores; Corning Costar, Corning, NY). The plates were incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO₂. Three replicate wells were used per group. Following incubation, the medium was removed from the upper chamber and cells in the upper chamber were scraped off with a cotton swab. The cells that had migrated to the lower surface of the membrane were fixed with 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet for 5 min. The number of migrating cells was calculated by analyzing five random fields under a microscope (200 \times magnification). Three independent experiments were performed.

Protein Extraction And Western Blot

Cells were washed with PBS and lysed with 500 μ L TNE buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol, and protease inhibitors) on ice. Lysates were then centrifuged for 15 min at 12,000 rpm. Proteins were denatured by heating at 95 °C for 5 min, and then underwent Western blot analysis. Equal amounts of protein (10 μ g per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were incubated in blocking buffer (5% non-fat milk in Tris-buffered saline with Tween 20 [TBST]) for 1 h, then incubated with the primary antibody overnight at 4 °C. Membranes were rinsed with TBST, incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 h at room temperature, and then analyzed by chemiluminescent detection.



B

Samples	n	RGS4 expression		P
		no/or low n	high n	
Normal	67	52	15	0.0002
Cancer	101	49	52	

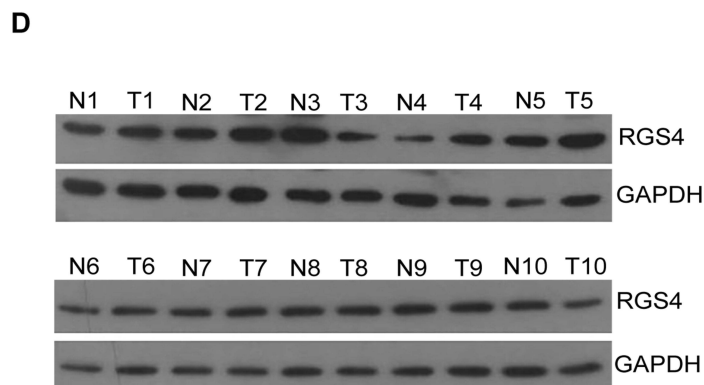
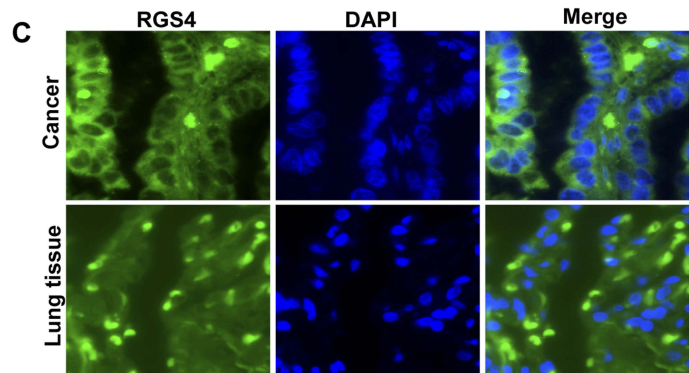


Figure 1 RGS4 was overexpressed in certain NSCLC samples. **(A)** Protein levels of RGS4 were assessed by immunohistochemistry (100x; RGS4 is indicated by a brown stain; normal tissue samples, as well as tissue samples with low and high expression of RGS4 are shown). **(B)** Distributions of low and high RGS4 expression in tumor samples and normal tissues based on immunohistochemical staining. **(C)** Immunofluorescence of RGS4 in tumor samples and normal tissue samples. **(D)** RGS4 protein levels in lung cancer tissue samples were determined by Western blot analysis; N: normal tissue; T: tumor sample. GAPDH was used as a loading control.

Animal Experiments

Female 4–5-week-old BALB/c nude mice were kept under specific pathogen-free conditions. Xenografts of human tumor cell lines were generated by subcutaneous injection of 5×10^6 tumor cells into the backs of mice. When tumors reached a diameter of 3–5 mm (ie, 10 days after injection), the mice were divided into different groups (six mice per group) for intraperitoneal injection of CCG-63802 (RGS4 inhibitor; MCE, NJ, USA) at a dose of 50 $\mu\text{g/g}$ body weight. Control mice were injected with PBS. The injections were repeated twice per week for 1 month. Tumor size was measured twice per week and tumor volume was determined according to the following equation: tumor volume = width² \times length \times ($\pi/6$). The experiment was independently performed three times by two researchers.

Statistical Analysis

All statistical analyses were performed using SPSS 13.5 software (SPSS Inc., Chicago, IL). Data were analyzed using Student's *t*-test and one-way analysis of variance followed by Tukey's post *hoc* analysis. The threshold for statistical significance was $P < 0.05$. Data are presented as the mean \pm standard deviation.

Results

RGS4 Is Overexpressed In Certain NSCLC Tissues

Due to the reported changes in RGS4 expression in various tumors,^{18–20} we first analyzed the RGS4 protein levels in NSCLC samples and examined its relevance to clinical parameters. We used an immunohistochemistry tissue microarray to detect RGS4 protein levels in 101 NSCLC tissue samples and 67 normal tissue samples. Of the NSCLC tissue, there was high RGS4 expression in 52 samples and low RGS4 expression in 44 samples, while the protein was completely undetectable in 5 samples. In contrast, moderate or low RGS4 protein levels were observed in the normal tissue samples. Representative images of RGS4 expression are shown in [Figure 1A](#). Statistical analysis revealed that RGS4 protein was significantly overexpressed in NSCLC tissue samples compared with normal tissue samples ([Figure 1B](#)). Additionally, high RGS4 levels were detected more frequently in adenocarcinoma samples (66.7%) than in squamous cell carcinoma samples (39.3%) ($P = 0.0062$; [Table 1](#)). The subcellular expression pattern of RGS4 in NSCLC samples was further investigated by immunofluorescence

Table 1 Relationship Between RGS4 Expression Level And Clinicopathologic Features

