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RASSF10 is epigenetically silenced and functions as a tumor suppressor in gastric cancer

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ABSTRACT

Ras association domain family (RASSF) proteins are encoded by several tumor suppressor genes that are frequently silenced in human cancers. In this study, we investigated *RASSF10* as a target of epigenetic inactivation and examined its functions as a tumor suppressor in gastric cancer. *RASSF10* was silenced in six out of eight gastric cancer cell lines. Loss or downregulation of *RASSF10* expression was associated with promoter hypermethylation, and could be restored by a demethylating agent. Overexpression of *RASSF10* in gastric cancer cell lines (JRST, BGC823) suppressed cell growth and colony formation, and induced apoptosis, whereas RASSF10 depletion promoted cell growth. In xenograft animal experiments, *RASSF10* overexpression effectively repressed tumor growth. Mechanistic investigations revealed that RASSF10 inhibited tumor growth by blocking activation of β-catenin and its downstream targets including c-Myc, cyclinD1, cyclinE1, peroxisome proliferator-activated receptor δ, transcription factor 4, transcription factor 1 and CD44. In conclusion, the results of this study provide insight into the role of RASSF10 as a novel functional tumor suppressor in gastric cancer through inhibition of the Wnt/β-catenin signaling pathway.

1. Introduction

Gastric cancer (GC) remains the second leading cause of cancer mortality worldwide [1]. In addition to multiple genetic alterations, recent studies indicate that epigenetic mechanisms, especially DNA methylation, are also associated with the development and progression of GC [2,3]. Promoter CpG methylation alters cancer-related gene expression and thereby modulates cancer pathways involved in cell proliferation, apoptosis and metastasis [4]. Several tumor suppressor genes associated with epigenetic alterations have been identified in GC, such as *RASSF1A*, *CHFR*, *CDH4*, and *CMTM3* [5–8], and genome-wide methylation screening has identified many genes in GC that are inactivated by DNA methylation [9,10]. However, the precise role of DNA methylation in GC remains unclear.

The RASSF (Ras association domain family) proteins comprise 10 members (RASSF1–10) [11–14], all of which share a conserved Ras association domain in the C-terminal (RASSF1–6) or N-terminal (RASSF7–10) region. All demonstrate biological properties compatible with a tumor suppressor function, and some are frequently inactivated or downregulated by promoter methylation during tumorigenesis [15–18]. RASSF10 is a recently identified member of the RASSF family, the gene for which is located on chromosome 11p15.2 [13]. Previous studies have revealed that *RASSF10* is frequently hypermethylated in leukemia, glioblastoma, thyroid and prostate carcinomas [19–22]. However, the expression of *RASSF10* and its role in GC remains unknown.

In the present study, we investigated *RASSF10* silencing by promoter hypermethylation in GC cells, and examined its role as a tumor suppressor, as well as the mechanisms responsible for its actions. The findings of our study suggest that epigenetic inactivation of RASSF10 plays a key role in gastric carcinogenesis.

2. Materials and methods

2.1. Cell lines

Eight GC cell lines (AGS, JRST, BGC823, MGC803, SNU1, KatoIII, MKN7, and MKN28) were obtained from the American Type Culture Collection (Manassas, VA, USA). The immortalized human gastric epithelial cell line GES1 was obtained from the Cancer Research Institute of Beijing, Beijing University, China. All cells were cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum. Cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere.

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2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was generated from 1 μg total RNA using SuperScript III (Invitrogen) and polyN primers. The PCR primers for *RASSF10* were 5′-CGCCATGGATCCTTCGGA-3′ (forward) and 5′-GCAGCCGTCCTCCAAAAGC-3′ (reverse). After PCR, the amplified products were electrophoresed in 2.5% Nuseive gels. The β-actin gene was used as an endogenous control. To analyze the restoration of *RASSF10* expression, AGS, JRST, KatoIII, and SNU1 cells were incubated for 96 h with the demethylating agent 2 μM 5-aza-2′-deoxycytidine (Sigma–Aldrich, St Louis, MO, USA) and then harvested for RT-PCR analysis.

