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SMC4 promotes the progression of cardia adenocarcinoma via regulation of the Wnt/β-catenin signaling pathway

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Abstract

Background: Structural maintenance of chromosome protein 4 (SMC4) is crucial for chromosome assembly and separation, but its role and mechanism in cardia adenocarcinoma (CA) are unknown.

Methods: SMC4 expression levels were initially detected by protein profiling in 20 CA tumour tissues and adjacent normal tissues. The level of SMC4 expression in CA was then evaluated using a Western Blot analysis. Cell proliferation was evaluated by CCK-8 and clone formation test, Scratch and transwell tests were used to investigate cell migration as well as invasion, while through the flow cytometry, we examined the cell apoptosis and progression of the cell cycle. The regulatory effects of the epithelial-mesenchymal transition (EMT) and the Wnt/β-catenin pathway were investigated using Western Blot. A tumorigenesis experiment was used to investigate the influence of SMC4 on tumor development in nude mice.

Results: This study showed overexpression of SMC4 in CA tissues and cells. SMC4 knockout significantly caused the inhibition of proliferation, migration, and invasion of CA cells, and inhibited tumor growth *in vivo* by stimulating the apoptosis and cell cycle arrest in the G0/G1 phase, In addition, down-regulation of SMC4 resulted in decreased expression of Bcl-2, Cyclin D1, CDK4, CDK6, β-catenin, phosphorylated GSK-3β, N-cadherin, and Vimentin, with an increased level of proteins i.e Bax, cleaved-caspase3, and E-cadherin. When SMC4 was overexpressed, these effects were reversed.

Conclusion: SMC4 can facilitate the biological progression of CA, suggesting that SMC4 could be a potential therapeutic target for the disease.

An earlier version of our study has been presented as a preprint in the following link: https://www.researchsquare.com/article/rs-698892/v1.

1. Introduction

One of the most prevalent malignant tumors of the digestive tract is gastric cancer (GC). Although the GC mortality and incidence in China have been on a downward trend, the increasing and aging population still leads to a substantial increase in the number of new cases every year[1]. Even now, GC remains one of the most prevalent and deadly tumors globally[2]. Helicobacter pylori infection is a significant risk factor for gastric cancer; nevertheless, helicobacter pylori eradication decreased overall gastric cancer incidence, it increased the incidence of cardia adenocarcinoma[3].

Cardia adenocarcinoma (CA) is a type of GC, and its tumor center is located within 5cm of the proximal and distal anatomical Cardia[4, 5]. CA has been on the rise in Asian countries in recent years, with China having a much greater prevalence of Siewert type II and type III tumors, while the prevalence of type I tumors is relatively low[6]. Surgery is still the most common treatment for CA, however, due to the high rates of local and distal recurrence, surgical treatment alone is usually ineffective[7]. The survival rate of patients can be enhanced if cancer can be discovered early. However, it is frequently advanced by the

time it is diagnosed. Therefore, the survival rate of patients with CA is still not high. Trastuzumab, ramoxicumab, and pemumumab are examples of targeted treatment that have been shown to be effective in patients with advanced or metastatic CA[8], indicating that targeted therapy is achievable to some extent. Therefore, more therapeutic targets and biomarkers must be recognized for CA patients to obtain early diagnosis and improved therapy, hence improving patients' overall survival rates.

Structural maintenance of chromosome protein 4 (SMC4) is a protein that belongs to the SMC family. Located in the 3 q25. 33 (https://www.ncbi.nlm.nih.gov/gene/10051)[9]. SMC4, the core subunit of condensation proteins I and II, is involved in chromosomal aggregation and mitotic sister chromatid separation, which has been linked to the regulation of cell cycle progression in various malignancies, as well as tumor growth and carcinogenesis[10, 11]. In recent years, there have been increasing reports on the SMC4 gene in colorectal cancer[12], liver cancer [11], lung adenocarcinoma[13], breast cancer[9], acute myeloid leukemia[10], and glioma[14] and other tumors. According to research findings, the SMC4 gene has a vital role in the occurrence and progression of known cancers, such as lung adenocarcinoma and glioma. However, its expression, potential role, and mechanism in CA are still unclear.

In this study, SMC4 expression was found to be higher in CA tissues and cell lines. SMC4 can enhance cell proliferation, invasion, and migration, promote cell cycle progression, inhibit cell apoptosis, and expedite EMT and tumor growth in xenografts. In addition, these regulatory effects of SMC4 on CA are likely to be realized through the regulation of the Wnt/β-catenin signaling pathway. Therefore, targeting the SMC4 gene and regulating the Wnt/β-catenin signaling pathway could be an effective approach to inhibit the progression of CA, and these results may provide a new target for CA treatment and research.

2. Materials And Methods

2.1 Protein profiling analysis

In the First Affiliated Hospital of Bengbu Medical College, 20 pairs of CA and paracancer normal tissue specimens were collected from 2018 to 2019. During the detection phase, mass spectrometry (QE plus) was used to detect the protein expression profile of each tissue sample, and then MaxQuant analysis software was used to identify the differentially expressed proteins. The diagnosis of CA was confirmed in all samples. All of the patients had provided informed consent and had not undergone any preoperative CA therapy.The study was approved by the Ethics Review Committee of the First Affiliated Hospital of Bengbu Medical College (BYYFY-2017KY21).

2.2 Cell lines and cell culture

Human gastric cancer cell lines BGC-823, SGC-7901, MGC-803, and MKN-45 were all purchased from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). Wuhan Procell Life Science & Technology Co., Ltd (Wuhan, China) provided the human gastric mucosal epithelial cell GES-1. These cells were grown at 37 °C in a humidified environment of 5% CO_2 in RPMI-1640 media (Gibco, USA)

supplemented with fetal bovine serum (10%, Gibco, USA) and streptomycin-penicillin (1%). The medium was changed every two days.