Clinicopathologic Features	Cases (n)	RGS4		P
		Low-Expression	High-Expression	
Age (years)				
≤ 60	54	26	28	0.937
> 60	47	23	24	
Sex				
Male	55	26	29	0.7848
Female	46	23	23	
Pathological grade				
I-II	53	24	29	0.4947
III	48	25	23	
Pathological Classification				
Adenocarcinoma	45	15	30	0.0062
Squamous cell carcinoma	56	34	22	
Lymph-node metastasis				
Present	37	15	22	0.2228
Absent	64	34	30	

analysis. As shown in [Figure 1C](#), RGS4 was mainly localized to the cytoplasm of tumor cells, while in normal lung tissues, the protein was mainly localized to the stroma. Western blot analysis indicated that the protein level of RGS4 was increased in 58.5% (24/41) of the NSCLC samples (eg, T1, T2, T4, and T5; [Figure 1D](#)), but decreased in 41.5% (17/41) of the NSCLC samples (eg, T3 and T10). These results suggested that RGS4 is significantly overexpressed in NSCLC samples compared with normal lung tissue samples.

RGS4 Knockdown Decreases H1299 And PC9 Cell Proliferation, But Not Migration And Invasion

The overexpression of RGS4 in NSCLC samples implied that the protein plays important roles in the development of NSCLC. To functionally characterize RGS4, we examined whether a deficiency in RGS4 affected cell activities, such as proliferation, clonogenicity, and migration. RGS4 was knocked down using a short-hairpin RNA (shRNA), then Cell Counting Kit-8 (CCK-8) assay was utilized to evaluate the effect of RGS4 knockdown on the proliferation of H1299 and PC9 cells. First, cells were transfected with RGS4 shRNA (shRGS4), and quantitative real-time (qRT)-PCR

confirmed the lack of RGS4 mRNA (Figure 2A and B). The CCK-8 assay results showed that the optical density (450 nm; OD450) values were significantly lower for the shRGS4 group compared to the negative control (NC) group at 48 h and 72 h, suggesting that RGS4 knockdown greatly inhibited cell proliferation (Figure 2C and D). In clonogenic assays, cells transfected with shRGS4 or shNC were plated out at low cell densities. Two weeks later, the ability to form colonies had decreased in the shRGS4 group by 47% relative to that in the shNC control group, indicating that RGS4 knockdown inhibited colony formation (Figure 2E and F).

To investigate the effect of RGS4 on H1299 and PC9 cell migration and invasion, we conducted wound healing and transwell assays. As shown in Figure 3A and B, the wound healing assay revealed that the migratory ability of H1299 and PC9 cells in the shRNA transfection group was not significantly affected at 24 h and 48 h post-wounding. Meanwhile, the cell invasion assay involving a transwell

system revealed a similar number of invading H1299 cells regardless of whether they were transfected with shRGS4 or the shNC control (Figure 3C and D). These data suggest that RGS4 regulates the development of NSCLC partially by promoting the proliferation of tumor cells.

RGS4 Knockdown Or Inhibition Promotes Apoptosis In H1299 And PC9 Cells

To address whether the decreased cell proliferation occurred due to increased apoptosis, we completed a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay to investigate whether RGS4 influences the apoptosis of H1299 and PC9 cells under serum starvation conditions. As shown in Figure 4A–C, there were only a few TUNEL-positive cells in the shRNA negative control (shNC) group. In contrast, the percentage of TUNEL-positive cells increased significantly at 40 h after shRGS4 transfection,