2.3. Methylation-specific PCR (MSP) and bisulfite genomic sequencing (BGS)

Genomic DNA was extracted from GC cell lines using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Bisulfite modification of DNA, and MSP were performed as described previously [23]. The MSP primer sequences for the *RASSF10* promoter were: 5′-GGGTATTTTGGGTAGAGTTAGAGTG-3′ (forward) and 5′-AAACAAACTAAAAAACAACTACAAC-3′ (reverse) for unmethylated reactions (126 bp); and 5′-GGGTATTTTGGGTAGAGTTAGAGC-3′ (forward) and 5′-AAACAAACTAAAAAACGACTACGAC-3′ (reverse) for methylated reactions (126 bp). BSG was performed to characterize the methylation density in the *RASSF10* promoter using the BigDye Terminator Cycle Sequencing kit version 1.0 (Applied Biosystems, Foster City, CA, USA). The primers were: 5′-TTTGGGTTTTGAGTTTTTTGTATTT-3′ (forward) and 5′-ACTACACTAACCCTATTCCCCC-3′ (reverse). Twenty-three CpG sites spanning the −331 and −74 bp regions were evaluated. Sequences were analyzed using SeqScape software (Applied Biosystems).

2.4. Lentivirus infection and oligonucleotide transfection

The *RASSF10* sequence was purchased from Origene Technologies (Rockville, MD, USA). *RASSF10* siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Constructs containing the *RASSF10* or *RASSF10* siRNA sequence were cloned into the lentivirus-based expression plasmid pCDH-CMV-MCS-EF1-coGFP constructs (System Biosciences, CA, USA). Virus particles were harvested 48 h after pCDH-CMV-*RASSF10* or pCDH-CMV-*RASSF10* siRNA transfection with the packaging plasmids pRSV/pREV, pCMV/pVSVG and pMDLG/pRRE into 293T cells using Lipofectamine 2000 reagent (Life Technologies, Inc., Grand Island, NY, USA). JRST and BGC823 cells were infected with recombinant lentivirus-transducing units plus 10 μg/ml Polybrene (Sigma, St Louis, MI, USA).

2.5. Cell proliferation and apoptosis assays

Cells were plated in 24-well plates at 5×10^3 cells per well and cultured for 1–4 days, respectively. Cells were trypsinized and counted with a Coulter counter (Beckman Coulter, Fullerton, CA, USA). For analysis of apoptosis, cells were stained with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI) and analyzed with a flow cytometer (FACScan®; BD Biosciences, Mountain View, CA, USA) equipped with CellQuest software (BD Biosciences). All assays were conducted in triplicate.

2.6. Colony-formation assay

Cells (1×10^3) were plated into 10-cm dishes and cultured for 2 weeks to allow colony formation. After 2 weeks, the cells were washed twice with phosphate-buffered saline (PBS), fixed with methanol/acetic acid (3:1, v/v), and stained with 0.5% crystal violet. The number of colonies was counted under the microscope. All experiments were performed in triplicate wells in three independent experiments.

2.7. Western blotting

Proteins from the cells were extracted in radio-immunoprecipitation assay buffer (Beyotime, China) and protein concentrations were determined using a BCA assay kit (Beyotime). Cell extracts (20–30 μg) were boiled with equal amounts of loading dye for 10 min and separated by 10% polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes (Hybond-ECL, GE HealthCare, UK). Membranes were blocked in PBS with 0.1% Tween 20 (PBS-T) containing 5% non-fat milk for 1 h, and then incubated with primary and secondary antibodies in PBS-T containing 5% non-fat milk. The following primary antibodies were used at the indicated dilutions: RASSF10, β-catenin, cyclinD1, PPARδ, cyclinE1, β-actin (1:1000; Abcam, UK); c-Myc, CD44, TCF-1, and TCF-4 (1:500; Santa Cruz Biotechnology). Primary antibody incubations were carried out overnight at 4 °C. The membranes

