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Original Article

TRIM29 functions as an oncogene in gastric cancer and is regulated by miR-185

Feng Qiu[1,2], Jian-Ping Xiong[2], Jun Deng[2], Xiao-Jun Xiang[2]

[1]*Affiliated Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, People's Republic of China;* [2]*Department of Oncology, The First Affiliated Hospital of Nanchang University, Nanchang 330006, People's Republic of China*

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Abstract: Tripartite motif-containing 29 (TRIM29) belongs to TRIM family of transcription factors and may function as an oncogene or a tumor suppressor depending on the tumor types. Overexpression of TRIM29 is frequently observed in gastric cancer but the underlying mechanisms remain largely unknown. In the present study, we investigated the function of TRIM29 in gastric cancer-derived cell line MGC803. RNAi-mediated silencing of TRIM29 resulted in significantly reduced cell proliferation and colony formation, as well as G1-S cell cycle arrest and apoptosis. Interestingly, expression levels of β-catenin, cyclin D1 and c-Myc were all downregulated in TRIM29 knockdown cells, indicating that TRIM29 is involved in regulating the activity of Wnt/β-catenin signaling pathway. Furthermore, based on target prediction and luciferase assay, we identified TRIM29 as a potential target of miR-185, which is frequently downregulated in gastric cancer. Over-expression of miR-185 in MGC803 cells inhibited TRIM29 expression and activity of Wnt/β-catenin signaling. Taken together, our results suggest that TRIM29 functions as an oncogene in gastric cancer and is regulated by miR-185.

Keywords: TRIM29, Wnt/β-catenin signaling, gastric cancer, miR-185

Introduction

Gastric cancer (GC) is the most frequent malignancy occurring in Southeast Asia and the second leading cause of cancer mortality worldwide [1]. Despite recent improvements in GC therapy, the disease remains a huge health burden due to the high recurrence rates and tumor metastases. Recent studies have identified the association of multiple genetic, epigenetic and environmental factors with the disease, however, the underlying molecular mechanisms that govern the survival and colonization of tumor cells has not yet been fully understood. Tripartite motif-containing 29 (TRIM29), is a member of the tripartite motif (TRIM) family characterized by the conserved RING finger, B-box, and coiled-coil domains [2]. The TRIM family has been implicated in a variety of physiologic processes including development, apoptosis and epithelial-mesenchymal transition (EMT) [2, 3]. Previous studies have identified TRIM29 as a potential oncogene in breast cancers [4, 5]. It has been shown that TRIM29 can facilitate tumor cell proliferation and invasion through stabilization of β-catenin [6]. The mechanisms may also involve deactivation of p53 activity and promoting cell survival by inhibiting proapoptotic genes regulated by p53 [7]. However, the function of TRIM29 could also be oncogenic in some tumors such as pancreatic cancers [4, 5], depending on cellular context and tumor types [4]. Accordingly, a possible role of TRIM29 in pathogenesis of gastric cancer remains to be explored. Here we investigated the function of TRIM29 in gastric cancer-derived cell line MGC803. We present data supporting a tumor suppressor effect of TRIM29 in gastric tumor cells. To determine the potential mechanism underlying how TRIM29 is regulated by miRNA, we searched for putative miRNAs binding at the TRIM29 3'UTR region. We have identified TRIM29 as a functional target of miR-185, which is down-regulated in GC cell lines. We find that miR-185 represses TRIM29 expression and significantly inhibited malignant behavior

via regulating Wnt/β-catenin signaling. The results showed an anti-growth effect of TRIM29, and identified its miRNA regulatory mechanism in gastric cancer.

Materials and methods

Cell lines and tissues

Human GC cell lines GES1, BGC823, and MGC803 were cultured in PRIM1640 medium with 10% fetal bovine serum (FBS) and 1% antibiotics. All cell lines were incubated at 37°C in 5% CO_2.

