Original Research

TRIM28 suppresses cancer stem-like characteristics in gastric cancer cells through Wnt/β-catenin signaling pathways

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Impact statement

In our work, we explored the effects of TRIM28 on gastric cancer (GC) stemness. And we found that TRIM28 knockdown promoted GC cell stem-like characteristics. Mechanistically, we illustrated that TRIM28 knockdown activated the Wnt/β-catenin signaling pathways. Our work makes contributions to elucidating the cellular mechanisms of GC development and identifying implicated-genes and associated driver mutations.

Abstract

The influences of TRIM28 on the gastric tumorigenesis together with potential molecular mechanisms remain to be studied. We aimed at exploring the important effects of TRIM28 on gastric cancer (GC) and uncovering underling molecular mechanisms. Through immunohistochemistry analysis of 20 pairs of GC and the peritumoral tissues, the expression level of TRIM28 was determined. A variety of assays were applied to explore the important roles of TRIM28 in GC. Western blotting and qRT-PCR analyses were used to analyze the association between TRIM28 and the Wnt/β-catenin signaling pathway. TRIM28 was highly expressed in GC tissues than peritumoral tissues. And high expression level of TRIM28 in GC was associated with good prognostic effects. *In vitro* functional assays suggested TRIM28 knockdown enhanced the proliferation and clone formation of GC cell. Moreover, TRIM28 knockdown enhanced the expression level of stemness markers, strengthened sphere-forming and drug-resistance properties of GC cells, suggesting important effect on GC cell stemness. Besides, our analysis showed that the Wnt/β-catenin signaling was involved in the effect of TRIM28 on GC cell stemness property, and blocking Wnt/β-catenin signaling pathway obviously rescued the promotion influence of TRIM28 knockdown. Overall, TRIM28 has an important influence on regulating the stem-like property of GC cell via Wnt/β-catenin signaling, suggesting TRIM28 a promising drug target and a potential predictor of prognosis.

Keywords: TRIM28, Wnt/β-catenin, gastric cancer, CD44, stemness, cancer progression

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Introduction

As the fifth most frequent cancer and the fourth leading cause of tumor-associated deaths worldwide[1], GC has a 33% five-year survival rate[2]. Although much effort has been put forth to improve prevention and treatment so as to reduce GC morbidity and mortality. Nevertheless, the outcomes of GC patients is unfavorable because its high metastasis and recurrence. Hence, exploring the underling mechanisms of GC development and identifying implicated-genes are in the emerging world today.

Because of its self-renewing capacity, cancer stem cells (CSCs) play important roles in progression, recurrence, and survival of malignancy[3]. Previous investigations have indicated that CSCs had profound influence on the development of GC[4]. Therefore, therapies targeting the CSCs may become potential strategies of advanced GC in the near future.

Tripartite motif containing 28 (TRIM28), also named as KAP1or TIF1β, belongs to the TRIM family[5]. In a stem cell model, TRIM28 is important for maintaining the pluripotent state[6]. Moreover, TRIM28-null mice die before the process of gastrulation[7]. Moreover, TRIM28 is significant for mouse embryonic stem cells differentiation[8,9]. However, TRIM28 has also been reported to antagonize adult erythrocytes differentiation[10]. Similar paradoxical effects of TRIM28 have also been observed in cancer. Through downregulating the p53 expression in lung cancer, TRIM28 plays oncogenic roles[11]. However, TRIM28 also inhibits proliferation of lung

Table 1. Primer sequences.

Primer	Sequence	
GAPDH	ATCAAGAAGGTGGTGAAGCA	AGACAACCTGGTCCTCAGTGT
TRIM28	AAGGACCATACTGTGCGCTCTAC	ACGTTGCAATAGACAGTACGTTCAC
CD44	TCCAGGCAACTCCTAGTAGT	CAGCTGTCCCTGTTGTCGAA
Nanog	AATACCTCAGCCTCCAGCAGATG	TGCGTCACACCATTGCTATTCTTC
Oct4	CTTGCTGCAGAAGTGGGTGGAGGAA	CTGCAGTGTGGGTTTCGGGCA
Sox2	AAATGGGAGGGGTGCAAAAGAGGAG	CAGCTGTCATTTGCTGTGGGTGATG
β-catenin	AAAGCGGCTGTTAGTCACTGG	CGAGTCATTGCATACTGTCCAT
MMP7	GAGTGAGCTACAGTGGGAACA	CTATGACGCGGGAGTTTAACAT
cyclin D1	CAATGACCCCGCACGATTTC	CATGGAGGGCGGATTGGAA
c-Myc	GTCAAGAGGCGAACACACAAC	TTGGACGGACAGGATGTATGC

cancer cells through downregulating E2F family members that is important for cell proliferation[12]. Previous studies indicate an oncogenic effect of TRIM28 on human GC[13]. Results from that study showed that high mRNA expression level correlated with obviously decreased survival[13]. However, the results also showed reduced TRIM28 mRNA expression level among 1/3 of the GC tissues; unfortunately, this important observation was not further explored[13]. These results indicate that TRIM28 might also have complex influences on human GC. Nevertheless, the biological effects of TRIM28 on GC stemness have not been fully studied.

