

Apigenin suppresses colorectal cancer cell proliferation, migration and invasion via inhibition of the Wnt/ β -catenin signaling pathway

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Abstract. Abnormal activation of the Wnt/ β -catenin signaling pathway has a significant role in human tumorigenesis. The search for potential anticancer drugs has included wide-spread screening of inhibitors of the Wnt signaling pathway. Recently, one of the most common flavonoids, apigenin, demonstrated potential anti-tumor effects on multiple human cancer cell lines, with low cytotoxicity and no mutagenic activity. However, the association between apigenin and the Wnt/ β -catenin signaling pathway remains to be elucidated. The results of wound healing and Transwell invasion assays revealed that apigenin was able to significantly suppress colorectal cancer cell proliferation, migration and invasion in a dose-dependent manner. An organoid culture assay revealed that apigenin was also able to suppress the growth of intestinal organoids. Furthermore, apigenin inhibited β -catenin/T-cell factor/lymphoid enhancer factor signaling activation, which was induced by LiCl in a dose-dependent manner. This inhibited β -catenin nuclear entry, and therefore the expression of Wnt downstream target genes. In conclusion, apigenin significantly suppressed colorectal cancer cell proliferation, migration, invasion and organoid growth by inhibiting the Wnt/ β -catenin signaling pathway.

Introduction

The Wnt signaling pathway is able to function via two routes: A canonical (Wnt/ β -catenin) pathway or a non-canonical Wnt pathway (1). In the canonical Wnt signaling pathway, when

the Wnt ligand does not bind to its receptors, the pathway is 'switched off'. In this case, β -catenin is degraded by a destruction complex comprising Axin, adenomatous polyposis coli and glycogen synthase kinase 3 β (Gsk3 β). β -catenin is phosphorylated in the cytosol, an event which is followed by ubiquitination, resulting in proteasomal degradation (2). When the Wnt pathway is 'switched on', cytosolic β -catenin is stabilized, resulting in β -catenin accumulation in the cytoplasm, and subsequent nuclear entry. Once inside the nucleus, β -catenin activates Wnt target genes by binding to transcription factors of the T-cell factor (TCF)/ lymphoid enhancer factor (LEF) family (3).

Wnt signaling is a crucial mechanism involved in the regulation of cell proliferation, differentiation and morphogenesis (4). Numerous studies have suggested that abnormal activation of the Wnt/ β -catenin signaling pathway may have a significant role in human tumorigenesis, including in colorectal cancer (CRC), melanoma and lung cancer (5-7). Therefore, the screening of inhibitors of the Wnt signaling pathway is significant in the search for potential anticancer drugs.

Apigenin is one of the most commonly occurring flavonoids, and is found in numerous plants, including parsley, onions, orange, tea, chamomile, wheat sprouts and certain condiments (8). Apigenin has exhibited potential for use in cancer prevention and therapy, and suppresses cell growth in a number of human cancer cell lines, including skin, colon, thyroid, breast, leukemia and prostate cancer cells (9-13). Although previous studies have indicated that the anti-carcinogenic mechanism of apigenin occurs via certain signaling pathways, including the nuclear factor- κ B, P53 and mitogen activated protein kinase pathways, to the best of our knowledge there have been no previous reports indicating a contributory role of apigenin in the Wnt/ β -catenin signaling pathway. Therefore, the present study aimed to investigate the effect of apigenin on the migration and invasion of CRC cells, as well as the inhibition of the Wnt/ β -catenin signaling pathway.

Materials and methods

Cell lines, cell culture and reagents. Apigenin was purchased from Sigma-Aldrich (St. Louis, MO, USA). A 10 mM solution of apigenin was prepared in sterile water, stored at -20°C and protected from light, and diluted to the required concentrations for each experiment. Human CRC cell lines (SW480 and

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HCT15) were purchased from the American Type Culture Collection (Manassas, VA, USA), cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA), supplemented with 10% heated-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 10 μ g/ml streptomycin sulfate and then held at 37°C in a humidified 5% CO₂ incubator. HEK293T cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Mitomycin C was purchased from Roche Diagnostics (Shanghai, China).