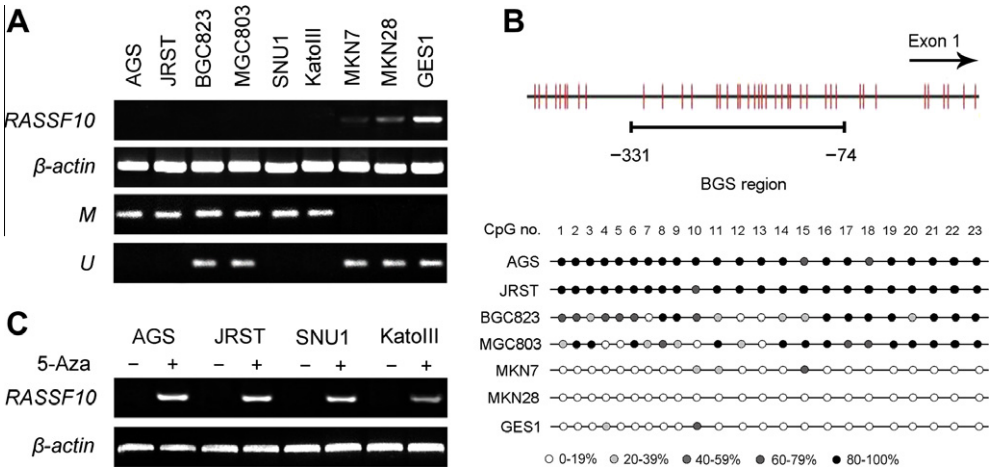

Fig. 1. Expression and methylation status of *RASSF10* in GC cell lines. (A) *RASSF10* was frequently silenced and methylated in GC cell lines, but was expressed and unmethylated in GES1 immortalized gastric epithelial cells. (B) The methylation status of the *RASSF10* promoter was confirmed by BGS. (C) Pharmacological demethylation with 2 μM 5-aza-2′-deoxycytidine restored *RASSF10* expression.

were then washed with wash buffer (1× PBS and 0.01% Tween-20) and incubated with a 1:2000 dilution of horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody. The membranes were washed with wash buffer and developed.

2.8. Luciferase reporter assay

TOPflash (TCF reporter) luciferase plasmid (Millipore, Watford, UK) and FOPflash scrambled vector (Millipore) were used. Cells were transiently transfected with 1 mg of TOPFlash or FOPFlash (scrambled control), or 0.1 mg pRLTK using Fugene HD (Roche, Basel, Switzerland), according to the manufacturer's instructions. Firefly and *Renilla* luciferase activities were assayed 24 h post-transfection. Firefly luciferase activity was normalized to *Renilla* luciferase activity as a transfection control. Promoter activity was expressed as luciferase activity relative to that obtained in FOPFlash (scrambled) control-transfected cells.

2.9. In vivo xenograft tumor growth in nude mice

Four-weeks-old, male Balb/c athymic nude mice were housed and manipulated according to the protocols approved by the Animal Research Committee at Shanghai Second Military Medical University. Approximately 5×10^6 BGC823-RASSF10 or control cells in log-phase growth were injected subcutaneously into the dorsal flank of the mice. The mice were housed for 25 days post inoculation. All mice were then euthanized and tumor tissues were removed by surgical excision. Tumor volume was measured every 5 days during the experimental period.

2.10. Statistical analysis

Data were expressed as the mean ± SD of at least three independent experiments. The differences between two groups were ana-