In the current work, the potential roles of TRIM28 may play in GC were further explored. We demonstrated high TRIM28 expression had good prognostic effect on GC. Knockdown of TRIM28 had a pivotal role in GC stemness and chemoresistance, in addition to promoting cell proliferation and clonogenicity. Mechanistically, TRIM28 knockdown promoted GC stemness through a novel modulative process on Wnt/β-catenin pathway.

Materials and methods

Tissue specimens and immunohistochemistry (IHC)

The IHC of the GC tissue microarry (HStmA020PG01, Outdo Biotech) was performed as previously reported[14]. And the IHC results were scored by an expert pathologist.

Kaplan-Meier Plotter database analysis

The Kaplan-Meier plotter, an online tool, was applied to determine the prognostic effect of TRIM28 on GC.

Cell culture

MKN28, HGC-27, BGC-823, and AGS are human GC cell lines, while GES-1 is a normal gastric cell line. To HGC-27, BGC-823, MKN28, and GES-1, they were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS)[15]. While for AGS, it was grown in F-12K containing 10% FBS[16].

Antibodies and reagents

Primary antibodies included anti-TRIM28 (ab22553) and anti-CD44 (ab189524) were purchased from Abcam, while anti-Nanog (#4903T), anti-Sox2 (#3579T), anti-Oct4 (#2840T), and anti-β-catenin (#8480) were purchased from Cell Signaling Technology. The 5-Fluorouracil (Fu) (ST1060) was from Beyotime Biotechnology. Stock solution of 5-Fu was prepared with dimethylsulfoxide (DMSO) at 25mg/mL. The Wnt/β-catenin inhibitor (KYA1797K) (S8327) was from Selleck. Stock solution of KYA1797K was prepared in dimethylsulfoxide (DMSO) at 25mM. KYA1797K could effectively degrade β-catenin at a concentration of 25μM (48 h). Negative control were cells treated with an equal volume of DMSO. Puromycin was purchased from MedChemExpress.

Viral transduction of cells

Lentiviral shRNA (GenePharma) was used to establish TRIM28-knockdown AGS cell lines. Lentiviral TRIM28 expression construct (GenePharma) was used to establish TRIM28-overexpressing HGC-27 cell line. The sequences of shRNA-1 and shRNA-2 was 5′-TAAGCACAGGTTTGGTCTCAG-3′ and 5′-TAAGAACTGGTACTGGTGGTC-3′. Stable TRIM28-knockdown (shTRIM28) or TRIM28-overexpressing (TRIM28) cells together with the corresponding controls were selected with puromycin (8μg/ml).

Quantitative real-time PCR (qRT-PCR)

The qRT-PCR was conducted as reported previously[14]. The primers for TRIM28, CD44, Nanog, Oct4, Sox2, β-catenin, MMP7, cylcin D1, and c-Myc were shown as Table 1. Notably, mRNA levels were all standardized to GAPDH.

Western blotting analysis

The western blotting analysis was conducted as reported previously[14]. And the expression levels of TRIM28, CD44, Nanog, Oct4, Sox2, and β-catenin were determined through western blotting analysis and were normalized based on GAPDH. The membrane was blocked and incubated in the primary antibody, followed by incubating in the secondary antibody.

CCK-8 assay

2000 GC cells were plated into the 96-well plates for determining GC cell viability based on the CCK-8 kit (Beyotime Biotechnology).

EdU proliferation assay

Staining of EdU was performed according to the specific protocols (RIBOBIO) and images from three fields were acquired.