2.3 Lentivirus Infection

Lentivirus expression constructs of SMC4 were obtained from GeneChem (Shanghai, China). The two SMC4 lentivirus knockout constructs, SMC4-RNAi1 and SMC4-RNAi2, were purchased from GenePharma (Shanghai, China). The sequences of shRNA of two knockout constructs were RNAi1,GGGUAUGCUUGAAUAUUUATT; RNAi2,GCCCAAGAAUGUGUAAACUTT. As mentioned earlier, the obtained SMC4 expression or knockout constructs were infected with the target cells, and purinomycin was used to screen out cells that stably expressed or knocked out the SMC4 gene.

2.4 CCK-8 assay

The required cells at a density of 4×10^3 cells were seeded in 96-well plates (Corning, NY, USA) and cultured in 5% CO_2 at 37 ^oC. CCK-8 was added to each well on days 1, 2, 3, and 4, and incubated at 37 ^oC for 2 h. The optical density (OD) was measured at 450 nm using a microplate reader (Bio-Rad, CA, USA, and the cell viability was determined.

2.5 Colony Formation Assay

The cells of the required groups were seeded on a 6-well plate at a density of 1.5×10^3 cells per well and cultures were incubated for 14 days at 37 ^oC and 5% CO_2. The fixation of cells was then performed with 4 % polyformaldehyde, then dyed with crystal violet, dried, and the colonies of more than 50 cells were counted.

2.6 Wound healing assay

Cells from different groups were seeded on 6-well plates at a density of 6×10^5 cells per well. When they grew to more than 90%, the cell surface was scraped with a sterile pipette tip and washed to remove cell debris. Imaging was performed under a microscope at 0 and 24 hours after treatment, respectively. The wound area was quantified using ImageJ and the healing rate was calculated to assess mobility after 24 hours.

2.7 Assays for migration and invasion

To detect cell movement and invasion, a transwell method was used. Medium supplemented with 10% fetal bovine serum was added to the lower chamber. Then the required cells were suspended in the media free with serum and injected into the upper chamber at a density of 1.5×10^4 per well. The cells were incubated at 37 °C in a humidified environment with 5% CO_2. After incubation for 24 hours, they were rinsed with PBS, subjected for fixation with 4% paraformaldehyde and crystal violet staining, and counted

under a microscope. The migration test was described above, while the invasion test was precoated with 50 ul of matrigel at the bottom of the upper chamber using the same steps as done in the migration test.

2.8 Flow Cytometry Analysis

For apoptosis, cells were typically digested with trypsin and washed. Annexin V-FITC/PI Cell Apoptosis Detection Kit (YEASEN Biotech Co., Ltd., Shanghai, China) was employed for dying the cells as directed by the manufacturer. Finally, through a Flow Cytometer (BD Biosciences) we detected stained cells, and the apoptotic data was analyzed with FLOWJO software (Tree Star, Inc, Ashland, OR).

Harvested cells were rinsed in cold PBS and fixed in 70% ethanol (pre-cooled) for at least 2 hours for the cell cycle. The cells were stained with Propidium iodide (Sigma, USA) after being rinsed again with pre-cooled PBS. Similarly, the stained cells were perceived by Flow Cytometer and the effects of SMC4 on various stages of the cell cycle were analyzed by FLOWJO software.

2.9 Western Blotting

RIPA cell lysate (Solarbio, Beijing, China) with protease and phosphatase inhibitors were used for extracting the total protein while its concentration was quantified with a BCA protein detection kit (Beyotime Institute of Biotechnology, China). After that, the protein separation was performed on an SDS-PAGE gel through the process of electrophoresis and shifted to PVDF membranes (Millipore, USA). Then we treated the membranes with primary antibody at 4 °C overnight after 30 minutes of rapid closure. After 2 hours of incubation with appropriate secondary antibodies labeled with Horseradish Peroxidase (HRP), the visualization of protein bands was carried out using ECL (Beyotime) method.

Antibodies against SMC4, α-tubulin, Bcl-2, Bax, caspase3, Cleaved-caspase3, CDK4, CDK6, Cyclin D1, P21, E-cadherin, Vimentin N-cadherin, (Proteintech, Wuhan, China), β-catenin, p-GSK3β, and GSK3β (Abcam, Cambridge, UK) were used in this research. The internal reference for the expression levels of these proteins was α-tubulin.

2.10 Xenograft tumor in nude mice

Male BALB/ C nude mice (Shanghai SLAC Laboratory Animals Co., Ltd, Shanghai, China) aged around 4 weeks were injected with MGC-803 cells (5×10^6 cells) from distinct groups. Every 5-6 days, the tumor's length (L) and breadth (W) were measured, and the tumor volume was determined using the formula $V = (\pi/6) \times L \times W^2$. After 27 days, the mice were euthanized, and the tumor was excised, weighed, and preserved. The experiment was approved by the First Affiliated Hospital of Bengbu Medical College's Ethics Committee (2020.069).

2.11 Statistical analysis

Through GraphPad Prism 7 (GraphPad Inc., La Jolla, CA, USA), all data were analyzed. The data was gathered from three separate experiments and shown as mean ± SEM. ANOVA was used to compare the

difference analysis, and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 SMC4 is overexpressed in CA

Protein spectrum analysis of 20 pairs of samples collected from CA and paracancer normal tissue showed that the level of SMC4 in CA patients was significantly higher than that in paracancer tissues (Figure 1 A). Subsequently, Western Blot showed the upregulation of SMC4 in all CA cell lines compared with GES-1 (Figure 1 B). Collectively, these results suggest the upregulation of SMC4 in human CA.