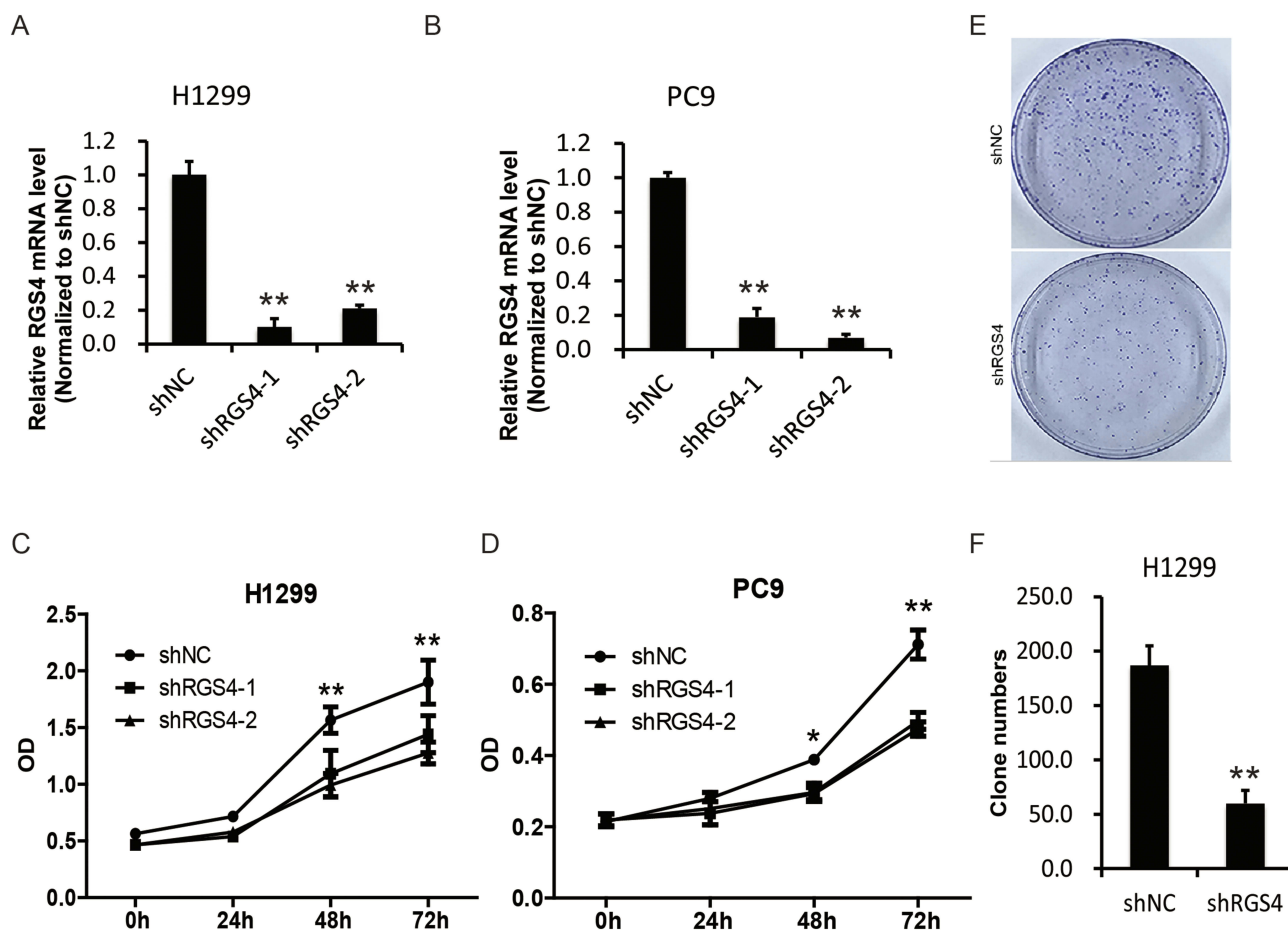


Figure 2 RGS4 promoted cell proliferation and colony formation in H1299 and PC9 cells. Relative RGS4 mRNA levels in H1299 (A) and PC9 (B) cells was determined by qRT-PCR. Data are shown as the mean \pm standard deviation (n = 3); ***P < 0.01. Proliferation of H1299 (C) and PC9 (D) cells was measured by CCK-8 assay; *P < 0.05; **P < 0.01. (E) Colony formation of H1299 and PC9 cells was analyzed by crystal violet staining. (F) Quantitative analysis was performed with the soft-agar assay. Data are shown as the mean \pm standard deviation (n = 3); **P < 0.01.

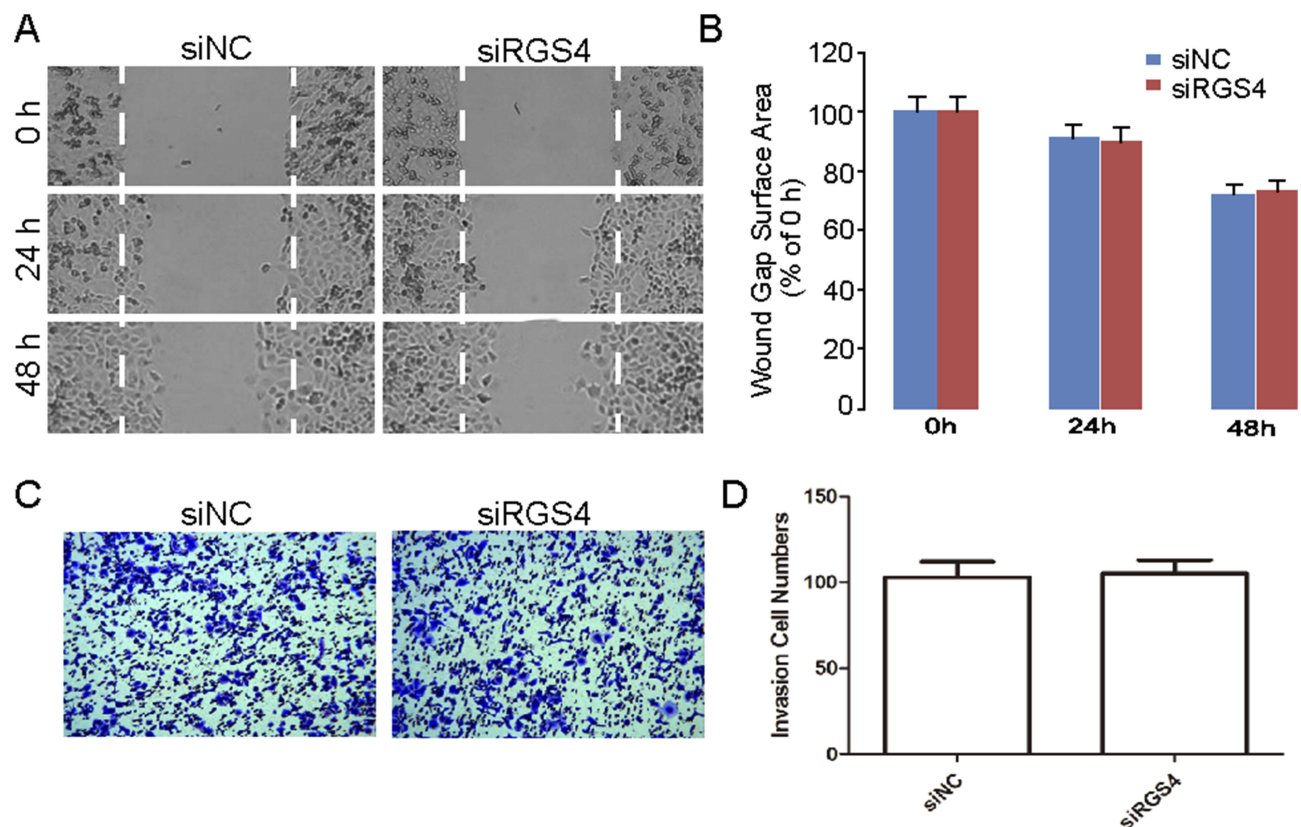


Figure 3 RGS4 did not affect H1299 cell invasion and migration. (A) The impact of RGS4 on H1299 migration in a wound healing assay. (B) Quantitative analysis of the wound healing assay. (C) The impact of RGS4 expression on H1299 cell invasion in a transwell assay. (D) Quantitative analysis of the transwell assay. Data are shown as the mean \pm standard deviation (n = 3).