Cell viability assay. SW480 and HCT15 human colorectal cancer cells (2x10⁴ cells/well) were treated with various concentrations (0, 5, 10, 20, 40 and 80 μ M) of apigenin for 48 h. Cell viability was determined by MTT assay (Sigma-Aldrich) using a Bio-Rad microplate reader (Model 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA) (14).

Wound healing assay. SW480 cells were cultured to full confluence in 6-well plates and subsequently incubated with 10 μ g/ml mitomycin C for 2 h to inactivate cell proliferation. Cells were washed with phosphate-buffered saline (PBS) and wounds were made with sterile pipette tips. DMEM supplemented with 0.5% FBS was added into wells with various concentrations (0, 20 and 40 μ M) of apigenin. Images of the cells were captured using an Olympus DX41 inverted microscope (Olympus Corp., Tokyo, Japan), following 10 h of incubation with the aforementioned reagents.

Transwell migration assay. Transwells (Corning Inc., Corning, NY, USA) were coated with 50 μ l growth factor reduced Matrigel (1:8 dilution; BD Biosciences, Franklin Lakes, NJ, USA) for 30 min in a cell incubator. The bottom chambers of the Transwells were filled with DMEM containing 0.5% FBS and the top chambers were seeded with mitomycin C inactivator, SW480 cells (4x10⁴ cells/well) in 100 μ l DMEM (0.5% FBS) and various concentrations (0, 20 and 40 μ M) of apigenin. Following an overnight incubation, the cells on the top surface of the membrane (non-migrated cells) were removed with a cotton swab and the cells which had migrated onto the bottom sides of the membrane (invasive cells) were then fixed with cold 4% paraformaldehyde and stained with 2% crystal violet (Beyotime Institute of Biotechnology, Inc., Shanghai, China). Images were captured using an Olympus DX41 inverted microscope.

Wnt/ β -catenin signal reporter assay. HEK293T and SW480 cells were plated at a concentration of 5,000 cells/well on white-bottomed 96-well plates. Cells were serum-starved overnight and co-transfected with 0.2 μ g TOPflash or FOPflash expression plasmids (EMD Millipore, Billerica, MA, USA) and 0.1 μ g pRL-TK (*Renilla* TK-luciferase vector; Promega Corp., Madison, WI, USA) as a control, using Lipofectamine[®] 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA). Cells were subsequently treated with LiCl (20 mM/ml) for 48 h prior to the measurement of luciferase activity. Luciferase activity was measured using a Glomax 96 Microplate Luminometer (Turner BioSystems, Sunnyvale, CA, USA). Firefly luciferase activity was normalized for transfection efficiency by dividing the results by the *Renilla* luciferase activity.

The TOP/FOP ratio was used as a measure of β -catenin-driven transcription. Average activity and standard deviations were derived from 8 transfected samples.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Final concentrations were determined using the Nanodrop DA-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA (1 μ g) was purified from genomic DNA using DNase I (Invitrogen Life Technologies) and reverse transcribed to complementary DNA (cDNA) using the QuantiTect W Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. The resulting cDNA product was then used as a template for PCR amplification. A 25 μ l qPCR reaction mix, consisting of 25 ng diluted cDNA, SYBR Green Dye (Qiagen) and 0.1 μ M of each qPCR primer pair, was amplified to obtain quantifiable expressions of Wnt downstream genes in SW480 cells. All qPCR was conducted in a Stratagene MxPro[™] 3005P (Agilent Technologies, Inc., Santa Clara, CA, USA). Primer sequences were as follows: Axin2 forward, 5'-CAACACCAGGCGGAACGAA-3' and reverse, 5'-GCCCAATAAGGAGTGTAAAGGACT-3'; C-myc forward, 5'-ATGGCCCATTAACAAAGCCG-3' and reverse, 5'-TTTCTGGAGTAGCAGCTCCTAA-3'; Cylind1 forward, 5'-GCTGCGAAGTGGAAACCATC-3' and reverse, 5'-CCTCCTTCTGCACACATTTGAA-3'; Ephb2 forward, 5'-AGAAACGCTAATGGACTCCACT-3' and reverse, 5'-GTGCGGATCGTGTTCATGTT-3'; Ephb3 forward, 5'-TGGGTAACATCTGAGTTGGCG-3' and reverse, 5'-TGGTATGTGCGGATGGGATTC-3'.