To investigate the SMC4 regulation effect on proliferation of CA cells, silenced control group cells (RNAi-Vector) and cells stably knocked out SMC4 (SMC4-RNAi1 and SMC4-RNAi2) of MGC-803 and BGC-823, as well as negative control group cells (Vector) and cells stably expressed SMC4 (SMC4) of MKN-45 were constructed first. Then the expression level of SMC4 in MGC-803, BGC-823, and MKN-45 cells stably transfected were identified by Western Blot. The findings exhibited the significantly reduced expression level of SMC4 in the RNAi1 and RNAi2 groups as compared to the RNAi-Vector group, whereas SMC4 in the overexpression group was higher than that in the Vector group (Figure 1 C-F).

3.2 SMC4 promotes the proliferation of CA cells and formation of xenograft tumors

Based on the constructed stably knocked out or overexpressed SMC4 cell lines, the CCK-8 assay was used to study the effect of SMC4 on cell proliferation and it was observed that the SMC4 knockout significantly reduced the proliferation capacity of two strains of cells compared with the RNAi-Vector group, while SMC4 overexpression promoted cell proliferation (Fig. 2 A-B). Consistent with this, cells knocked out of SMC4 showed a significantly reduced colony-forming capacity, while the results were reversed when SMC4 was overexpressed (Fig. 2 C-D). In short, these results demonstrated that the proliferation of CA cells after SMC4 overexpressing was significantly promoted.

The xenograft tumors have been generated in nude mice, to evaluate the influence of SMC4 on tumor development and formation. The findings demonstrated that tumors generated by CA cells with down-regulated SMC4 in nude mice were smaller in size, lighter in weight, and had a slower tumor development rate than the control group (Figure 2 E-G).

3.3 SMC4 promotes CA cells’ invasion as well as migration

To reveal the regulation effect of SMC4 on the migration of CA cells, we conducted a wound-healing experiment. The knockout of SMC4 was found to prevent CA cells from migrating, whereas overexpression had the reverse effect (Fig. 3 A-C). At the same time, the transwell migration experiment can further confirm the above findings. Additionally, the transwell invasion assay showed that SMC4 knockout reduced cell invasion ability compared with the RNAi-Vector group, while SMC4 upregulation promoted the cells invasion (Figure 3 D-F). These outcomes indicated that SMC4 can facilitate the CA cell's migration and invasion.

3.4 SMC4 inhibits CA cells' apoptosis

The effect of SMC4 on cell apoptosis was subsequently investigated, and Annexin V/PI staining exhibited a significant rise in apoptosis rates in both lines after SMC4 knockdown than the RNAi-vector group, while the rate of apoptosis of CA cells decreased when SMC4 was overexpressed (Figure 4 A-C). The inhibitory effect of SMC4 on apoptosis was also validated by Western Blot analysis of apoptosis-related proteins. It was found that knocking down SMC4 resulted in downregulation of anti-apoptotic protein Bcl-2 with enhancement in the pro-apoptotic proteins Bax, cleaved-caspase3 levels. Upregulation of SMC4 had the opposite effect on apoptosis-associated proteins (Fig. 4 D-F).

3.5 SMC4 promotes the CA's cell cycle progression.

Simultaneously, we used PI labeling and flow cytometry to discover the influence of SMC4 on cell cycle progression. In SMC4 knockout cells, a significant increase in G0/G1 phase cells was detected, with a reduced number of S and G2/M phase cells, suggesting that SMC4 inhibition may induce G0/G1 phase arrest. Whereas the overexpression of SMC4 had the reverse effect (Fig. 5 A-C). Furthermore, Western Blot results revealed that when SMC4 was knocked out, the expression levels of proteins involved in cell cycle CDK4, CDK6, and Cyclin D1 were reduced. Whereas the overexpression of SMC4 had the reverse effect (Figure 5 D-F), confirming that SMC4 inhibition can cause cell cycle arrest in the G0/G1 phase. Collectively, we found that SMC4 inhibited apoptosis of CA cells and induced cell cycle progression.

3.6 SMC4 regulates the EMT process and Wnt/β-catenin signaling pathway in CA cells

We used Western Blot to assess the expression of the protein which is related to EMT in various cells following either SMC4 deletion or overexpression. The results showed that following SMC4 down-regulation, the expression of interstitial markers Vimentin and N-cadherin was dramatically reduced, but the expression of epithelial marker E-cadherin was dramatically elevated. Whereas the overexpression of SMC4 had the reverse effect (Figure 6 A-C). Together these findings suggest that SMC4 can promote the epithelial-mesenchymal transformation of CA cells.

To learn more about SMC4's impact on CA cells, we examined how it affects the proteins involved in the Wnt/β-catenin signaling pathway. We found that the levels of both β-catenin and p-GSK3β proteins were significantly reduced in CA cells after SMC4 knockout, while GSK3β protein levels remained unchanged. However, the overexpression of SMC4 showed opposite effects (Figure 6 D-F), suggesting that SMC4 induces activation of the Wnt/β-catenin pathway in CA. In nutshell, SMC4 can promote the EMT process and activate the PI3K/ AKT signaling pathway.

4. Discussion

CA is a heterogeneous disease with unknown Its etiology. However, it might be associated to lifestyle, gastroesophageal reflux disease (GERD), epigenetics, or other variables[15]. Because of the biological and pathological distinctions between esophageal cancer (EC) and gastric cancer (GC), the oncology

principles of EC and GC cannot simply be translated to CA, and its treatment remains a contentious issue[16]. Targeted therapy has increasingly become a significant therapeutic strategy for several malignancies, although it has not been successful in treating CA. As a result, determining the right target for the development of molecular targeted treatment for CA is critical.