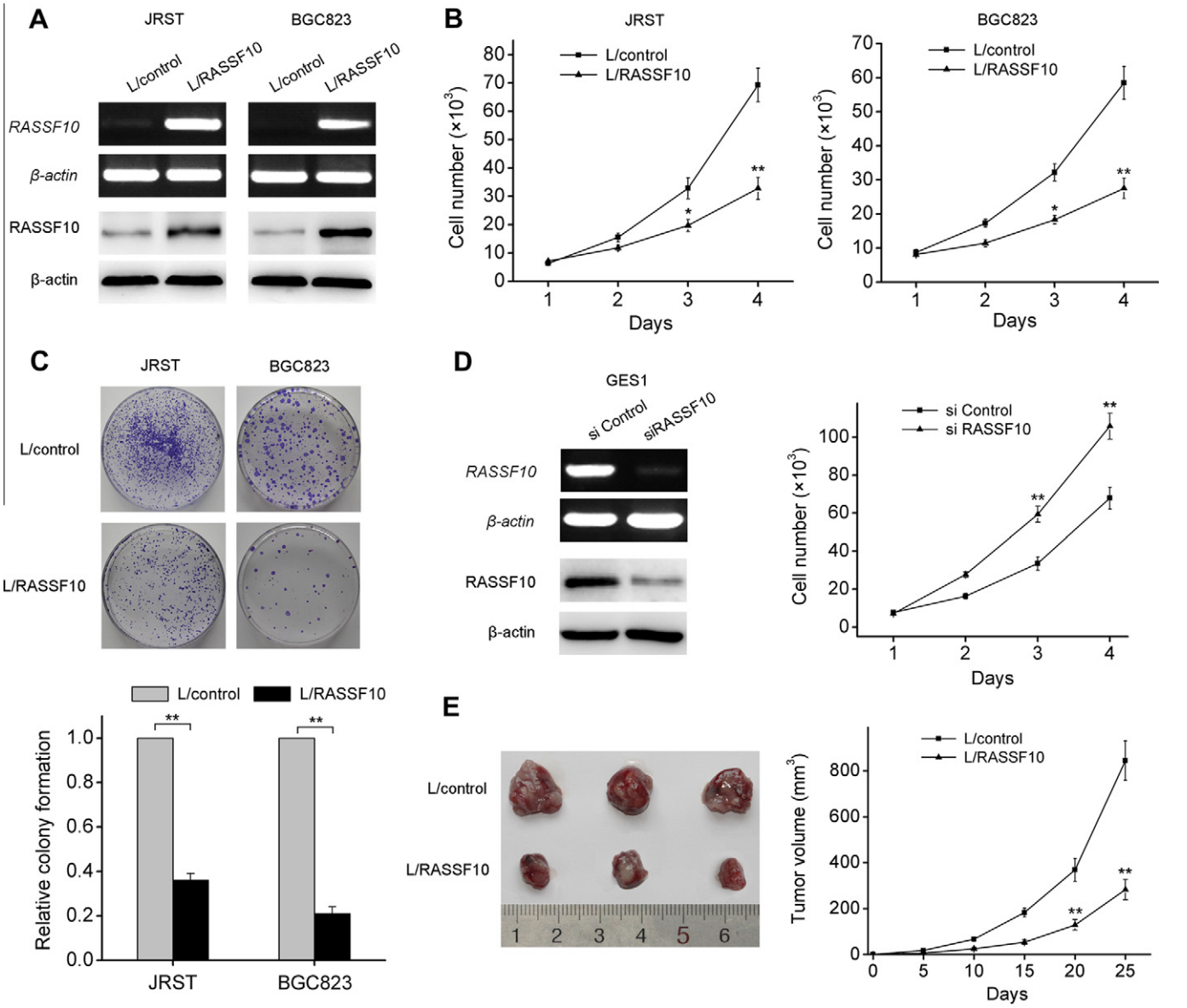

Fig. 2. *RASSF10* overexpression suppressed the growth and tumorigenicity of GC cells. (A) Expression levels of the RASSF10 gene and protein in JRST and BGC823 cells infected with *RASSF10*-overexpressing lentivirus (L/RASSF10) and control lentivirus (L/control) were measured by RT-PCR and Western blotting, respectively. (B) Cell growth was suppressed by *RASSF10* in JRST and BGC823 cells. (C) Overexpression of *RASSF10* inhibited colony formation. (D) *RASSF10* deletion promoted cell growth in GES1 cells. (E) Xenograft tumors recovered from three representative nude mice (left). Increase in tumor volume (right).