implying that the inhibition of RGS4 expression promoted cell apoptosis. This was confirmed by Western blot analysis, in which several apoptosis-related proteins were detected (Figure 4D). Moreover, the active pro-apoptotic proteins caspase-3 and Bax were significantly more abundant in H1299 cells at 48 h after the shRGS4 transfection compared to shNC control cells. Meanwhile, the level of the anti-apoptotic protein Bcl-2 was considerably lower in the shRGS4 group compared to the shNC group ($P < 0.05$) (Figure 4E and F). These data indicated that RGS4 knockdown induced apoptosis in H1299 cells by activating caspase-3 and increasing Bax production.

RGS4 Knockdown Inhibits AKT Signaling Pathway In H1299 Cells

Studies have shown that AKT signaling pathway is abnormally activated during the progression of NSCLC. We investigated the role of RGS4 on AKT signaling pathway in H1299 cells. As shown in Figure 5, RGS4 knockdown inhibited the level of p-AKT/AKT as well as the expression of its downstream protein, Cyclin D1 and P70 ($P < 0.05$).

These results indicated that RGS4 inhibited the AKT signaling pathway in H1299 cells.

RGS4 Knockdown Increases miR-16 Expression And Inhibits BDNF-Tropomyosin Receptor Kinase B (TrkB) Signaling In H1299 Cells

We first examined the relationship between RGS4 and miR-16, and found that RGS4 knockdown by shRNA resulted in upregulated miR-16 expression (Figure 6A). To investigate whether RGS4 functions through miR-16, we co-transfected H1299 and PC9 cells with a miR-16 inhibitor and shRGS4, then analyzed caspase-3 activation. As shown in Figure 6B–F, the miR-16 inhibitor abolished the effects of shRGS4 on caspase-3 activation and Bax expression, suggesting that RGS4 knockdown activates apoptosis through the induction of miR-16. However, a previous study found that miR-16 regulates RGS4 mRNA and protein levels in schizophrenia.²⁹ We next investigated whether miR-16 also regulates RGS4 expression in NSCLC cells. The miR-16 and BDNF