SMC4 is a subunit of the protein-encoding "chromosomal structure maintenance" and is also necessary for promoting the development of cells from the G1 phase to the S phase[17]. In addition, SMC4 and SMC2 are the core components of the condensed protein complex, which are involved in the assembly and separation of chromosomes and are closely associated with the progression of the cell cycle and even the incidence and development of tumors[18]. Recent studies have shown that SMC4 overexpression can significantly promote proliferation, invasion, migration ability of glioma cells by activating the TGFβ/Smad signal[14]. Other research has found that SMC4 deletion inhibits lung cancer cell growth and invasion, and acts as an independent prognostic factor[13]. In colorectal cancer, SMC4 knockout can also inhibit invasion, migration, proliferation, and cell cycle progression, and promote apoptosis[12]. However, the intrinsic relationship between SMC4 and CA remains unclear. In the current study, protein profiling revealed that SMC4 was overexpressed in CA tissues. At the same time, higher expression of SMC4 mRNA and protein were observed in CA cells than normal gastric epithelial cells, and up-regulation of SMC4 significantly promoted cell proliferation, invasion, migration phenotype, reduced apoptosis, and induced cell cycle progression and tumor formation *in vivo*. We believe that SMC4 can promote the tumorigenicity of CA cells and play an oncogene-like role in the occurrence and development of CA.

EMT is a reversible biological process that temporarily changes epithelial cells into quasi mesenchymal cells. Epithelial cells gradually lose their pebble epithelial appearance and become fusiform mesenchymal cells during this phase[19]. Intercellular adhesion changes are common in EMT, and down-regulation of E-cadherin is thought to be a key marker, along with overexpression of particular mesenchymal markers such N-cadherin and Vimentin[20]. Recent studies have shown that EMT actively participated in the incidence and progress of a variety of tumors, leading to highly invasive, drug-resistant, and stem-cell characteristics of cells, and easy formation of metastasis in distant organs, thus leading to cancer recurrence and metastasis[21]. It has been proved that some genes regulate the EMT process in CA and affect its progress[22], while whether SMC4 has a regulatory relationship with EMT in CA is not clear yet. Therefore, to further explore the role of SMC4 in CA, we studied the influence of knockdown or overexpression of SMC4 on EMT-related proteins in CA. We found that down-regulation of SMC4 could significantly increase the epithelial marker E-cadherin expression, while the expression of interstitial marker N-cadherin and Vimentin was significantly decreased. The results were reversed when SMC4 was overexpressed. These findings suggest that SMC4 can promote the process of epithelial-mesenchymal transformation of CA cells, and it can be further speculated that SMC4 may have a certain promoting effect on the metastasis and recurrence of CA.

The Wnt/β-catenin signaling pathway, one of the most important signaling pathways in normal physiological activities, is involved in embryogenesis, organogenesis, and internal environment

stability[23]. The major molecule in this pathway is β-catenin. In the absence of a Wnt ligand, the ubiquitin-proteasome system keeps β-catenin at a low level. When Wnt is activated, β-catenin accumulates in the cytoplasm and is then transferred to the nucleus[24]. SMC4 knockdown resulted in lower levels of β-catenin and p-GSK3 associated proteins in the Wnt/β-catenin signaling pathway in CA cells, whereas the opposite was true when SMC4 was overexpressed. As a consequence, SMC4 in CA cells may cause the Wnt/β-catenin signaling pathway to be activated. The literature revealed that the Wnt/β-catenin signaling pathway is abnormally activated in different cancers, including CA, and the abnormal activation of this pathway can regulate cell proliferation, invasion, migration, and EMT processes[22, 25, 26]. Therefore, the promotion of SMC4 on cells proliferation, migration, invasion ability, cycle progression, EMT process and tumor formation of CA, and the inhibition of cell apoptosis may be achieved via regulation of Wnt/β-catenin signaling pathway. The internal regulation mechanism of SMC4 on Wnt/β-catenin in CA cells still needs to be further studied.

In conclusion, these findings imply that SMC4 has a promoting effect on the onset and progression of CA, which could be achieved by activating the Wnt/β-catenin signaling pathway, suggesting that SMC4 may be a potential prognostic and therapeutic target for CA.

Declarations

Data Availability

All data generated or analysed during this study are included in this published article.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics approval and consent to participate

The present study was granted ethical approval by the Institutional Review Board of Bengbu Medical College. Written informed consent was obtained from all participants involved in the study. This research was conducted in accordance with the Declaration of Helsinki. Animal experiments were as per the “Regulations on the Administration of Laboratory Animals” of the State Council of the People’s Republic of China and the “Detailed Rules for the Administration of Medical Laboratory Animals” of the Ministry of

Health. The certificate number BYYFY- 2017KY21 and 2020.069 were used for the collection of clinical samples and animal experimentations, respectively.