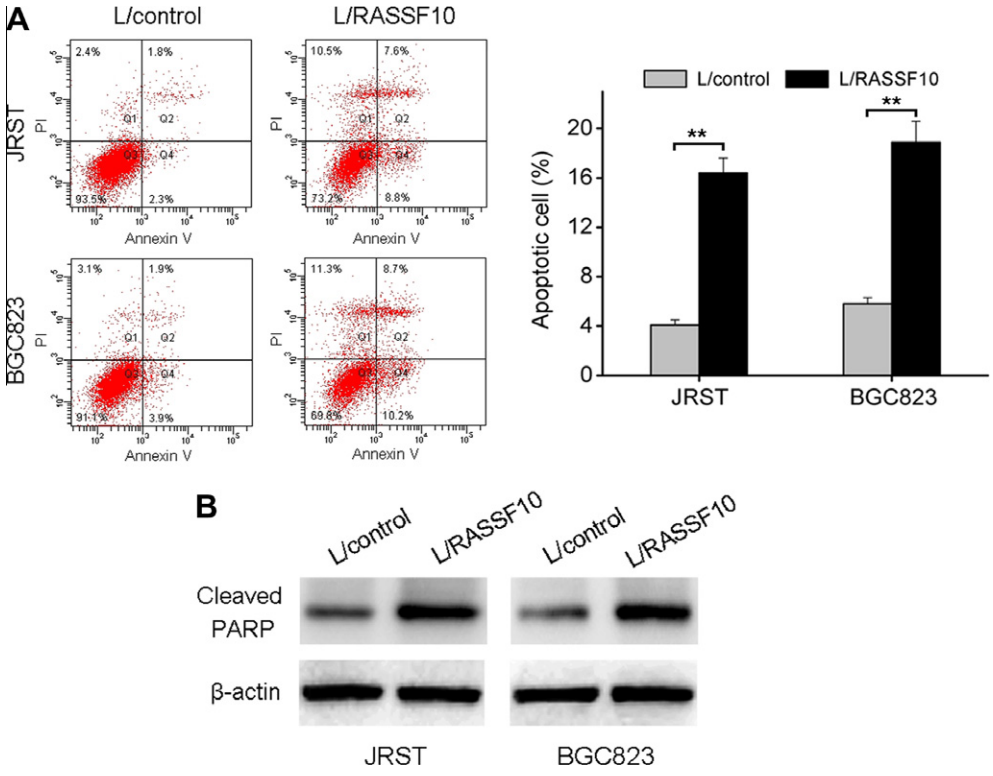

Fig. 3. *RASSF10* induced apoptosis of GC cells. (A) Apoptosis was determined by flow cytometry analysis of Annexin V/PI double-stained cells. *RASSF10* induced apoptosis in JRST and BGC823 cells. (B) *RASSF10* induced protein expression of cleaved poly-(ADP-ribose) polymerase in JRST and BGC823 cells, measured by Western blotting.

lyzed using Student's *t*-tests. Differences were considered to be statistically significant when $P < 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$.

3. Results

3.1. Epigenetic silencing of RASSF10 gene expression in GC cells

To explore the expression and significance of RASSF10 in GC carcinogenesis, we measured the gene expression levels of *RASSF10* in a panel of GC cell lines by RT-PCR. *RASSF10* expression was significantly reduced in six out of eight (75%) GC cell lines compared with GES1 immortalized gastric epithelial cells (Fig. 1A). We then investigated the involvement of promoter methylation in *RASSF10* silencing using MSP, and found that the *RASSF10* promoter was fully or partially methylated in the same six cell lines (Fig. 1B). These findings were validated by BGS; consistent with the results of MSP, dense methylation was observed in the methylated cell lines (KatoIII, JRST, AGS and SNU1), but not in the un-methylated lines (MKN7, MKN28 and GES) (Fig. 1D). To confirm that promoter methylation was responsible for *RASSF10* silencing, we treated four methylated cell lines with the demethylating agent 5-aza-2′-deoxycytidine, which resulted in re-expression of *RASSF10* mRNA in all of the cell lines (Fig. 1D). Taken together, these results indicate that *RASSF10* promoter methylation was significantly correlated with reduced *RASSF10* expression in GC cells.