Protein isolation and western blotting. Standard western blotting was performed for protein expression analyses (15). Cells were washed twice with cold PBS and lysed on ice in radioimmunoprecipitation assay buffer containing proteinase inhibitors (Roche Diagnostics). Protein lysates were resolved on 10% SDS polyacrylamide gel, transferred to nitrocellulose membranes (Beyotime Institute of Biotechnology, Inc.) and blocked in 0.1% Tween 20 and 5% bovine serum albumin (BSA) in Tris-buffered saline. Proteins were probed with rabbit anti- β -catenin monoclonal antibody (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) and rabbit anti- β -actin antibody (1:10,000; Sigma-Aldrich) overnight at 4°C. The membrane was washed and visualized by incubation with horseradish peroxidase-conjugated anti-rabbit IgG polyclonal secondary antibodies for 1 h. Signals were detected by enhanced chemiluminescence (Odyssey, LI-COR Biosciences, Lincoln, NE, USA).

Isolation of intestinal crypts and organoid culture. C57BL/6 mice were purchased from the Laboratory Animal Center (Shanghai, China). An equal number of male (n=3) and female (n=3) mice were kept in specific pathogen-free housing, fed a normal diet and drinking water, and submitted to a 12-h light/dark cycle and temperatures of 18-23°C. All animal experiments conformed to the regulations drafted by Association for Assessment and Accreditation of Laboratory Animal Care in Shanghai and were approved by the Ethics Committee of Suzhou University (Suzhou, China). The small

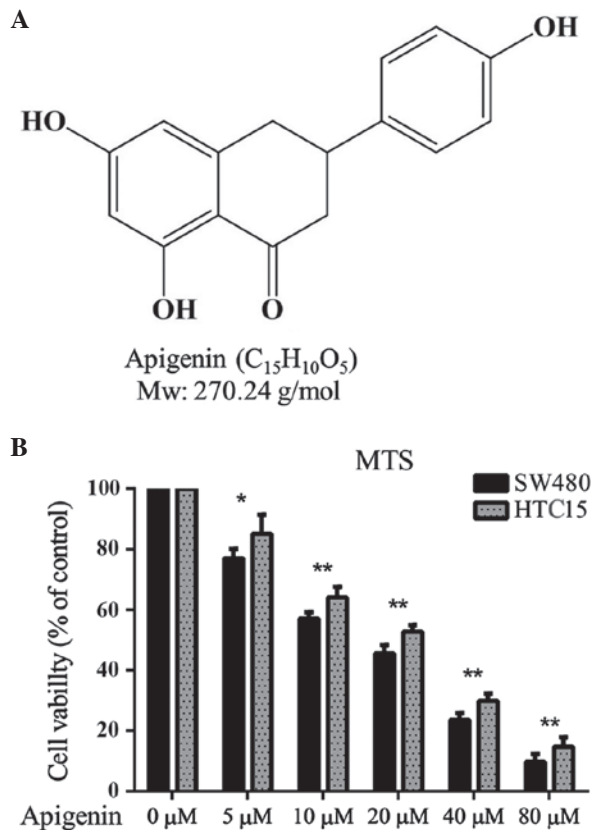


Figure 1. Apigenin inhibits cell viability in SW480 and HCT15 colorectal cancer cells. (A) Chemical structure of apigenin with molecular weight 270.24 g/mol. (B) Apigenin inhibits SW480 and HCT15 cell viability in a dose-dependent manner. SW480 and HCT15 cells (2×10^4 cells/well) were starved with 0.1% fetal bovine serum medium and then treated with various concentrations (0, 5, 10, 20, 40 and 80 μ M) of apigenin for 48 h. Cell viability was quantified by MTT assay. Values are expressed as the mean \pm standard error of three independent experiments with six duplicates. * $P < 0.05$ and ** $P < 0.01$ vs. control.