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Figures

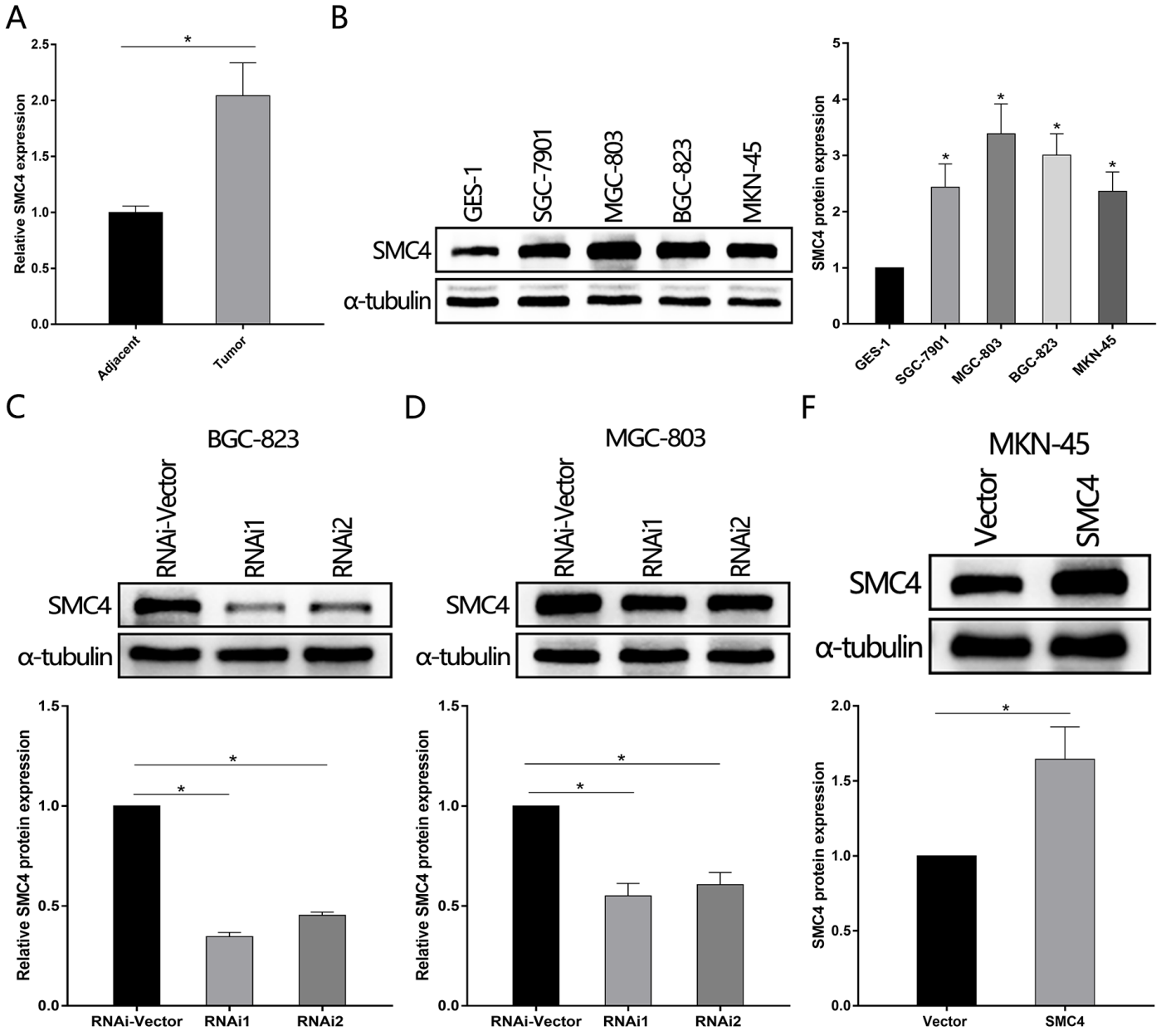

Figure 1

SMC4 was highly expressed in CA A. Protein profiling was used to analyze the expression of SMC4 in CA and paracancer tissues. B: Western Blot was utilized to evaluate the level of SMC4 protein expression in CA cells. C-F: The expression of SMC4 in different CA cells transfected with RNAi-Vector, SMC4-RNAi1, SMC4-RNAi2, and Vector, SMC4 was analyzed by Western Blot.