Cell transfection

TRIM29 siRNA and miR-185 mimics were created, and the nonsense sequence vector (NC) served as a negative control. All of the vectors were synthesized by GenePharma, Shanghai. MGC803 cells were transfected with Lipofectamine 2000 (Invitrogen). For real-time PCR analysis of miR-185 and TRIM29 expression, total RNA was isolated using Trizol Reagent (Invitrogen), reverse transcribed using a reverse transcription kit, and subjected to real-time PCR using a SYBR Green-real -time PCR master mix kit (Invitrogen). Real-time PCR was performed using a Step One plus Real-Time PCR System (Applied Biosystems). For PCR amplification, the following primers were used: TRIM29, 5'-CTATGTGAACAACTACACGAACAG-3' (forward) and 5'-TGTCAGGTACATGGAGTATCTCTTCAT-3' (reverse); GAPDH, 5'-ACCCACTCCTCCACCTTTGAC-3' (forward) and 5'-GTCCACCACCCTGTTGCTGTA-3' (reverse). Values are average of triplicates ± S.D.

Cell proliferation assay

MTT experiment was used following the manufacture's protocol. Briefly, cells were plated by 2×10^2 cells/well in 96-well plates and cultured for up to seven days. 10 μl of MTT solution was added to each well, and incubated for 4 h at 37°C. Absorbance at 450 nm was read on a microplate reader. All experiments were performed in triplicate.

Colony formation assay

A total of 3×10^2 cells were plated into 6-cm plates. Cells were fixed with methanol and stained with 0.1% crystal violet after 10 days. The number of colonies with ≥ 50 cells/colony was counted. All experiments were performed in triplicate. Cell cycle assay and apoptosis by flow cytometry was performed using propidium iodide DNA staining (Invitrogen). Cellular apoptosis was measured using Annexin-V -PE/7-AAD apoptosis detection kit (BD Biosciences) based on the manufacturer's instructions. Cells undergoing apoptosis were Annexin V -PE positive and 7-AAD negative. All experiments were performed in triplicate.

Western blotting

Each group of cells was seeded into 6-well plates, the cells were allowed to grow until 100% confluence, and then lysed in lysis buffer on ice. Proteins were separated by 12% SDS-PAGE, and blotted onto nitrocellulose membranes. Membranes were blocked with 10% non-fat milk powder at room temperature for 1 h, and incubated overnight with the primary antibodies TRIM29, β-catenin, c-myc, and CyclinD1 (Abcam, USA). After three 15 min washes in TBS-T, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, and then washed again in TBS-T. The membranes were then developed with an ECL plus Western blotting detection system.

Luciferase reporter assay

293T cells were seeded into 24-well plates at 2×10^5 cells/per well. TRIM29-3'-UTR was amplified by primers 5'-CTCGGATCCGTGAATGGGAGATGGGTG-3' (Forward), 5'-GATATCGTCAGCAGATGCCTGGAC-3' (reverse) and cloned into pmirGLO Dual-Luciferase miRNA Target Expression Vector using EcoRV and BamHI. Mutant TRIM29-3'-UTR was generated by replacing TCTCTCCA with AGAGAGGT. Both vectors were co-transfected with 100 nM miR-185 mimics or a negative control using Lipofectamine 2000 (Invitrogen). Renilla and firefly luciferase activities were measured by a Dual-Luciferase Reporter Assay (Promega) at 48 h after transfection.

Statistical analysis

Data from at least three independent experiments were expressed as means ± standard deviation (SD). The differences between groups were analyzed using the Student's t-test when only two groups were present, or by one-way analysis of variance (ANOVA) when more than two groups were compared. A value of $P <$