intestine was separated from mice aged 3-5 weeks following sacrifice by cervical dislocation. After several washes with cold PBS, tissues were divided into 5 mm sections. Intestinal crypts were selectively isolated as previously described (16); however, certain modifications were made to the subsequent procedures and media in the organoid culture. Briefly, crypts were further dissociated into single cells using Accutase® (Innovative Cell Technologies, Inc., San Diego, CA, USA) treatment for 10 min at 37°C and seeded on polymerized Matrigel (BD Biosciences). Serum-free media optimized for organoid culture containing epidermal growth factor (EGF; Peprotech, Inc., Rocky Hill, NJ, USA), R-Spondin1 (R&D Systems, Inc., Minneapolis, MN, USA) and Noggin (Peprotech, Inc.) were routinely supplemented with Y27632 (Wako, Osaka, Japan) and Jagged-1 (AnaSpec, Inc., Fremont, CA, USA) to support the survival of single cells and the proliferation of stem cells (14). The medium was replaced every 2 days.

Immunofluorescent staining and confocal microscopy. Cells were fixed using methanol and blocked with 5% BSA. Cells were subsequently stained with anti- β -catenin monoclonal antibody (1:1,000, Cell Signaling Technology, Inc.), followed by fluorescein isothiocyanate (FITC)-conjugated goat

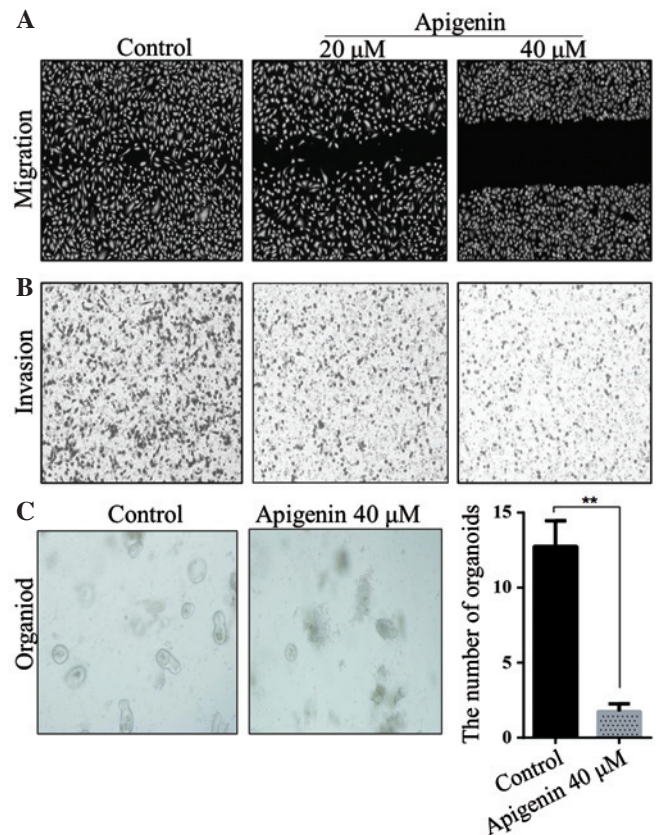


Figure 2. Apigenin inhibits the migration and invasion of SW480 cells, as well as the growth of intestinal organoids. (A) Apigenin inhibited SW480 cellular migration in a dose-dependent manner. SW480 cells were allowed to grow to full confluence in six-well plates, inactivated with 10 ng/ml mitomycin C for 2 h, wounded with a pipette tip and treated with or without various concentrations (20 or 40 μ M) of apigenin in medium supplemented with 0.5% fetal bovine serum. Following incubation, the migrated cells were quantified by manual counting. (B) Apigenin inhibited the invasion of SW480 cells. SW480 were seeded in the upper chamber of a Transwell and treated with various concentrations (20 or 40 μ M) of apigenin. (C) Following ~8-10 h, the invasive SW480 cells passed through the membrane and were counted. Values are expressed as the mean \pm standard error of three individual experiments performed in duplicate. ** $P < 0.01$ vs. control. Apigenin inhibited the growth of intestinal organoids at a concentration of 40 μ M (magnification, $\times 100$).