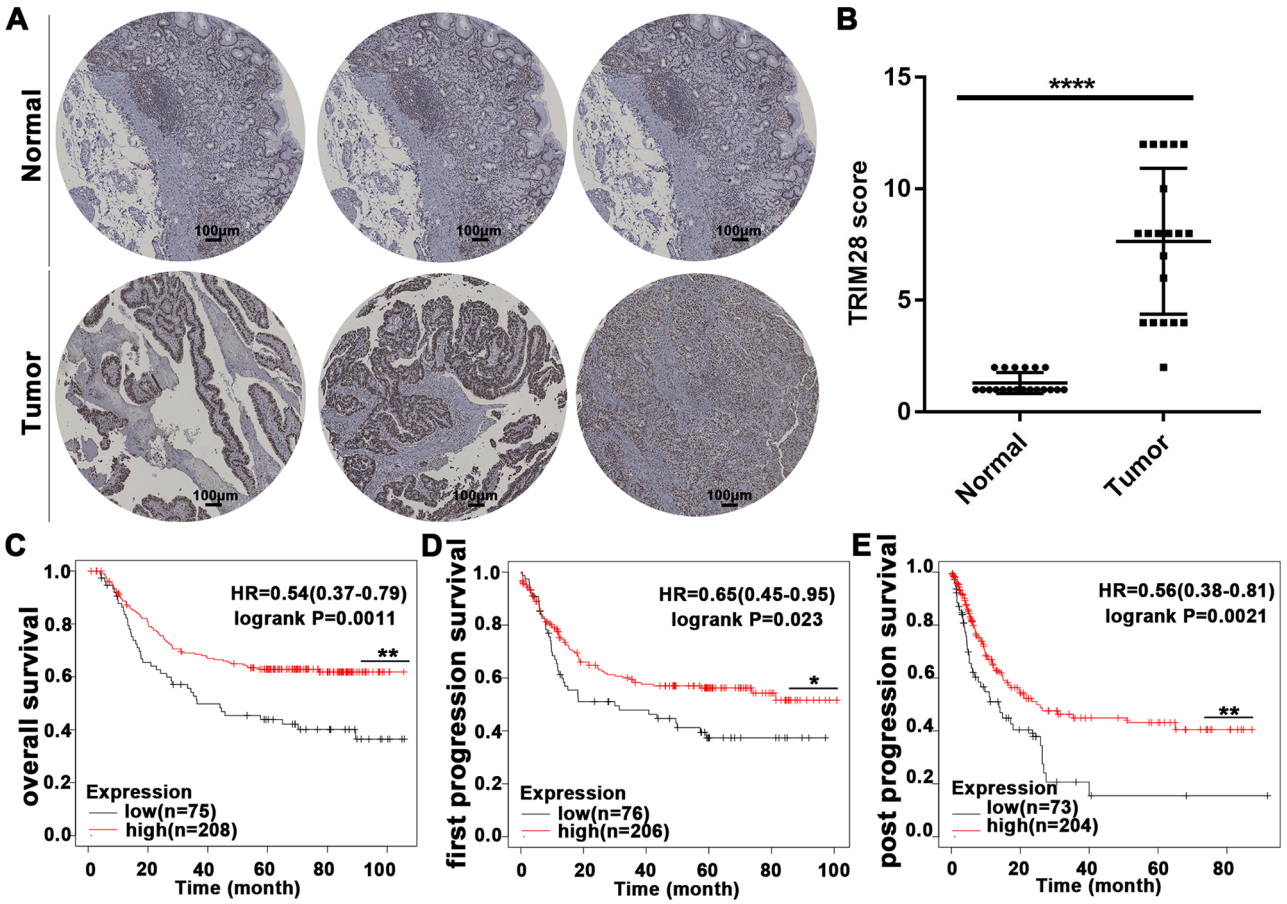

Figure 1. TRIM28 was upregulated in human GC tissues and related to good prognosis of GC. A IHC images of GC and corresponding peritumoral tissues of tissue microarrays. **B** IHC scores of GC tissue microarrays.**C-E** Kaplan-Meier Plotter analysis displayed that the overall survival ($P = 0.0011$, **C**), first progression survival ($P = 0.023$, **D**), and post progression survival ($P = 0.0021$, **E**) rates of high TRIM28 expression group were obviously higher compared with that of the low expression group. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

Then, the EdU-positive and total cells were counted with ImageJ to calculate the EdU-positive cell proportion.

Colony-formation assay

Six-well plates were plated with 1000 GC cells/well. When observable colonies formed, the GC cells were treated as previously reported[17], and counted by using ImageJ.

Tumorsphere-formation assay

The steps of tumorsphere-formation experiment were as follows[18,19]. The GC cells were seeded into the ultra-low-attachment plates with an appropriate density with the DMEM/F12 medium containing B27 supplement (2%), bFGF (10ng/mL), and EGF (20ng/mL). Seven days later, tumorspheres of 50μm or bigger in diameter were counted by using ImageJ.

Statistical analysis

Statistical analysis were conducted using GraphPad Prism 9.0. Comparison across multiple groups was carried out using one-way analysis of variance, while comparison between two groups was done using student's t-tests (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

Results

TRIM28 was upregulated in gastric cancer and indicated good prognosis

The gastric tissue microarrays contained 20 GC tissues and 20 peritumoral tissues and were applied to determine TRIM28 protein level. Results in Fig. 1A-B indicated that TRIM28 was highly expressed in GC tissues relative to the peritumoral tissues ($P < 0.0001$). Moreover, based on a publically available database (GSE62254), the association between TRIM28 level and patient survival were explored. The results showed that high TRIM28 expression was associated with better patient survival (Fig. 1C). Furthermore, high TRIM28 also correlated with first progression survival (Fig. 1D) or post progression survival (Fig. 1E). All the aforementioned results indicated that TRIM28 could have a tumor suppressor influence on GC development.