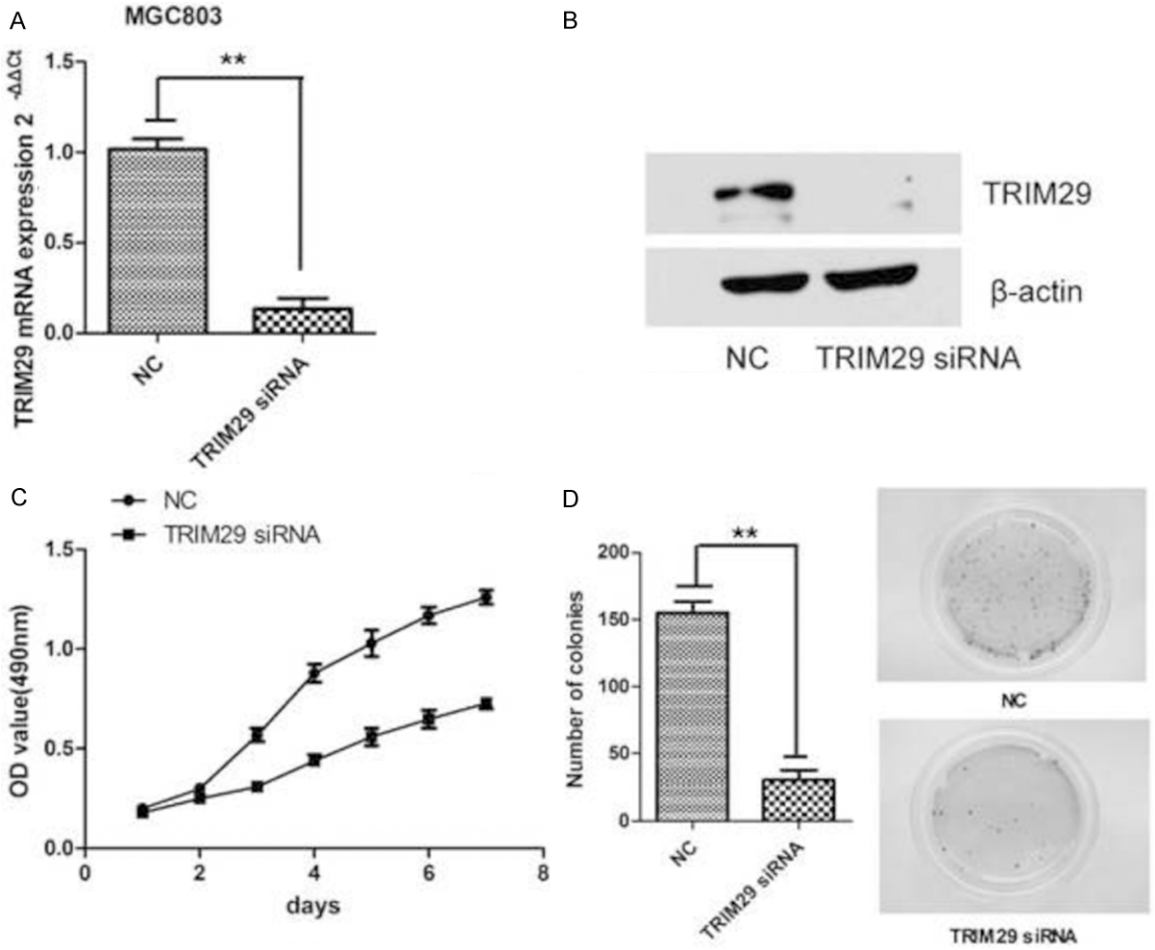

Figure 1. TRIM29 silencing in gastric cancer-derived cell line MGC803 strongly inhibited cell proliferation and colony formation. A. TRIM29 siRNA decreased the mRNA expression of TRIM29. B. TRIM29 siRNA decreased the protein expression of TRIM29. C. MGC803 growth curve after TRIM29 siRNA, NC siRNA transfection. The growth index was assessed at 1, 2, 3, 4, 5, 6, and 7 days. D. 3×10^2 cells were plated into 6-cm plates. Ten days later, cells were fixed with methanol and stained with 0.1% crystal violet. All experiments were performed in triplicate. $^{**}P < 0.01$.

0.05 was considered to indicate statistical significance.

Results

TRIM29 silencing inhibits cell proliferation, colony formation, and induces G1-S cell cycle arrest and apoptosis

We used siRNA to knockdown TRIM29 expression in GC cell MGC803. As shown in **Figure 1A** and **1B**, TRIM29 mRNA and protein levels were significantly downregulated in knockdown cells. We then carried out a proliferation assay to investigate the potential role of TRIM29 as an oncogene in GC carcinogenesis. In the MGC803 cell line, TRIM29 siRNA significantly decreased cell proliferation (**Figure 1C**, **1D**). We further investigated the effect of TRIM29 silencing on the cell cycle and apoptosis using flow cytometry. TRIM29 silencing resulted in cell cycle arrest demonstrated by enhanced percentage of cells in G1/G0 phase and decreased percentage of cells in S phase (**Figure 2A**). Interestingly, knockdown of TRIM29 resulted in enhanced apoptosis of MGC803 cells (**Figure 2B**). These results suggest that TRIM29 may act as an oncogene in GC by facilitating cell cycle progression and reducing apoptosis.