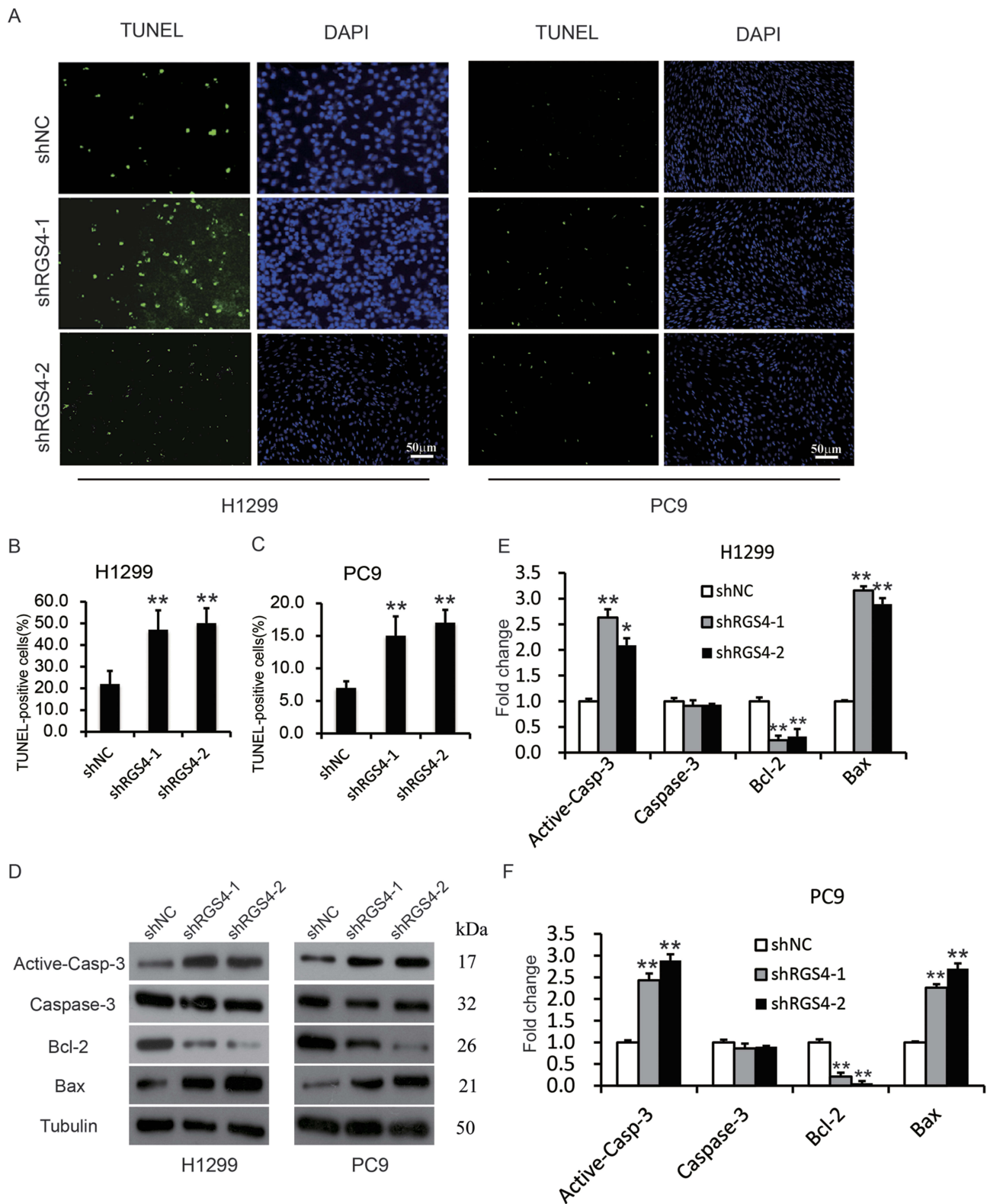


Figure 4 RGS4 inhibited cell apoptosis in H1299 and PC9 cells. Morphological assessment of H1299 and PC9 apoptosis by TUNEL assay. (A) Representative photomicrographs of TUNEL staining. Percentage of TUNEL-positive cells in H1299 (B) and PC9 (C) cell lines (n = 3). (D) Apoptosis-related protein levels in H1299 cells transfected with shNC or shRGS4 were determined by Western blot analysis. The relative protein levels in H1299 (E) and PC9 (F) cells were analyzed. *P < 0.05; **P < 0.01 considered statistically significant.

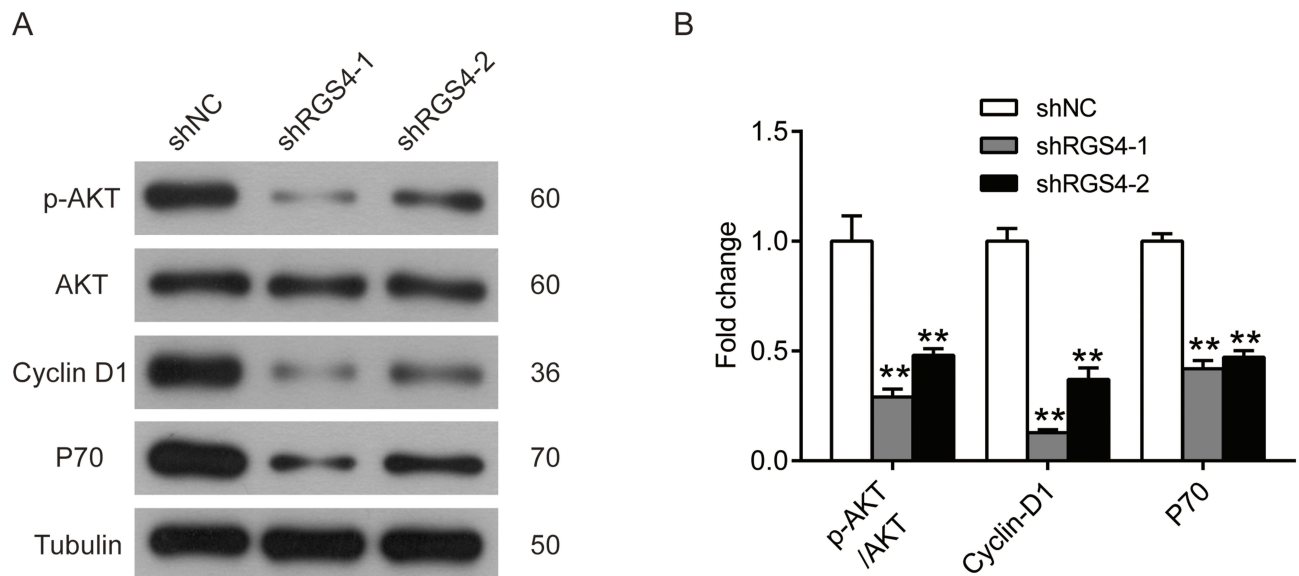


Figure 5 RGS4 promoted the AKT signaling pathway in H1299 cells. (A) The expression levels of AKT signaling pathway related protein were determined by Western blot analysis. (B) The relative protein levels in H1299 cells were analyzed. ** $P < 0.01$.

expression levels were determined for the NSCLC and normal tissue samples. As shown in Figure 6J, there was a significant decrease in BDNF expression and an increase in miR-16 expression in NSCLC tissue samples compared to normal tissue samples. Interestingly, we also observed that the presence of the miR-16 inhibitor significantly increased the RGS4 transcript level in H1299 cells (Figure 7A). Together, these data imply that the increased RGS4 mRNA levels in NSCLC cells may be due to decreased miR-16 expression.

We hypothesized that RGS4 knockdown induces miR-16 expression, ultimately leading to suppressed BDNF expression. Our results showed that RGS4 knockdown indeed decreased BDNF expression along with the phosphorylation of TrkB, which is the receptor of BDNF, suggesting that the BDNF-TrkB signaling pathway may be a downstream target of RGS4 (Figure 6G–I). Moreover, BDNF expression was significantly upregulated by the inhibition of miR-16 (Figure 7B). We also found that K252a, which is a specific inhibitor of Trk, suppressed the effects of the miR-16 inhibitor on cell proliferation (Figure 7C, E and F). In summary, RGS4 and miR-16 may regulate each other's production in a feedback loop and may control the downstream BDNF-TrkB signaling pathway in NSCLC cell lines (Figure 7D).

CCG-63802 Inhibits The Growth Of Human Xenografted Lung Cancer Cells

To assess whether *RGS4* knockdown inhibits cell proliferation in vivo, we established a xenograft lung cancer

model in nude BALB/c mice, and investigated the effects of CCG-63802, which is an RGS4 inhibitor. A suspension containing 5×10^6 H1299 cells was injected subcutaneously into 4–5-week-old nude mice. When tumors were sufficiently established, the mice received either intraperitoneal injections of CCG-63802 (50 $\mu\text{g/g}$ body weight) or PBS twice a week for 6 weeks. The tumor volume and mean weight were significantly lower in mice treated with CCG-63802 compared to PBS-treated control mice (Figure 8).