TRIM28 knockdown promoted GC cell proliferation and clonogenicity

The finding that high TRIM28 level was positively related to GC good prognosis indicated that TRIM28 could play an anti-proliferative effect. And to test this hypothesis, we determined TRIM28 mRNA level in four GC cells (MKN28, HGC-27, BGC-823, and AGS) and GES-1. TRIM28 was found

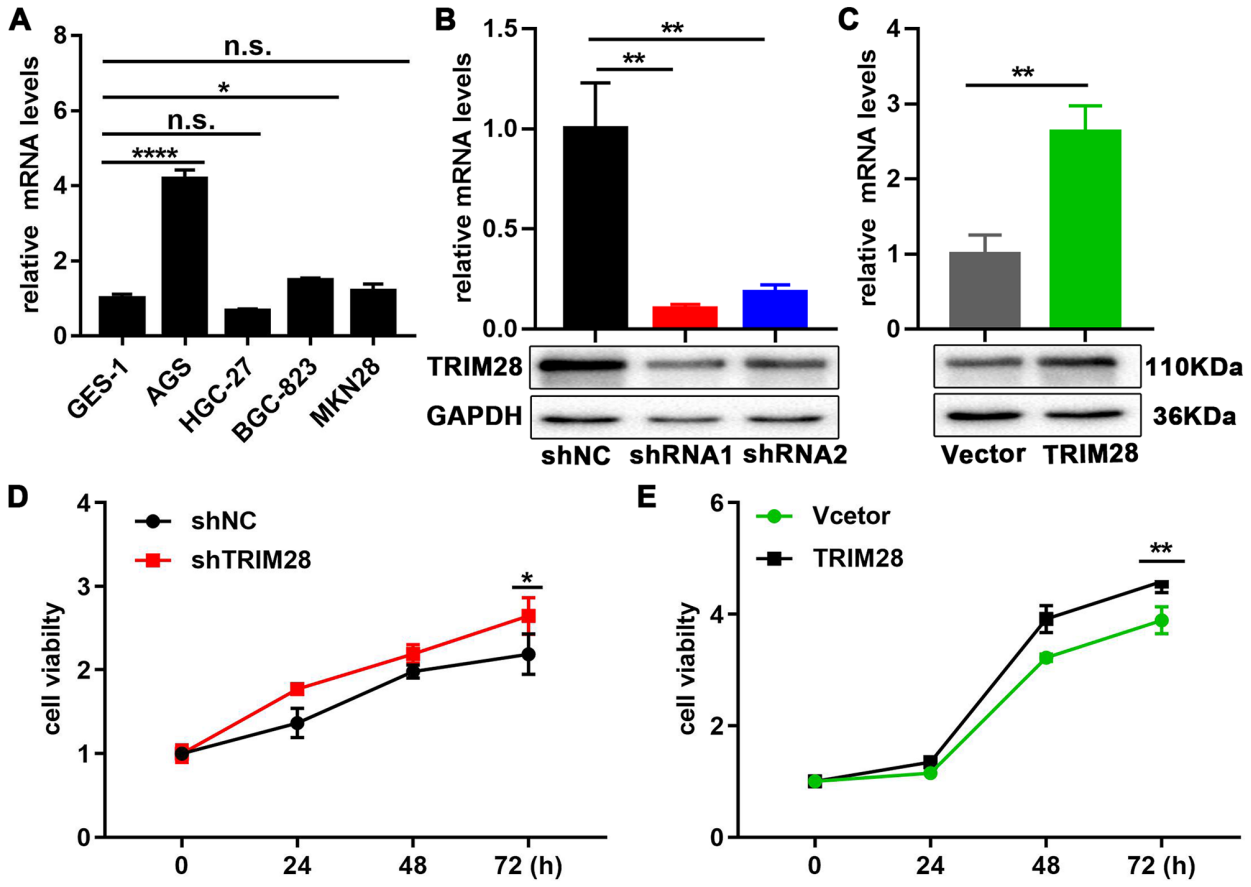

Figure 2. TRIM28 knockdown promoted GC cell proliferation. A The baseline mRNA level of TRIM28 in GES-1 and GC cells. **B-C** The efficiencies of TRIM28 knockdown in AGS cells **(B)** and overexpression within HGC-27 cells **(C)**. **D-E** CCK8 experiments showed TRIM28 knockdown increased cell viability in AGS cells and overexpression of TRIM28 within HGC-27 cells suppressed cell viability. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

to be obviously upregulated in AGS cells in comparison with that in the GES-1 cell (Fig. 2A). According to the baseline levels of TRIM28, the AGS cell line with the highest levels of TRIM28 was chosen to construct TRIM28-knockdown cell line. While the HGC-27 GC cell line with the lowest levels of TRIM28 was chosen to generate TRIM28-overexpression cell line. Both qRT-PCR and western blotting assays were used to determine the efficiencies of knockdown together with overexpression (Fig. 2B-C). The knockdown efficiency of shRNA1 was higher than shRNA2 and was selected for the subsequent experiments (termed shTRIM28). The CCK8 assays showed that TRIM28 knockdown in AGS cells displayed enhanced cell proliferation (Fig. 2D), whereas TRIM28-overexpressing HGC-27 cells showed significantly decreased proliferation (Fig. 2E). The EdU proliferation assay showed that TRIM28 knockdown increased EdU incorporation in AGS cells, while TRIM28 overexpression decreased EdU incorporation in HGC-27 cells (Fig. 3A-B). Similarly, the colony-forming assays showed that TRIM28 knockdown in AGS cells exhibited a significantly higher number of colonies than control cells, whereas TRIM28-overexpressing HGC-27 cells showed significantly decreased colonies (Fig. 3C-D).