TRIM29 knockdown results in reduced activity of Wnt/β-Catenin signaling pathway in gastric cancer cells

One possible downstream mediators of TRIM29 is Wnt/β-Catenin signaling pathway [8]. Previous study has shown that TRIM29 expres-

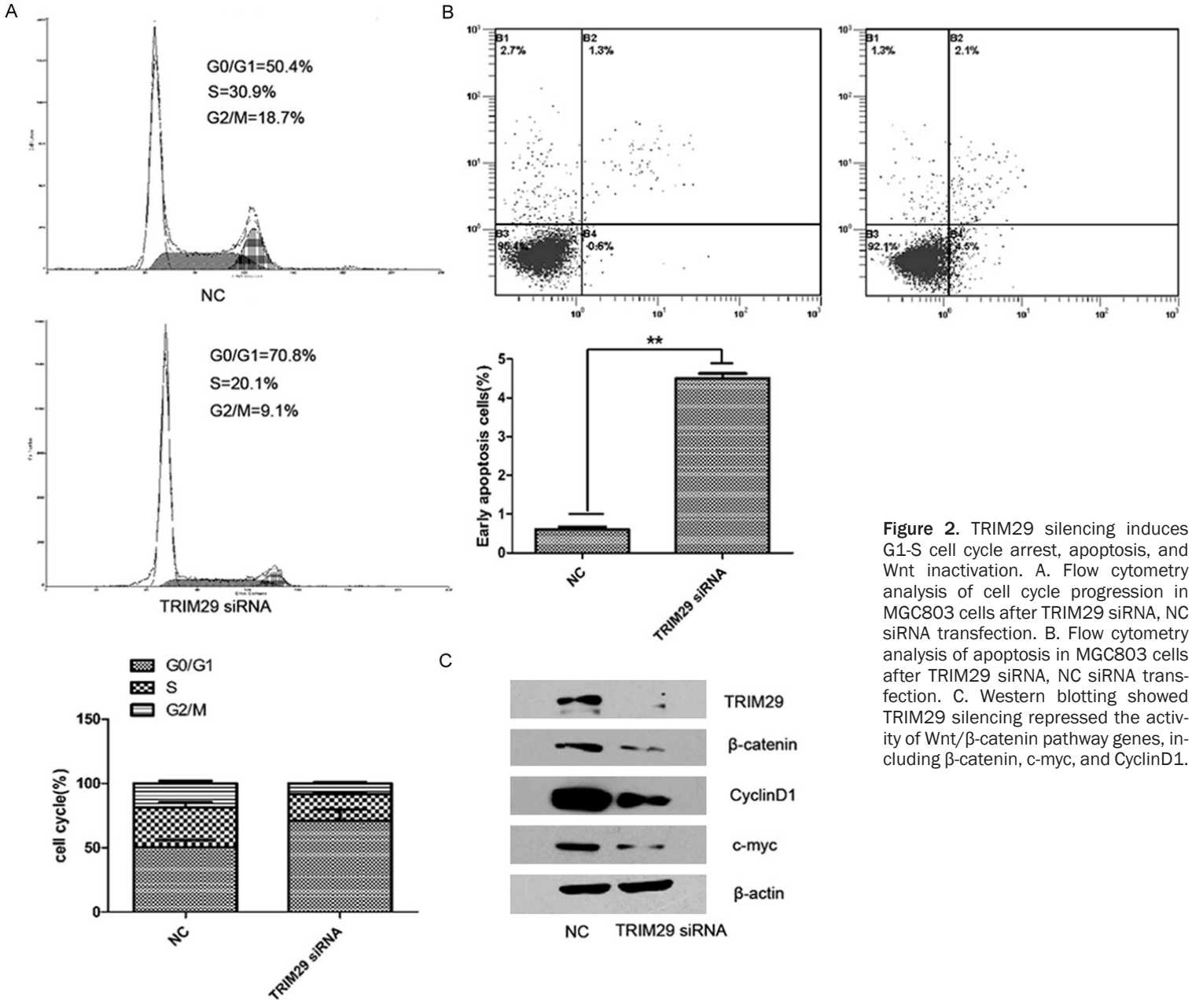

Figure 2. TRIM29 silencing induces G1-S cell cycle arrest, apoptosis, and Wnt inactivation. A. Flow cytometry analysis of cell cycle progression in MGC803 cells after TRIM29 siRNA, NC siRNA transfection. B. Flow cytometry analysis of apoptosis in MGC803 cells after TRIM29 siRNA, NC siRNA transfection. C. Western blotting showed TRIM29 silencing repressed the activity of Wnt/β-catenin pathway genes, including β-catenin, c-myc, and CyclinD1.