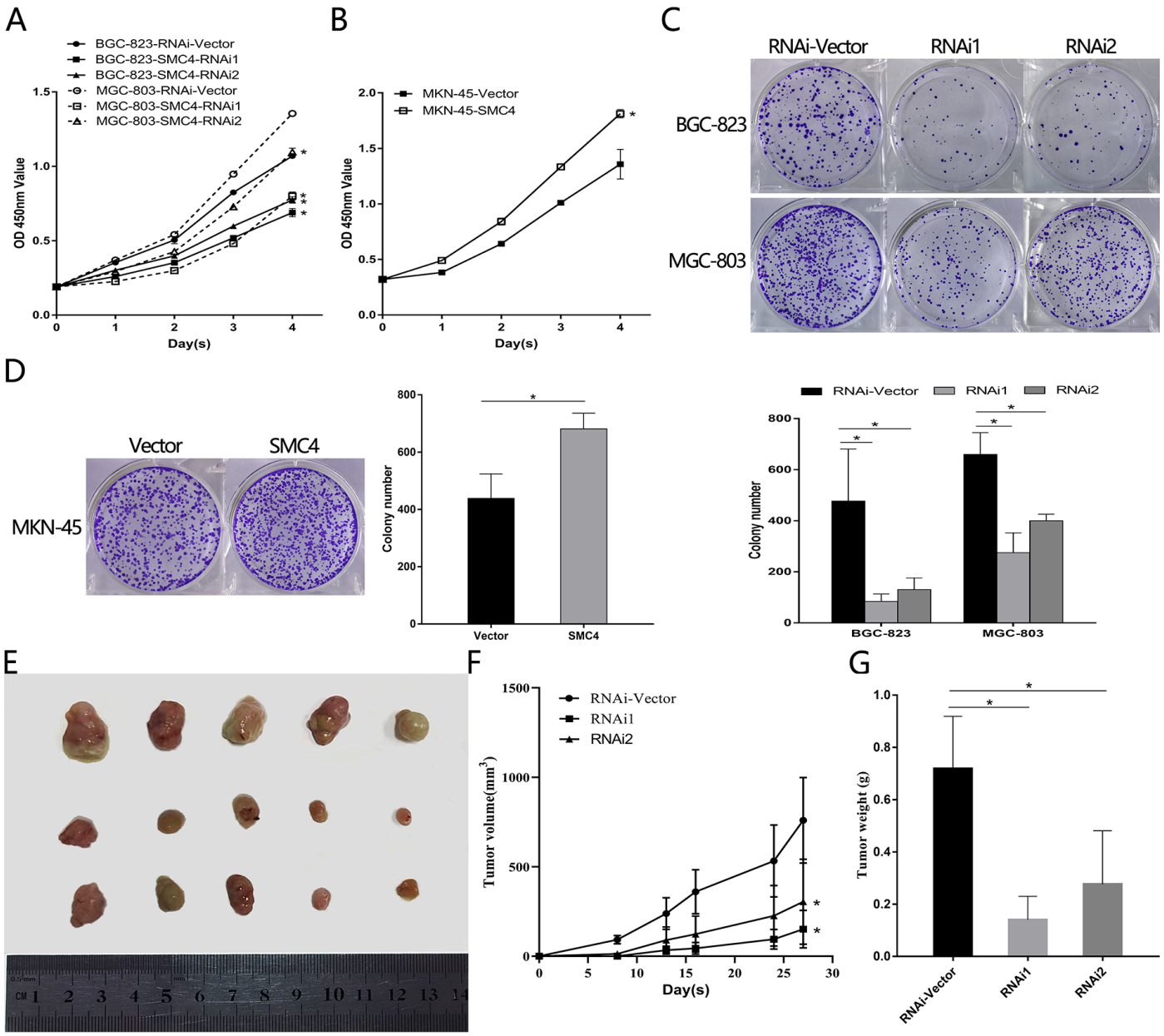

Figure 2

SMC4 promoted the CA cell's proliferation and the formation of xenograft tumors A-B: According to the CCK-8 assay, knockout and overexpression of SMC4 significantly reduced and improved the cell proliferation capability, respectively. C-D: Knockout and overexpression of SMC4 reduced and increased the average number of colonies in the clone formation test, respectively. A tumorigenesis experiment was performed on nude mice using MGC-803 cells of the control group and the stable knockout group. E: The tumor size was measured after 27 days. F: The tumor growth curve. G: The weight of the tumor.

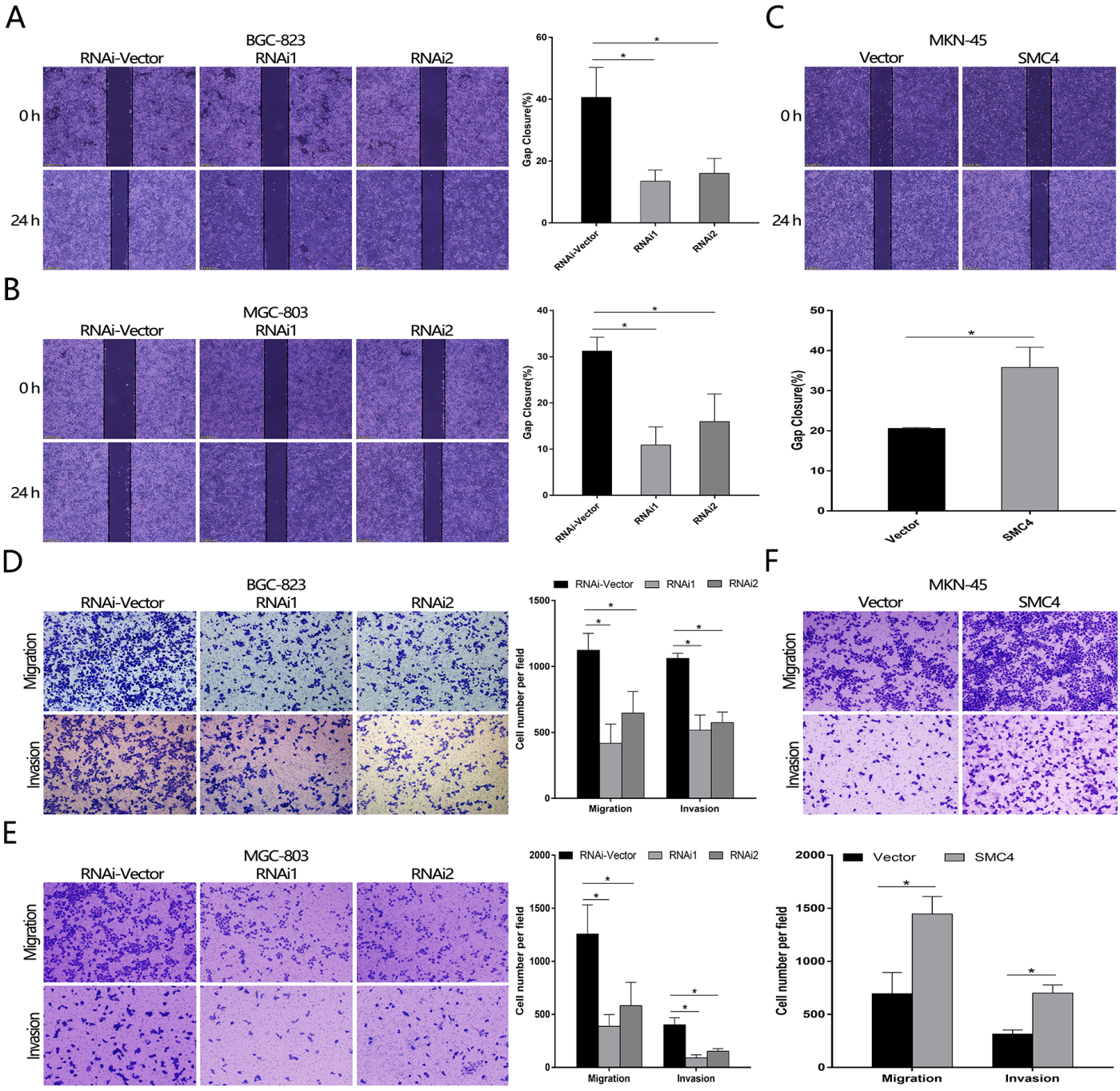

Figure 3

CA cells migrated and invaded more when SMC4 was overexpressed A-C: the wound-healing experiment looked at the effect of SMC4 deletion and overexpression on the migration of the CA cell lines. D-F: The effect of SMC4 knockout and up-regulation on the invasion and the migration capability of CA cells was evaluated by the Transwell experiment.