TRIM28 knockdown promoted stemness and resistance to drugs in GC cells

We further examined TRIM28 expression on GC cell stemness and resistance to drugs. We observed that TRIM28 knockdown in AGS cells had a significant higher number of tumorspheres over seven days, whereas TRIM28-overexpressing HGC-27 cells showed significant decreased tumorspheres (Fig. 4A). Then, qRT-PCR showed that AGS-shTRIM28 cells had higher level of stemness markers (including CD44, Nanog, Oct4, and Sox2) compared with those in AGS-shNC cells (Fig. 4B). While HGC-27-TRIM28 cells displayed lower level of these stemness markers (Fig. 4C). The protein level of these stemness markers were further validated via western blot analysis. The results showed AGS-shTRIM28 and HGC-27-Vector cells had higher protein level of stemness markers compared with those in AGS-shNC and HGC-27-TRIM28 cells (Fig. 4D-E), which were consistent with the results of qRT-PCR. Besides, we found that low levels of TRIM28 in AGS cell line increased resistance to 5-Fu (Fig. 4F), a commonly used chemotherapeutic drug for GC. While high levels of TRIM28 in HGC-27 cell line decreased resistance to 5-Fu (Fig. 4G). Thus, we revealed that TRIM28 downregulation promoted GC stemness and resistance to drugs.

TRIM28 knockdown promoted stemness via the Wnt/β-catenin pathway

During GC progression, the Wnt/β-catenin signaling pathway is shown to be important for the maintenance of GC cell stemness. Hence, we determined whether TRIM28 knockdown activated Wnt/β-catenin signaling pathway to