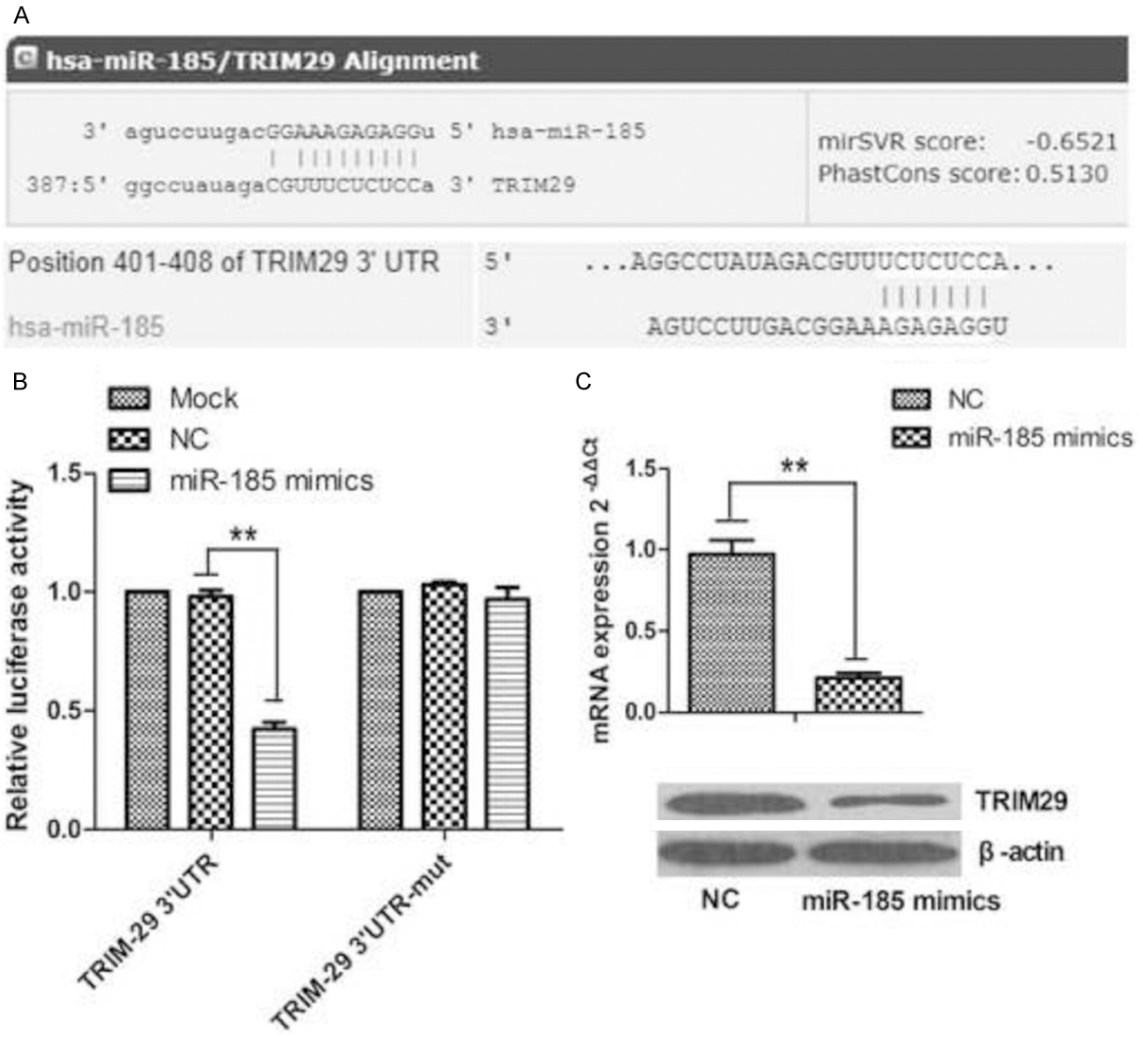

Figure 3. TRIM29 identified as a direct target of miR-185. A. TargetScan and Miranda showed TRIM29 to be identified as a direct target of miR-185. B. TRIM29-3'UTR-WT and TRIM29-3'UTR-MUT reporter luciferase activity in 293T cells treated with miR-185 mimics (100 nM), or NC. C. In the MGC803 cell line, TRIM29 was negatively regulated by miR-185 at both the transcriptional and protein levels. All experiments were performed in triplicate. $^{**}P < 0.01$.