Discussion

Several studies have demonstrated that RGS4 inhibits GPCR signaling through its GAP activity and plays a role in carcinogenesis.^{13,15–20,26,30,31} Additionally, RGS4 has regulatory roles in the cardiovascular system^{32,33} and central nervous system.^{34–36} The results of numerous studies suggest that RGS4 proteins also play a role in cancer cells.^{18,20,37} In the present study, we observed that the expression of *RGS4* was upregulated in NSCLC samples, and the distribution of RGS4 differed between normal and NSCLC tissues. RGS4 was localized to the cytoplasm¹⁶ in tumor cells but to the stroma in normal cells, suggesting that RGS4 might induce some stroma-like characteristics of cancer cells. Therefore, the diversity in the genetic background of the cell lines may explain the differences in these results. To our knowledge, this is the first report describing the translocation of RGS4. *RGS4* expression

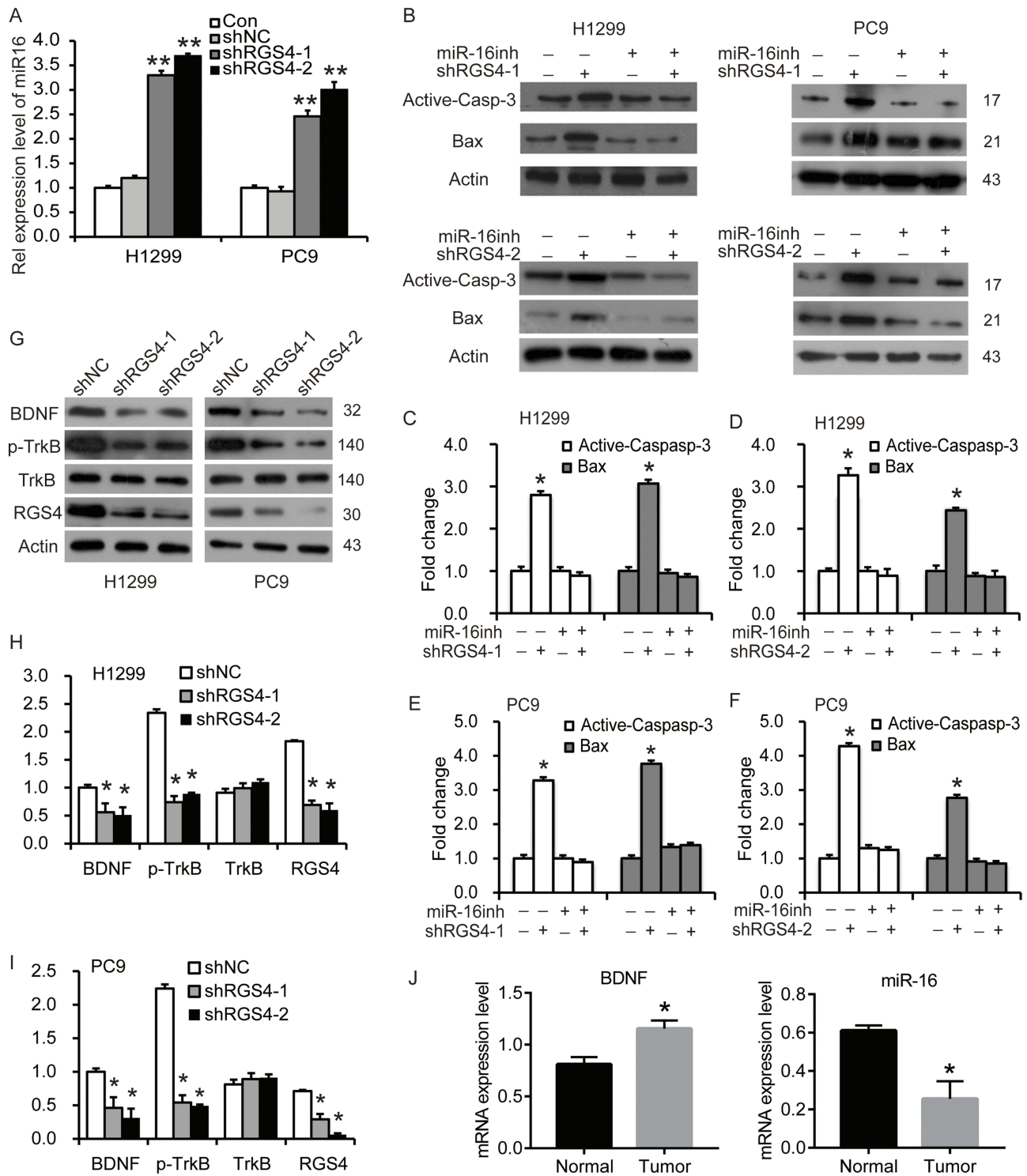


Figure 6 RGS4 affected the expression and function of miR-16 and BDNF. **(A)** H1299 cells were transfected with shNC or shRGS4. Two days later, relative miR-16 expression levels in H1299 cells were analyzed via qRT-PCR assay. Data are shown as the mean \pm standard deviation ($n = 3$); * $P < 0.05$, ** $P < 0.01$ (compared with the shNC group); Student's t -test. **(B)** Apoptosis-related protein levels in H1299 and PC9 cells were determined by Western blot analysis. Cells were co-transfected with the miR-16 inhibitor and shRNA for 48 h. **(C-F)** Quantitative analysis of the apoptosis-related protein levels. **(G)** H1299 cells were transfected with shNC or shRGS4. Two days later, BDNF, phosphorylated TrkB, and total TrkB levels were assessed by Western blot analysis. Actin was used as a loading control. **(H and I)** Quantitative analysis of the BDNF, phosphorylated-TrkB, and total TrkB levels. **(J)** Quantitative analysis of the BDNF and miR-16 levels in normal and tumor tissue samples. * $P < 0.05$, ** $P < 0.01$.

and localization of the encoded protein might be important in cancer development.