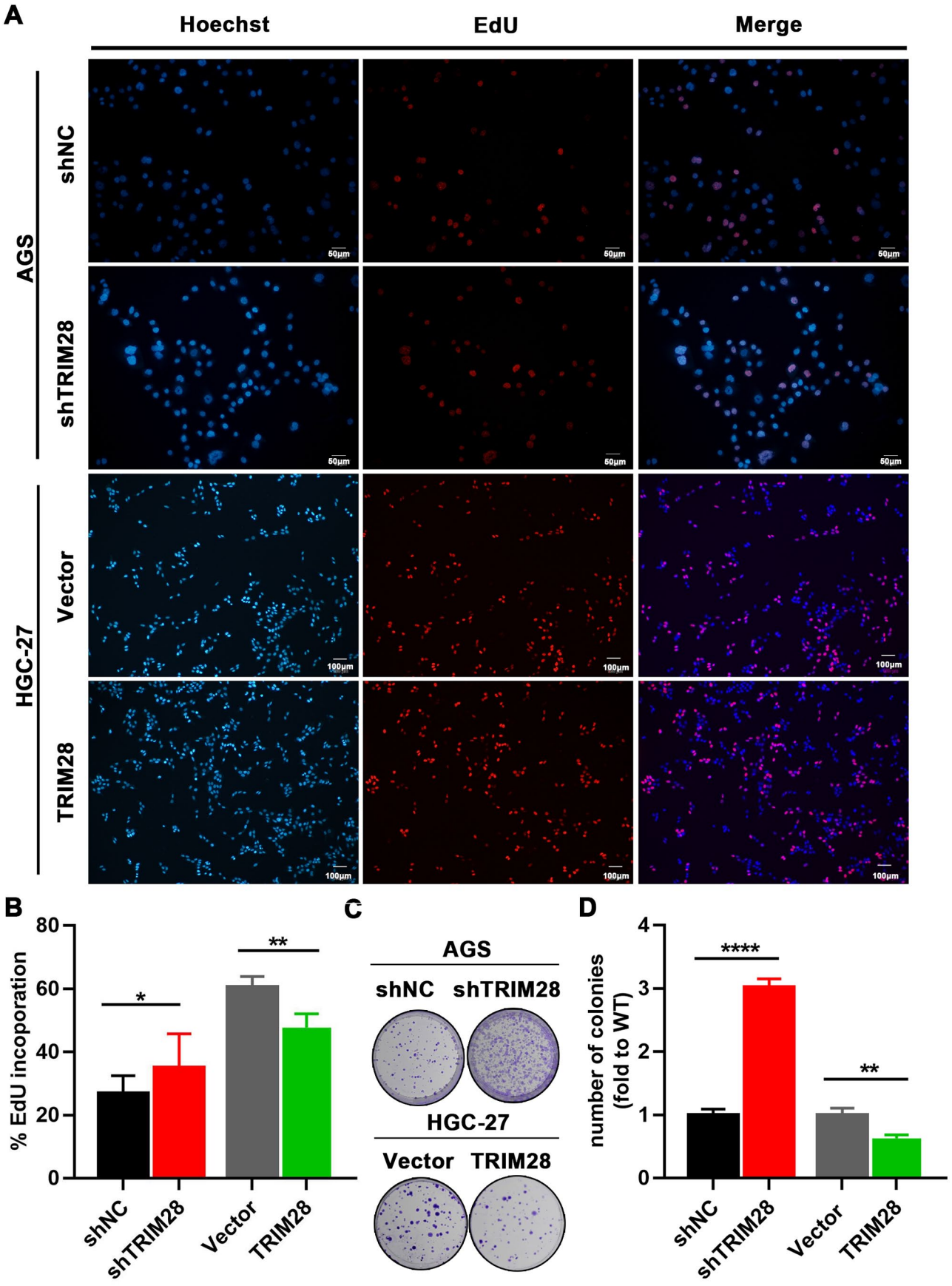

Figure 3. TRIM28 knockdown promoted EdU incorporation and clonogenicity of GC cells. A-B EdU assays demonstrated that knockdown of TRIM28 facilitated EdU incorporation in AGS cells and overexpression of TRIM28 within HGC-27 cells suppressed EdU incorporation. **C-D** Knockdown of TRIM28 increased cell clonogenicity in AGS cells and overexpression of TRIM28 within HGC-27 cells inhibited cell colony-forming . $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