sion correlated with elevated β-catenin levels in pancreatic cancer [5]. In line with this observation, siRNA-mediated silencing of TRIM29 resulted in significantly reduced expression of β-catenin in MGC803 cells (**Figure 2C**). Moreover, previous results have also shown that Activation of Wnt/β-catenin promotes G1 progression via up-regulation of cyclinD [2, 9]. The Wnt/β-catenin pathway is also involved in apoptosis through the regulation of c-myc activation [10, 11]. Consistent with these results, we found that expression of cyclinD and c-myc were all downregulated in cells lacking TRIM29. These results suggest that activation of Wnt/β-catenin signaling might be required for TRIM29's oncogenic effects.

TRIM29 is identified as a functional target of miR-185The mechanisms by which TRIM29 expression is regulated in gastric tumor remain unclear

One possible regulator of TRIM29 expression is miRNA [12]. Abnormal expression of miRNA has been implicated in various cancers [13]. We searched for potential miRNAs targeting sites in the TRIM29 3'UTR region using TargetScan6.2 [14] and Miranda [15]. Among the candidates (**Figure 3A**), miR-185 was selected for the further analysis. To determine whether TRIM29 is a direct target of miR-185, we cloned a part of the 3'UTR region of TRIM29 containing the potential miRNA binding site into a dual-lucifer-