anti-mouse immunoglobulin G (Merck Millipore, Hong Kong, China). Cells were then washed four times with PBS. Nuclear staining was achieved with DAPI. Cells were imaged with a TCS SP2 spectral confocal system (Leica Microsystems GmbH, Wetzlar, Germany). All experiments were conducted according to the antibody manufacturer's instructions.

Statistical analysis. Data are presented as the mean \pm standard error, and statistical comparisons between groups were made using Student's t-test. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Apigenin inhibits CRC cell line proliferation. Apigenin is one of the most common flavonoids and has a molecular weight of 270.24 g/mol (Fig. 1A). The *in vitro* anti-proliferative effect of apigenin on the SW480 and HCT15 colorectal cancer cell lines was determined using a standard MTT proliferation

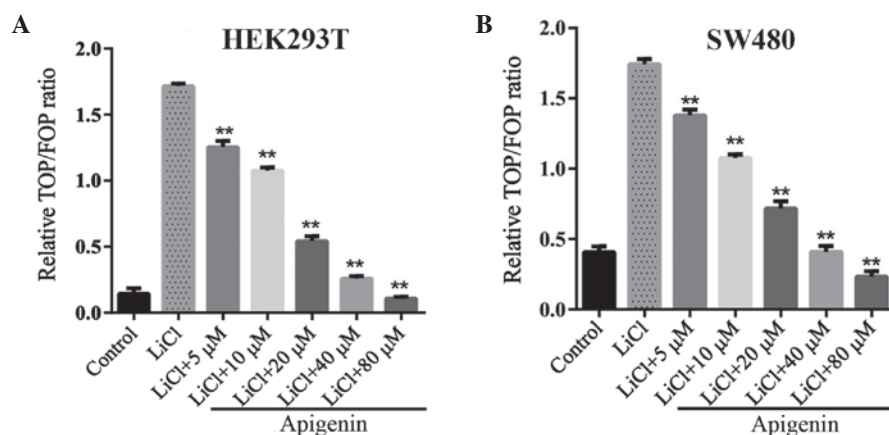


Figure 3. Apigenin inhibits β -catenin/TCF/LEF signal activation. Stimulation with LiCl (20 mM) significantly increased the transcription of the Wnt luciferase reporter in (A) HEK293T and (B) SW480 cells. Apigenin significantly inhibited LiCl-induced β -catenin/TCF/LEF signal activation in a dose-dependent manner. Values are expressed as the mean \pm standard error of three individual experiments performed in duplicate. ** $P < 0.01$ vs. control.