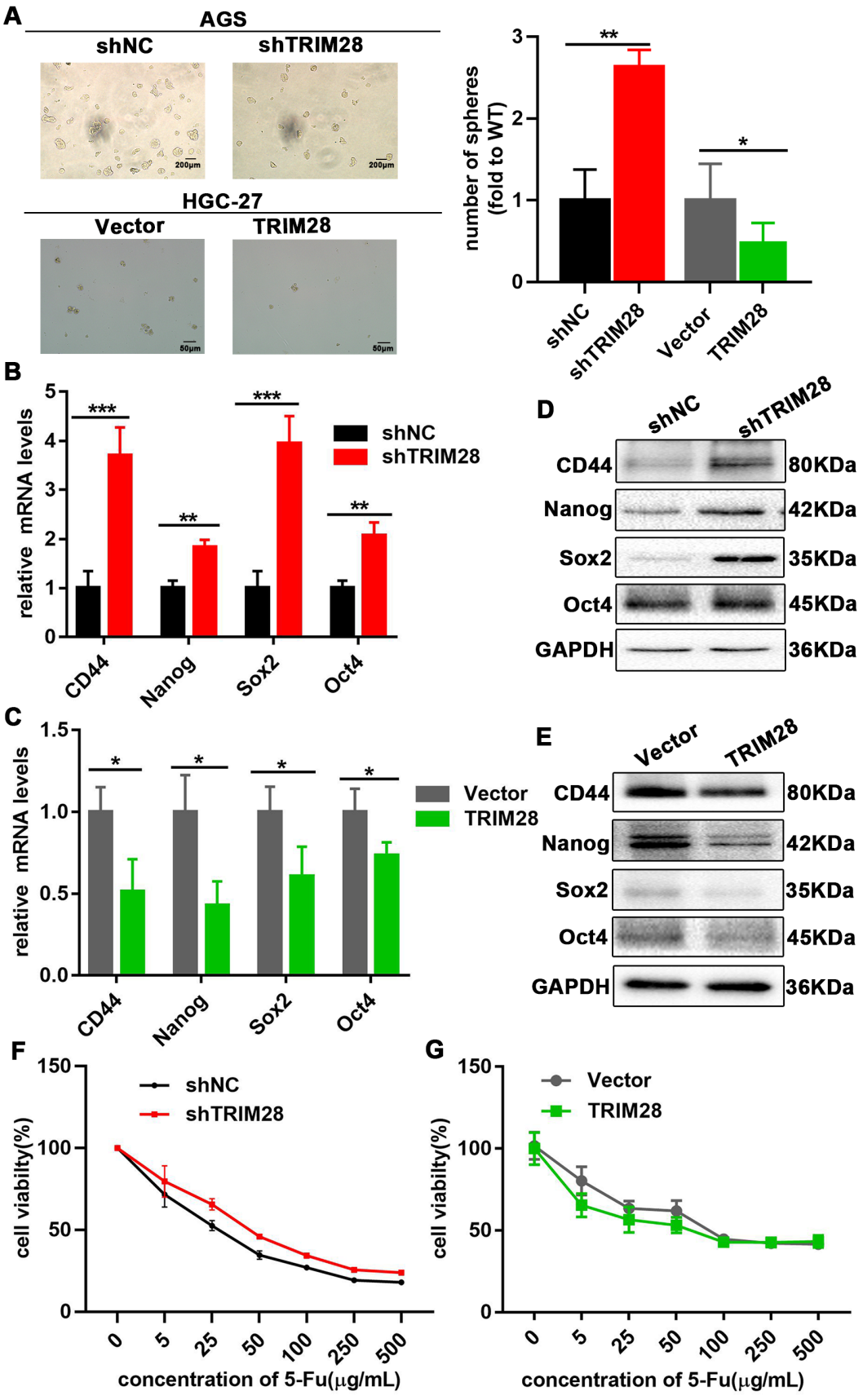

Figure 4. TRIM28 knockdown upregulated GC cell stemness, and resistance to drugs. A GC cell stemness was upregulated by TRIM28 knockdown within AGS cell line but decreased via TRIM28 overexpression within HGC-27 cell line. **B-C** The qRT-PCR analysis for expression of stem cell markers in TRIM28-knockdown AGS cells and cell control, together with in TRIM28-overexpressing HGC-27 cells and cell control. **D-E** Protein level of stemness markers within TRIM28-knockdown AGS cells and cell control, together with in TRIM28-overexpressing HGC-27 cells and normal control. **F** TRIM28-knockdown AGS cells showed significantly lower sensitivity to 5-Fluorouracil (Fu) than control cells. **G** TRIM28-overexpressing HGC-27 cells showed significantly highly sensitivity to 5-Fu than control cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

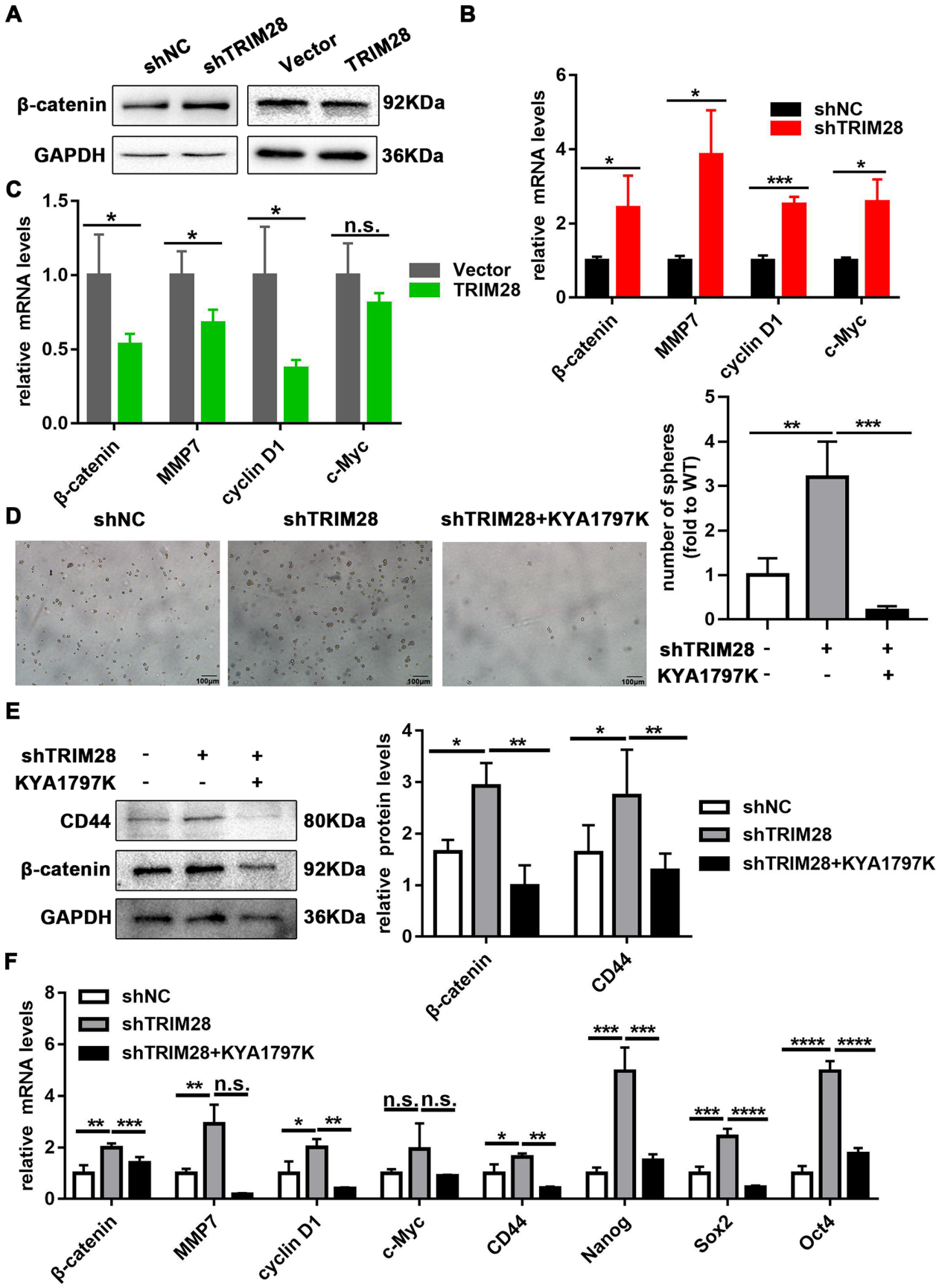

Figure 5. Effects of TRIM28 knockdown on cancer stemness are mediated by the Wnt/β-catenin signaling. A β-catenin protein within TRIM28-knockdown AGS cells and normal control, as well as in TRIM28-overexpressing HGC-27 cells and normal control. **B-C** qRT-PCR analysis for expression of β-catenin, MMP7, cyclin D1, and c-Myc. **D** Sphere formation assay in TRIM28-knockdown AGS cells and normal control with or without exposure to KYA1797K. **E** β-catenin and CD44 expression within TRIM28-knockdown AGS cell line and normal control with or without incubation with KYA1797K. **F** qRT-PCR analysis of β-catenin, MMP7, cyclin D1, c-Myc, CD44, Nanog, Sox2, and Oct4 in TRIM28-knockdown AGS cells and normal control with or without incubation with KYA1797K. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