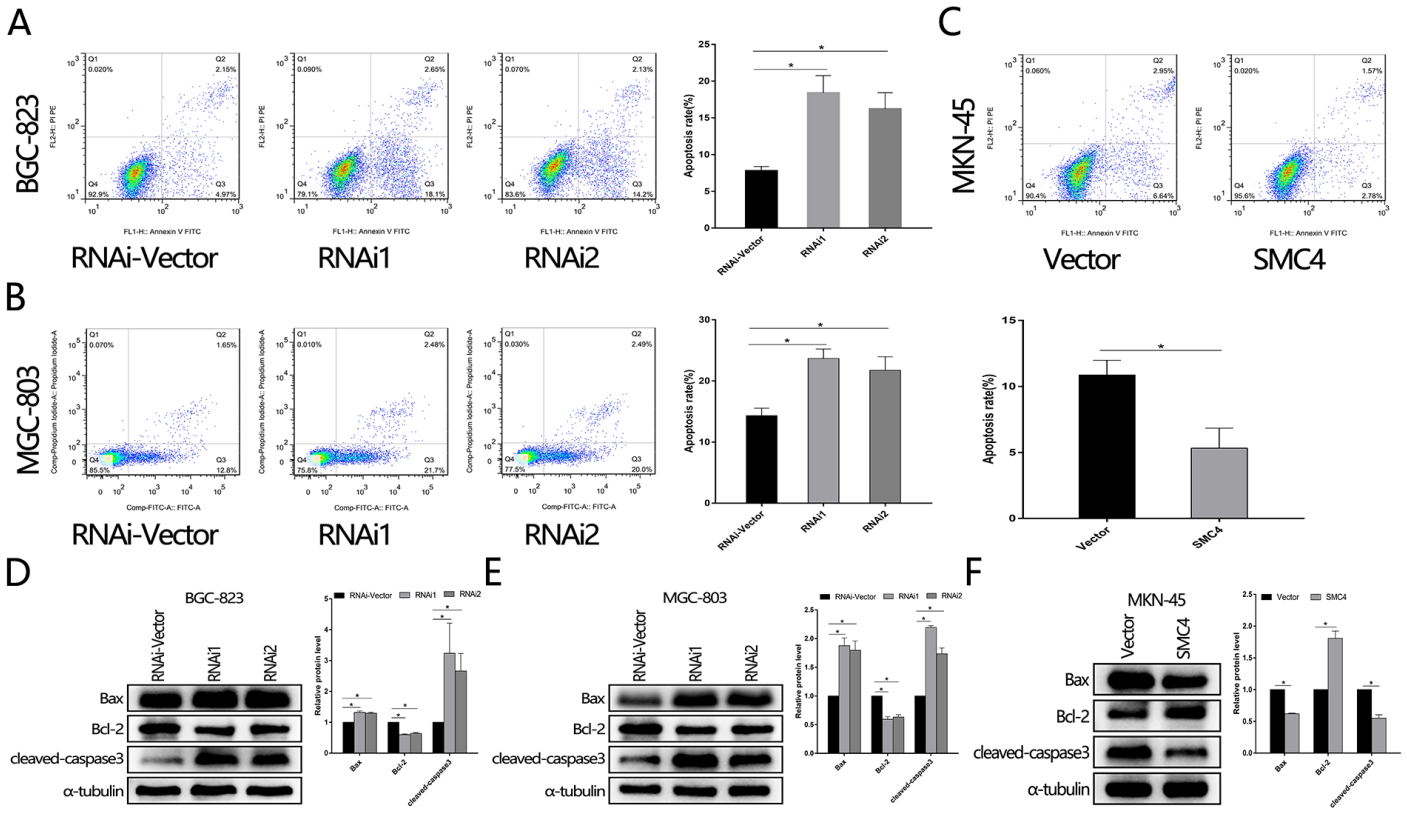

Figure 4

SMC4 can inhibit the apoptosis of CA cells A-C: A representative summary bar and image of the effect of SMC4 knockout and up-regulation on apoptosis in CA cells from the flow cytometry Annexin V-FITC / PI sting experiment. D-F: Expression of proteins involved in apoptosis Bcl-2, Bax, and cleaved- caspase3 after SMC4 knockout and overexpression.