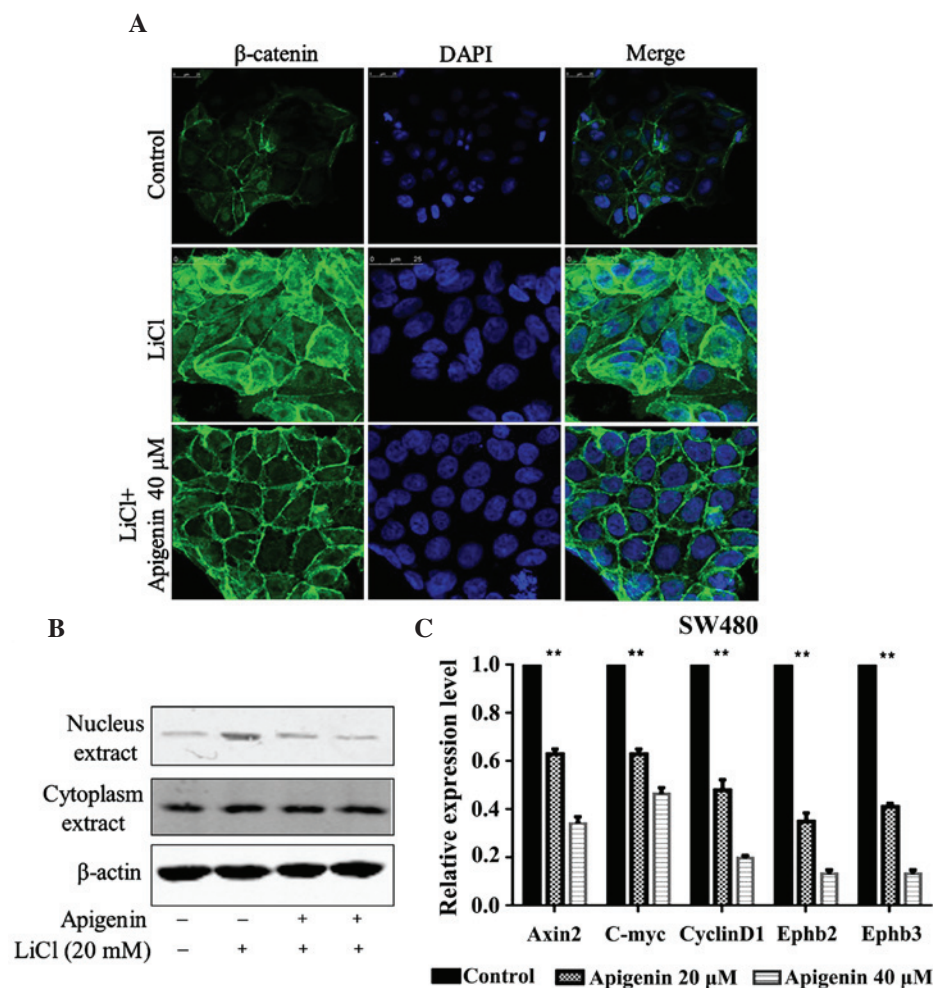


Figure 4. Apigenin inhibits β -catenin entry into the nucleus and thus the expression of Wnt downstream genes. (A) Apigenin treatment decreased LiCl-activated β -catenin nuclear entry in the HCT15 colorectal cancer cell line. (B) Apigenin treatment decreased the LiCl-activated expression of β -catenin in nuclear extracts, but had no effect on the expression in cytosolic extracts of SW480 cells. (C) Apigenin significantly decreased the expression levels of Wnt downstream target genes, including Axin2, C-myc, CyclinD1, Ephb2 and Ephb3, in SW480 cells in a dose-dependent manner. Values are expressed as the mean \pm standard error of three individual experiments performed in duplicate. ** $P < 0.01$ vs. control.

assay. The results revealed that apigenin significantly reduced SW480 and HCT15 cell proliferation at an IC_{50} of 18.17 μ M and 23.57 μ M, respectively. These data suggest that apigenin

may exhibit anti-CRC effects (Fig. 1B). The inhibitive action of apigenin was greater on SW480 cells and therefore these cells were used in subsequent experiments.

Apigenin inhibits migration and invasion of SW480 cells and growth of intestinal organoids. Tumor migration and invasion are essential steps in tumorigenesis (17). The effects of apigenin on the chemotactic motility of SW480 cells were determined using wound-healing migration and Transwell cell invasion assays. As shown in Fig. 2A and B, apigenin significantly inhibited SW480 cell migration and invasion at 20 and 40 μ M. Furthermore, the inhibition of apigenin on the migration of SW480 cells occurred in a dose-dependent manner. Intestinal organoid culture *in vitro* requires multiple cytokines, particularly Wnt signaling pathway cytokines. Without Wnt signaling pathway activation, organoids are unable to grow effectively (18). Therefore, 40 μ M apigenin was added to the intestinal organoid culture system and it was observed that the growth of organoids was significantly inhibited (Fig. 2C).