promote GC cell stemness. Through the western blotting experiment, the β-catenin protein in TRIM28-knockdown or TRIM28-overexpressing cells were respectively determined. Fig. 5A showed that TRIM28 downregulation elevated total β-catenin protein levels in AGS cell lines. While, overexpression of TRIM28 suppressed the protein level of β-catenin within HGC-27 cell lines. Consistent with the results of western blotting assays, the mRNA expression of β-catenin was significantly increased upon TRIM28 knockdown in AGS cells and were decreased after TRIM28 overexpression in HGC-27 cells, (Fig. 5B-C). Similarly, the mRNA expression of MMP7, c-Myc, and cyclin D1, which were downstream target genes of β-catenin, were significantly increased upon TRIM28 knockdown and were decreased after TRIM28 overexpression (Fig. 5B-C).

To further confirm that the GC cell stemness enhanced by TRIM28 knockdown was through activating the Wnt/β-catenin signaling pathway. KYA1797K, an inhibitor against Wnt/β-catenin pathway, was used for rescuing the roles of TRIM28 knockdown in stemness and found that KYA1797K was able to rescue the effects of TRIM28 knockdown using the sphere assay (Fig. 5D), consistent with the western blot and qPCR analyses in AGS cells (Fig. 5E-F). Our findings suggest that TRIM28 regulates cancer stemness through Wnt/β-catenin pathway.

Discussion

We have explored the important influence of TRIM28 on cancer for over 20 years. However, it is hard to unambiguously conclude whether TRIM28 is an oncogenic agent or tumor suppressor because the effect of TRIM28 on cancer is complicated. Several studies indicate that TRIM28 may play oncogenic roles when it is overexpressed in tumor, which might advance tumor growth and metastasis via promoting EMT[20], mediating metabolic switch in stressed conditions[21], regulating the activity or stability of p53 or other proteins[11,22,23] which corporately enable tumor cells to smoothly across the cell cycle even when the genome includes many errors. However, for certain tumor cells, TRIM28 downregulate pro-apoptotic genes, indicating that TRIM28 also promotes cancer cell survival[12,24,25].

We investigated the crucial influence of TRIM28 on GC progression and its potential molecular mechanisms. TRIM28 was highly expressed in GC, and its elevated expression level was related to increased overall survival. This finding was in accordance with several investigations that showed TRIM28 was highly expressed in tumor tissue relative to the peritumoral tissues. Besides promoting the proliferation rate of GC cells, TRIM28 knockdown was especially found to facilitate pluripotent phenotype through the activation of Wnt/β-catenin signaling. In all, our results strongly indicated that TRIM28 may paly anti-tumor effects on GC cells.

Growing evidence has suggested the significant influence of Wnt/β-catenin signaling on maintaining tumor stemness property, which is considered as emerging hallmarks of cancer. A large number of papers have indicated that β-catenin level and its activity are associated with GC stemness. SNHG11 resulted in GSK-3β ubiquitination and activated the Wnt/β-catenin signaling, which facilitated GC stemness[26]. In GC, RORβ downregulates the cell stemness through antagonizing the Wnt/β-catenin pathway[27]. MARCH1 facilitates GC growth and promotes the GC cell stem-like characteristics via activating the Wnt/β-catenin signaling pathway[28]. It is worth noting that upregulating the activation of β-catenin might be potential models for investigating the cellular mechanism of GC and evaluating anti-tumor targeting drugs. Therefore, antagonizing the β-catenin activity by TRIM28 could play an anti-tumor role.

Our results suggested that TRIM28 was overexpressed in primary GC and its high protein level was associated with favorable prognosis. Moreover, in addition to facilitating GC cell proliferation and clonogenicity, TRIM28 knockdown was found to be a key modulator of GC cell stemness through activating Wnt/β-catenin signaling pathway. These results strongly indicated that TRIM28 could be prognostic factor for GC and it might have a tumor suppressing role in GC cells. Further study is clearly warranted for the molecular mechanisms involving TRIM28 overexpression in GC, especially the target with which it interacts when regulating cancer cell biological function. Hence, our research provides obvious elucidation to the molecular mechanisms through which TRIM28 partnered in cancer cell stem-like characteristics, at least with respect to GC, and TRIM28 provides new perspectives for research.

AUTHORS' CONTRIBUTIONS

S.Z. and M.L. designed the research and revised the manuscript. T.N. and M.Z. carried out the experiment and data analysis, and prepared the draft. Z.W., N.Z. partially conducted the IHC experiments. S.Z. partially revised the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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