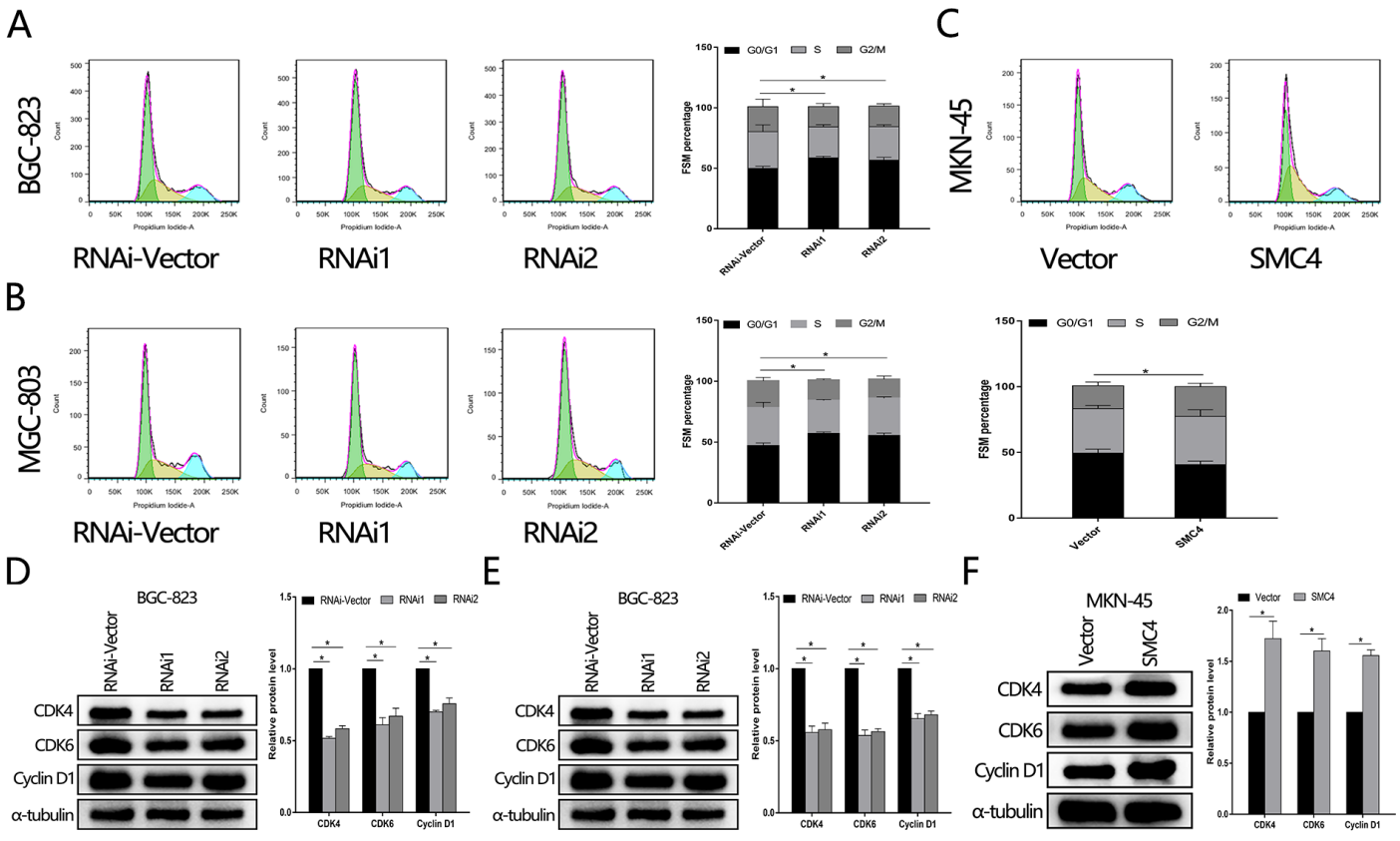

Figure 5

SMC4 can promote the cell cycle progression of CA cells A-C: A representative image of flow cytometry PI training and a summary histogram of the effect of stripping and overexpressing SMC4 on the cell cycle of different CA are shown. D-F: Expression of cyclin-related proteins CDK6, CDK4, and Cyclin D1 after SMC4 knockout and up-regulation.

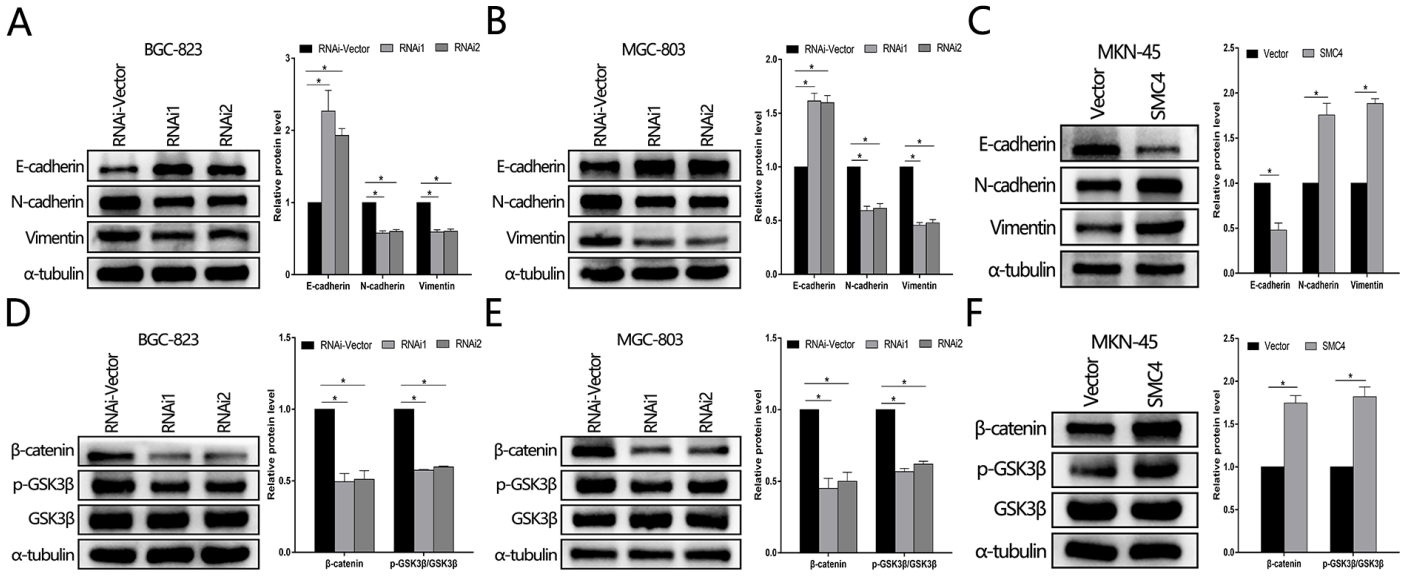

Figure 6

SMC4 regulates the EMT process and Wnt/β-catenin signaling pathway of CA cells A-C: Expression of EMT-related markers N-cadherin, E-cadherin, and Vimentin after SMC4 knockout and overexpression in CA cells. D-F: The expressions of Wnt/β-catenin signaling pathway-related proteins β-catenin□p-GSK3β in CA cells were evaluated by Western Blot after SMC4 knockout and overexpression.