3.2. RASSF10 overexpression inhibited cell growth in vitro and in vivo

To investigate the role of *RASSF10* in GC cell growth, we performed *in vitro* gain-of-function analyses by overexpressing *RASSF10* in two silenced GC cell lines (JRST and BGC823) using a lentiviral vector. RASSF10 expression levels were increased after transfection, as shown by RT-PCR and Western blotting, respectively (Fig. 2A). Overexpression of *RASSF10* significantly inhibited the growth of JRST and BGC823 cells compared with the corresponding controls (Fig. 2B). *RASSF10* overexpression also suppressed colony-formation in both the cell lines (Fig. 2C). Accordingly, GES1 cells with depleted *RASSF10* showed increased cell growth (Fig. 2D). We subsequently investigated the effects of *RASSF10* expression on the tumorigenic potential of GC cells in vivo. BGC823 cells overexpressing *RASSF10* were injected subcutaneously into nude mice and the animals were closely monitored for tumor growth. The average tumor volume was significantly reduced in *RASSF10*-overexpressing tumors compared with vector control tumors (Fig. 2E). These results indicate that RASSF10 plays a suppressor role in gastric carcinogenesis.

3.3. RASSF10 induced apoptosis in GC cells

We performed cell apoptosis assays to clarify the mechanism whereby RASSF10 inhibits cell growth. Overexpression of *RASSF10* provoked a robust apoptotic response in JRST and BGC823 cells (Fig. 3A). Consistent with these apoptosis result, *RASSF10* overexpression also enhanced the expression of cleaved poly-(ADP-ribose) polymerase, a key apoptosis-related protein, in both JRST and BGC823 cells (Fig. 3B).

3.4. RASSF10 inhibited Wnt/β-catenin signaling pathway

To explore the molecular mechanisms of tumor suppression by RASSF10, we examined the canonical Wnt signaling pathway, which is known to be aberrantly activated in GC and is important for the proliferation, survival and metastasis of GC cells. Overexpression of *RASSF10* in JRST and BGC823 cells reduced TCF/LEF transcriptional activity (Fig. 4A), indicating that the canonical Wnt signaling pathway was altered by *RASSF10* overexpression. *RASSF10* overexpression also resulted in nuclear localization of β-catenin in JRST and BGC823 cells (Fig. 4B). We examined the