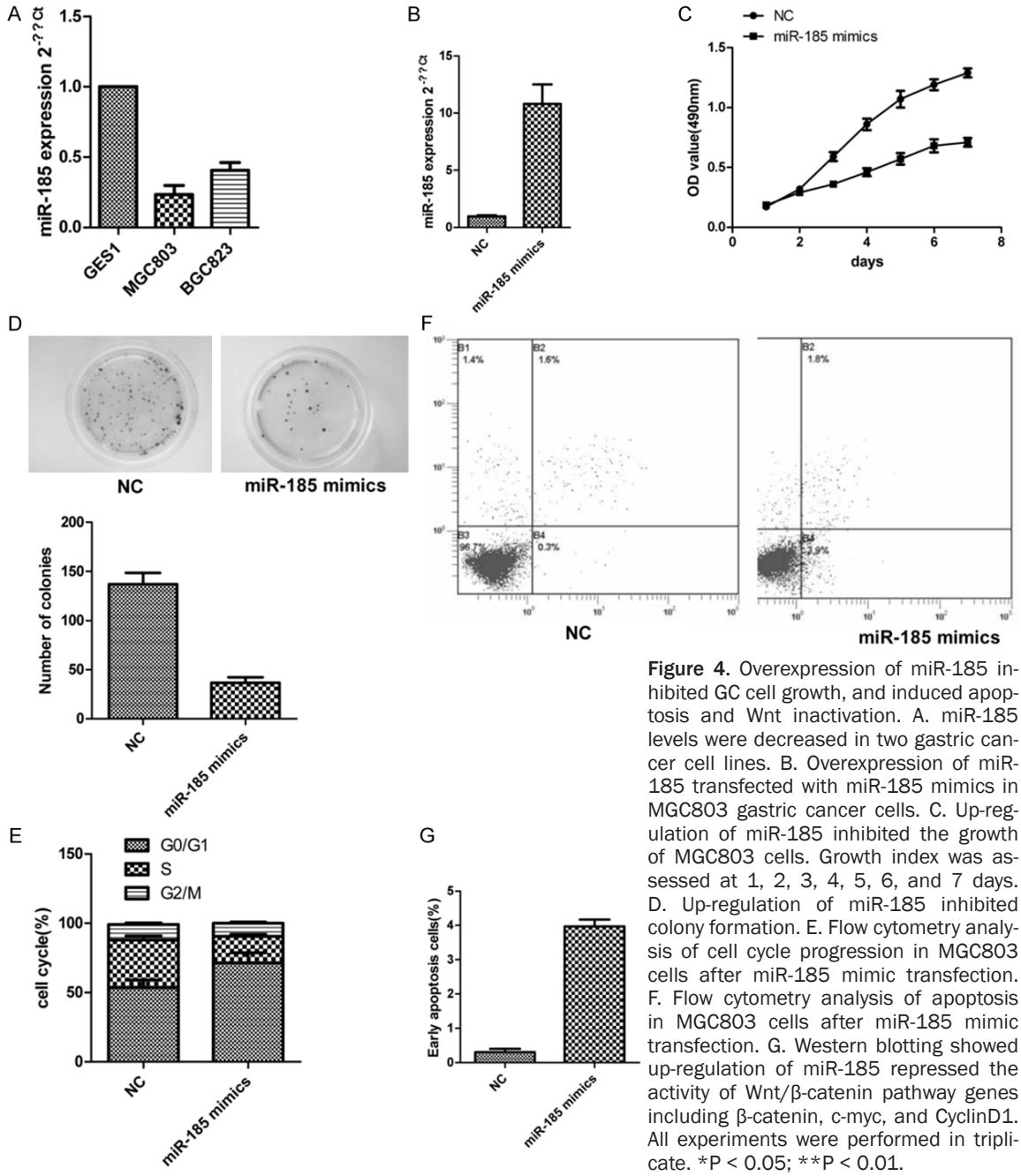

Figure 4. Overexpression of miR-185 inhibited GC cell growth, and induced apoptosis and Wnt inactivation. A. miR-185 levels were decreased in two gastric cancer cell lines. B. Overexpression of miR-185 transfected with miR-185 mimics in MGC803 gastric cancer cells. C. Up-regulation of miR-185 inhibited the growth of MGC803 cells. Growth index was assessed at 1, 2, 3, 4, 5, 6, and 7 days. D. Up-regulation of miR-185 inhibited colony formation. E. Flow cytometry analysis of cell cycle progression in MGC803 cells after miR-185 mimic transfection. F. Flow cytometry analysis of apoptosis in MGC803 cells after miR-185 mimic transfection. G. Western blotting showed up-regulation of miR-185 repressed the activity of Wnt/β-catenin pathway genes including β-catenin, c-myc, and CyclinD1. All experiments were performed in triplicate. *P < 0.05; **P < 0.01.

ase reporter. Co -transfection of this vector with a miR-185 mimics resulted in significant reduced luciferase activity. In contrast, the reporter with mutant miRNA binding site was largely consistent to miRNA (**Figure 3B**). In the MGC803 cell line, TRIM29 was regulated negatively by miR-185 mimics at both the transcriptional and protein levels (**Figure 3C**). These results indicate that TRIM29 is a direct target of miR-185.

MiR-185 reduces cell proliferation and induces apoptosis in human GC. We then investigated the biological role of miR-185 in GC cell lines

Reduced expression of miR-185 levels was observed in two different GC cell lines, MGC803 and BGC823, as compared with gastric cell GES-1 (**Figure 4A**). Transfection of miR-185 mimics resulted in significantly enhanced

expression of miR-185 inMGC803 GC cells (**Figure 4B**). The results showed that overexpression of miR-185 inhibited cell proliferation and colony formation, and induced G1-S cell cycle arrest and apoptosis (**Figure 4C-F**). These results suggest that miR-185 may have a tumor suppressing role in GC development and progression. We then examined whether miR-185 inactivated the Wnt/β-catenin pathway through targeting TRIM29. As expected, the results revealed that miR-185 up-regulation repressed the activity of Wnt/β-catenin pathway genes including β-catenin, c-myc, and CyclinD1 (**Figure 4G**).

Discussion

Accumulating evidence indicates that TRIM29 and abnormal miRNA expression may be common molecular mechanisms involved in the development of cancers. Therefore, better understanding the function of TRIM29 and miRNAs in the pathogenesis of GC may lead to more effective cancer therapies. In the present study, we have shown for the first time that the miR-185-TRIM29 axis regulates GC cell growth and apoptosis through Wnt/β-catenin pathway inactivation in vitro.

Recent study has shown TRIM29 functions as an oncogene in GC, and the overexpression of TRIM29 mRNA is associated with poor prognosis and worse survival [3]. However, the mechanism of action of TRIM29 in GC remains largely unknown. A study showed that TRIM29 functions as an oncogenic in pancreatic cancer through Wnt-pathway activation and beta-catenin stabilization [5]. Another report found that TRIM29 protein binds p53 and antagonizes p53-mediated functions [9]. The current study showed that down-regulation of TRIM29 reduced the proliferative ability of GC cells. In addition, down-regulation of TRIM29 in GC cells blocks cell cycle progression by arresting cells in G0/G1. Moreover, down-regulated TRIM29 induced apoptosis of GC cells. To explore the mechanisms involved in TRIM29-mediated tumor growth in GC, we detect the Wnt/β-catenin pathway after TRIM29 knockdown.