Apigenin inhibits β -catenin/TCF/LEF signal activation. The TOPflash/FOPflash luciferase assay is frequently used for the analysis of β -catenin/TCF/LEF signal activation (19). The TOPflash construct contains two repeats of three optimal copies of the TCF/LEF binding sites, upstream of a thymidine kinase minimal promoter that is able to direct the transcription of the luciferase reporter gene. FOPflash, which contains mutated TCF/LEF binding sites, is used as a negative control (20). The Gsk3 β inhibitor LiCl activates the Wnt pathway; therefore, stimulation with LiCl (20 mM) significantly increased the transcription of the Wnt luciferase reporter in HEK293T and SW480 cells. Subsequently, the effect of apigenin treatment on HEK293T and SW480 cells and expression of the Wnt luciferase reporter was investigated. Treatment with various concentrations of apigenin and LiCl (20 mM) inhibited β -catenin/TCF/LEF signal activation in a dose-dependent manner (Fig. 3A and B).

Apigenin suppresses β -catenin nuclear entry and the expression of Wnt downstream genes. Wnt signaling activation requires β -catenin nuclear entry (1). In order to determine how apigenin inhibits the Wnt signaling pathway, immunofluorescent staining was used to examine β -catenin nuclear entry in HCT15 cells and western blotting was used to examine the expression of β -catenin in SW480 nuclear and cytoplasmic extracts following LiCl treatment, as well as treatment with various concentrations of apigenin. Apigenin was found to effectively inhibit LiCl-activated β -catenin nuclear entry (Fig. 4A) and markedly decrease LiCl-activated expression of β -catenin in nuclear extracts. However, apigenin had no effect on β -catenin expression in cytosolic extracts (Fig. 4B). The expression levels of Wnt downstream target genes, including Axin2, C-myc, CyclinD1, Ephb2 and Ephb3, were also detected. Apigenin significantly decreased the expression levels of Wnt downstream target genes in a dose-dependent manner (Fig. 4C).

Discussion

In the United States CRC is the third most common type of cancer, and is second only to lung cancer in terms of mortality, resulting in ~50,000 mortalities/year (21). Tumorigenesis in sporadic CRC is typically initiated by an inactivating mutation in the APC gene which induces aberrant activation of the Wnt

signaling pathway (22). Therefore, inhibition of Wnt signaling may contribute to the treatment of CRC.

Numerous Wnt signaling inhibitors, each of which function at various stages in the Wnt/ β -catenin pathway, have been reported to be used for the treatment of cancer (23-26). Mesd C-terminal region peptide, similarly to the full-length Mesd protein, was reported to inhibit Wnt 3A- and Rspodin1-induced Wnt/ β -catenin signaling in LRP5- and LRP6-expressing cells, suppress Wnt/ β -catenin signaling in HS578T human breast cancer cells and PC-3 prostate cancer cells, and inhibit cancer cell proliferation (27). Calcimycin, a Wnt signaling inhibitor, targets the expression of S100A4 and provides a functional strategy for the restriction of cell motility in colon cancer cells (28). Thus, the results of the present study suggested that apigenin, as a natural compound, may inhibit β -catenin/TCF/LEF signaling activation, which was induced by LiCl in a dose-dependent manner, preventing β -catenin nuclear entry and therefore the expression of Wnt downstream target genes.

Apigenin has been identified in numerous plants. Recently, it was reported that apigenin may have potential anti-tumor effects on multiple human cancer cell lines with low cytotoxicity and no mutagenic activity (20,29,30). In a study of apigenin as a potential treatment for CRC, apigenin demonstrated inhibition of colorectal cellular migration and invasion. It was shown to suppress the growth of CRC xenografts through decreased phosphorylation of AKT, as well as phosphorylation and upregulation of FADD expression (31,32). In the present study, apigenin significantly suppressed CRC cell proliferation, migration, invasion and intestinal organoid growth.

In conclusion, apigenin significantly suppressed CRC cell proliferation, migration and invasion in a dose-dependent manner. Additionally, apigenin inhibited β -catenin/TCF/LEF signaling activation, which was induced by LiCl in a dose-dependent manner. Finally, apigenin inhibited β -catenin nuclear entry and thereby prevented the expression of Wnt downstream target genes.

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