We also found that decreasing the protein levels of *RGS4* or inhibiting its activity restricted cell proliferation

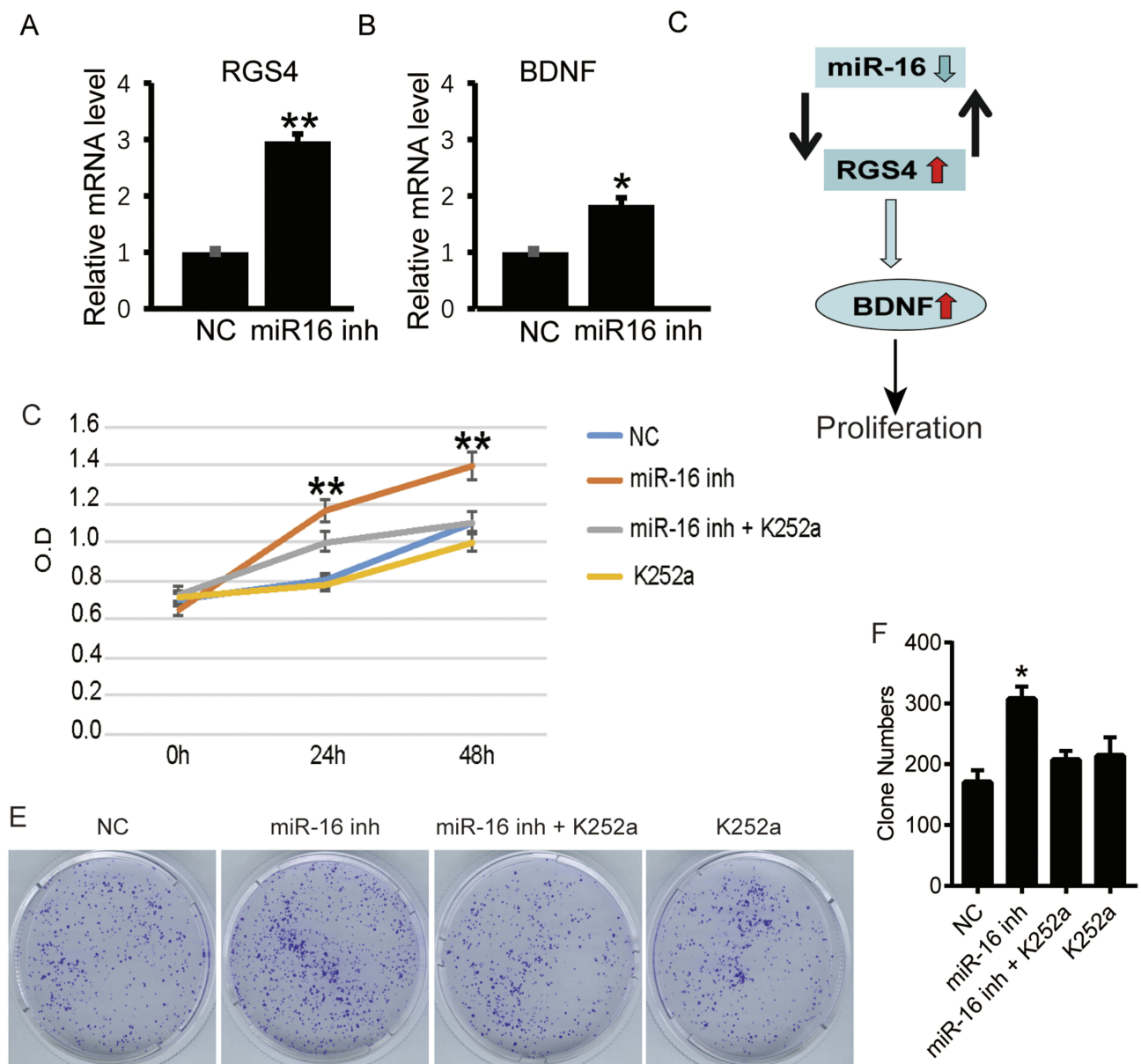


Figure 7 RGS4 and BDNF expression levels were affected by miR-16. Relative RGS4 (A) and BDNF (B) expression levels in H1299 cells transfected with the NC (scrambled sequence) or miR-16 inhibitor were determined by qRT-PCR. Data are shown as the mean \pm standard deviation ($n = 3$). * $P < 0.05$, ** $P < 0.01$. (C) Proliferation of H1299 cells transfected with NC, miR-16 inhibitor, Trk inhibitor, or miR-16 inhibitor and Trk inhibitor was assessed by CCK-8 assay; ** $P < 0.01$. (D) Simplified model depicting the negative regulatory cycle of RGS4 and miR-16 in H1299 cells. (E) Colony formation of H1299 cells was analyzed by crystal violet staining. (F) Quantitative analysis was performed with the soft-agar assay; * $P < 0.05$.

and induced apoptosis in H1299 and PC9 cells, without affecting cell migration and invasion. Although Cheng et al reported that RGS4 is a tumor suppressor in A549 cells,¹⁶ we observed that RGS4 promoted H1299 and PC9 cell proliferation. Therefore, the diversity in the genetic background of different cancer cell lines may influence gene functions. appears that RGS4 function may differ depending on the cellular context.