The Wnt/β-catenin pathway participates in a wide number of cellular processes including cell growth, proliferation, invasion, apoptosis, differentiation, drug-resistance, and metabolism [10, 11, 16, 17]. Deregulation of the Wnt/β-catenin pathway is common in many cancers, especially in GC. Loss of APC function, inactivation of Axin, and activation of beta-catenin mutations results in the cellular accumulation of β-catenin [6]. Upon translocation to the nucleus, beta-catenin serves as an activator of T-cell factor (Tcf)-dependent transcription leading to an increased expression of several specific target genes including c-Myc and cyclin D1 [8]. In the current study, we have shown that silencing of TRIM29 repressed the activity of the Wnt/β-catenin pathway as well as expression of c-Myc and cyclin D1 in GC cells, which might possibly explain the arrest of cell cycle and proliferation in cells lackingTRIM29.

Although many studies have investigated the function of TRIM29 in different cancer types, however, how expression of TRIM29 itself remain largely unknown. Gene expression is regulated at multiple levels; chromatin structure [18, 19], transcriptional regulation [20], RNA export [21], mRNA stability [22, 23], protein translation [24, 25] and other regulatory processes are all playing critical roles in regulating protein production. MiRNAs are small non-coding RNAs which suppress translation or promote the degradation of target mRNA by directly targeting 3'-untranslated regions (3'-UTRs) [13, 14]. Abnormal miRNA expression has been reported in many types of human cancers, and plays a vital role in tumorigenesis and function as tumor oncogenes or suppressors [12-14]. Many miRNAs have been shown to influence the process of GC proliferation, invasion, apoptosis, metastasis, EMT, and drug resistance [5, 11, 26, 27]. For example, previous studies show that miR-21 is overexpressed in various cancers, and functions as an oncogene by targeting tumor-suppressor genes including PTEN, PDCD4, RECK [28-30]. Let-7 family targeting of the oncogenes mammalian target of RAS, GAB2 and FN1 inhibit the proliferation and invasion of breast cancer [31, 32]. Among the candidates, miR-185 was selected for the further analysis. miR-185 is localized in frequently altered chromosomal regions, and overexpression of miR-185 results in cell cycle arrest [33]. It has been shown that miR-185 mediates its tumor suppressor function by regulating cell-cycle proteins and the Six1 transcriptional targets c-myc and cyclinA1 in cancer cells [34]. A recent study showed that miR-185 is a prognostic biomarker for the prediction of

survival and relapse in GC [35]. Another report found restoration of GKN1 protein suppresses GC cell growth by inducing endogenous miR-185 that directly targets the epigenetic effectors DNMT1 and EZH2 in GC cells [26]. In addition, RUNX3 (Runt-related transcription factor) is involved in the activation of miR-185 at the transcriptional level, and miRNA-185 regulates chemotherapeutic sensitivity in GC by targeting apoptosis repressor with caspase recruitment domain (ARC) [36]. The current study showed that up-regulation of miR-185 repressed TRIM29 expression and significantly inhibited malignant behavior via Wnt inactivation. These results prove that miR-185 plays a vital role in GC.

In summary, the results of the current study showed that downregulation of TRIM29 inhibited cell proliferation and colony formation, and induced G1-S cell cycle arrest and apoptosis. Also, TRIM29 silencing repressed the activity of Wnt/β-catenin pathway genes in vitro. More importantly, TRIM29 was identified as a direct target of miR-185, which is down-regulated in GC cell lines. Over-expression of miR-185 repressed the TRIM29 expression in GC and significantly inhibited malignant behavior via Wnt inactivation. The miR-185-Trim29-Wnt/β-catenin signaling axis may provide novel insights into the molecular mechanisms of GC progression, and may lead to new treatment for GC.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Feng Qiu, Affiliated Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, People's Republic of China; Department of Oncology, The First Affiliated Hospital of Nanchang University, Nanchang 330006, People's Republic of China. E-mail: qiufengncu@gmail.com

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