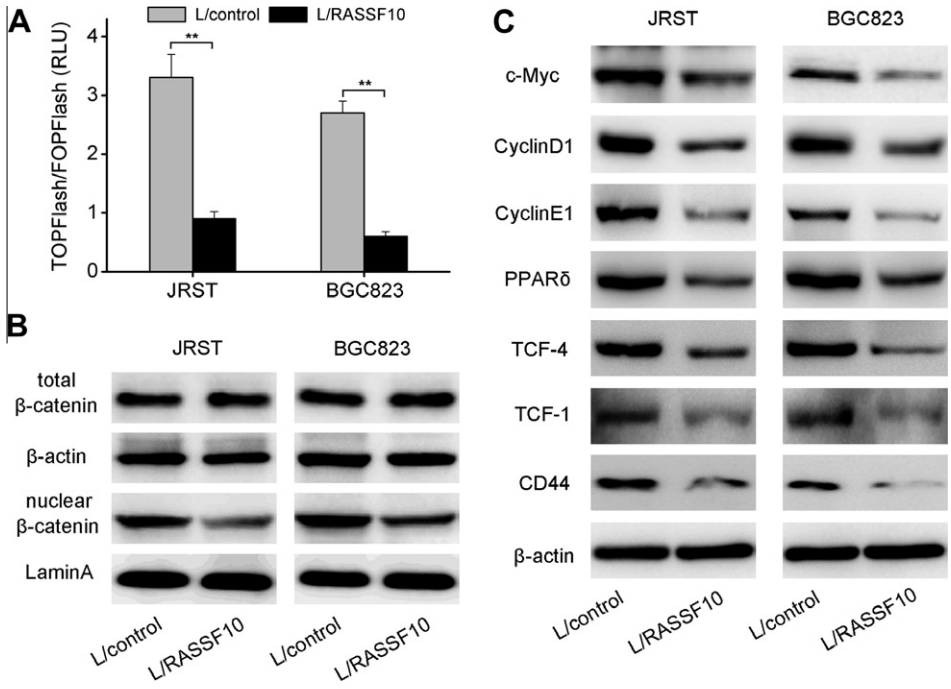

Fig. 4. *RASSF10* regulated Wnt/β-catenin signaling pathway. (A) Overexpression of *RASSF10* reduced TCF/LEF transcriptional activity in JRST and BGC823 cells. (B) Overexpression of *RASSF10* reduced nuclear β-catenin levels, but total levels remained constant. (C) Expression levels of c-Myc, cyclin D1, cyclin E1, PPARδ, TCF-1, TCF-4, and CD44 in JRST and BGC823 cells infected with L/control or L/RASSF10, examined by Western blotting.

potential effects of RASSF10 on the β-catenin downstream target genes c-Myc, cyclinD1, cyclinE1, peroxisome proliferator-activated receptor δ, TCF-1, TCF-4, and CD44. Levels of these molecules were all reduced in JRST and BGC823 cells stably overexpressing *RASSF10* (Fig. 4C). These findings demonstrated that RASSF10 suppressed GC cell growth through inhibiting the Wnt/β-catenin signaling pathway.

4. Discussion

Epigenetic inactivation of tumor suppressor genes is a fundamental event in the pathogenesis of malignant tumors. In this study, we showed that gene for the recently identified RASSF member *RASSF10* was frequently silenced in GC cell lines. Downregulation of *RASSF10* expression was closely associated with aberrant promoter methylation, as evaluated by MSP and validated by BGS. In addition, *RASSF10* expression could be restored by pharmacological demethylation, suggesting that promoter methylation was a predominant regulatory mechanism of *RASSF10* inactivation in GC.

RASSF10 has been reported to function as a tumor suppressor in several cancers including glioblastoma, thyroid and prostate cancers [20–22]. To reveal the role of RASSF10 in GC carcinogenesis, we tested its effects on cell growth *in vitro* and in vivo. Our results showed that overexpression of *RASSF10* significantly inhibited cell growth and colony formation in silenced GC cell lines (JRST and BGC823), and suppressed tumorigenesis in nude mice. In contrast, siRNA-mediated *RASSF10* deletion in normal gastric epithelial GES1 cells significantly promoted cell growth. Overexpression of *RASSF10* significantly increased apoptosis, as verified by upregulation of cleaved poly (ADP-ribose) polymerase by RASSF10. These results indicate that RASSF10 functions as a tumor suppressor in gastric carcinogenesis.

The results of this study also demonstrated that *RASSF10* overexpression inhibited the Wnt/β-catenin signaling pathway in GC cells. This pathway plays an important role in cancer development and progression by regulating cell proliferation and apoptosis [24–26]. In this study, *RASSF10* overexpression reduced nuclear accumulation of β-catenin, as well as increasing TCF/LEF transcriptional activity. We further explored the molecular mechanisms of growth inhibition induced by *RASSF10* by examining the expression levels of the β-catenin downstream genes c-Myc, cyclinD1, cyclinE1, peroxisome proliferator-activated receptor δ, TCF-1, TCF-4 and CD44 [27–30], and demonstrated that the protein levels of all these molecules were significantly suppressed. All these results indicate that the inhibition of GC cell growth by RASSF10 is mediated, at least in part, by inhibition of the Wnt/β-catenin signaling pathway.

In summary, the results of this study demonstrated that *RASSF10* is a functional tumor suppressor gene in GC, and is mainly inactivated by promoter methylation. RASSF10 contributes to the suppression of tumorigenesis by promoting cell apoptosis and decreasing cell proliferation through inhibiting the Wnt/β-catenin signaling pathway. RASSF10 might thus serve as a novel molecular target for the detection and treatment of GC and other human cancers.

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