MicroRNAs are small non-coding RNA molecules containing about 22 nucleotides and contribute to RNA

silencing and the post-transcriptional regulation of gene expression.³⁸ In addition, microRNAs have been reported to be involved in cancer development and metastasis,³⁹ with changes in miR-16 expression detected in multiple cancers.^{4,6,12,13,16-20} Previous investigations have demonstrated the role of miR-16 as a tumor suppressor that is inhibited in several malignancies.^{40,50} miR-16 expression is downregulated in tumor tissue and cell lines in NSCLC⁴¹ with ectopic miR-16 expression significantly inhibiting cell proliferation and colony formation.²⁹ We

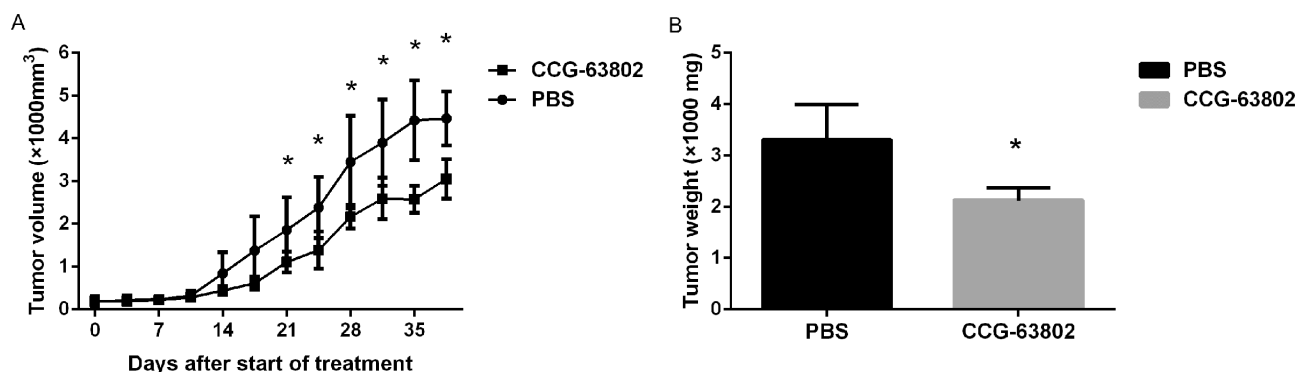


Figure 8 The RGS4 inhibitor restricted the growth of human xenografted lung cancer cells. **(A)** Tumor volume was significantly lower in mice treated with CCG-63802 compared to control mice. **(B)** Tumor weight was significantly lower in mice treated with CCG-63802 compared to control mice; * $P < 0.05$.

found that shRNA-induced *RGS4* knockdown resulted in an increase in miR-16 expression. Furthermore, inhibition of miR-16 was associated with upregulated *RGS4* expression, and the effects of *RGS4* silencing on cell proliferation and apoptosis were abolished. Indeed, *RGS4* expression is required for the proliferation of both normal and cancer cells, such as bladder carcinoma cells⁵¹ and lung airway smooth muscle cells.³⁷ Our data provide information regarding a previously unknown molecular mechanism in which RGS4 promotes NSCLC cell proliferation by regulating miR-16 expression. The expression of miR-16 is downregulated in NSCLC tissue samples and cell lines as well as in other malignancies.^{35,37,40–50} In NSCLC, miR-16 suppresses tumor development by inhibiting cell proliferation and promoting apoptosis.^{29,41} However, Cheng et al recently proposed that RGS4 is a tumor suppressor. Their study revealed that low *RGS4* expression levels are associated with poor survival in NSCLC patients, implying that RGS4 inhibits NSCLC progression.¹⁶ Similar findings have been reported in other cancers (eg, breast cancer cells) in which RGS4 suppresses cell migration and invasion, and the proteasome-mediated degradation of RGS4 leads to a metastatic phenotype.¹³ Meanwhile, RGS4 helps mediate glioma cell invasion as a downstream target of mTOR complexes.^{18,20} However, in this study, we found that upregulation of *RGS4* expression plays an important role in the proliferation of malignant tumors. Therefore, RGS4 may have dual functions in carcinogenesis depending on the cellular context and downstream targets. Future studies should aim to identify the factors controlling *RGS4* expression and its functions.

BDNF, which is a member of the neurotrophin superfamily, has been implicated in the pathophysiology of the

nervous system. A recent study reported that the autocrine activity of BDNF, induced by the STAT3 signaling pathway, causes prolonged TrkB activation and promoted the proliferation of human NSCLC cells.⁵² Our results showed that RGS4 regulated the expression of *BDNF*, which is one of the targets of miR-16.²⁹ The *BDNF* 3'-untranslated region is targeted by miR-16, which affects the cisplatin-regulated inhibition of SH-SY5Y cell growth.⁵³ Once activated, the BDNF-TrkB pathway typically affects neuronal survival and functions by regulating neuronal development and plasticity, long-term potentiation, and apoptosis.⁵⁴ The identification of *TrkA*, *TrkB*, and *TrkC* gene fusions and other oncogenic alterations in many tumor types implies that *Trk* gene family members are associated with human cancers.⁵⁵ Moreover, *BDNF* is reported to promote the proliferation of human NSCLC cells, and is associated with poor prognosis in NSCLC patients. Furthermore, *BDNF* is also involved in the tumorigenicity and malignant progression of invasive tumor cells in many other malignancies.⁴⁸ Our data suggested for the first time that RGS4 promotes NSCLC cell proliferation through BDNF-TrkB signaling, which was confirmed in an experiment involving the TrkB inhibitor K252a (Figure 6C).

Conclusion

In summary, the results of this study suggest that the role of RGS4 depends on miR-16 expression. Therefore, we propose that a vicious cycle of RGS4 and miR-16 expression ultimately leads to the activation of BDNF and tumor cell proliferation (Figure 6D). Our data provide new information regarding the molecular mechanisms underlying processes in NSCLC development, in which RGS4 promotes NSCLC cell proliferation by regulating miR-16 expression and the BDNF-TrkB signaling pathway.

Ethics Approval And Informed Consent

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Research Ethics Committee of Chinese PLA General Hospital and with the 1964 Helsinki declaration and its later amendments. All written informed consent to participate in the study was obtained from NSCLC patients for samples to be collected from them. The assay involving tissue microarray was also approved by the Research Ethics Committee of Chinese PLA General Hospital. All animal experiments were performed in compliance with the guidelines for the care and use of laboratory animals and were approved by the Research Ethics Committee of Beijing Center For Physical and Chemical Analysis.

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Disclosure

The authors report no conflicts of interest in this work.

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