Co-expression of Axin and APC gene fragments inhibits colorectal cancer cell growth via regulation of the Wnt signaling pathway

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Abstract. Adenomatous polyposis coli (APC) and Axin interactions serve an important role in colorectal cancer (CRC) pathogenesis. The aim of the present study was to assess the combined effects of Axin and APC co-expression in CRC cells, and to determine the underlying mechanisms involved. SW480 cells were divided into the following groups: Untransfected (SW480 group), transfected with pEGFP-N_3plus pCS2-MT (SW480/vector-vector), transfected with pEGFP-N_3-APC5 (SW480/APC5), and transfected with pEGFP-N_3-APC5 pluspCS2-MT-Axin (SW480/APC5-Axin). APC5 and Axin mRNA levels were determined by reverse transcription-polymerase chain reaction. MTT assays and flow cytometry analysis were performed to assess cell growth and cell cycle distribution, respectively. Quantitative PCR and western blot analyses were conducted to evaluate the mRNA and protein levels, respectively, of Wnt signaling effectors, including β-catenin, c-myc and survivin. Successful transfection of SW480 cells was determined with APC and APC-Axin plasmids as indicated by the green fluorescence signals. Notably, SW480/APC5 cell growth was inhibited by 40.33%, and cells co-expressing APC5 and Axin demonstrated 61.27% inhibition of cell growth compared with SW480 control cells. The results demonstrate that APC5 may induce G1/S arrest in SW480 cells, and Axin may enhance cell growth arrest induced by APC5. The mRNA and protein levels of β-catenin, c-myc and survivin were significantly reduced in SW480/APC-Axin cells when compared with the SW480/APC group. In conclusion, co-expression of APC5 and Axin genes significantly downregulated Wnt signaling in human SW480 CRC cells and inhibited cell growth, when compared with cells transfected with APC5 alone. These results may provide experimental evidence to support combined gene therapy in CRC.

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Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors in China. It is ranked as the fifth most commonly diagnosed cancer type, and is the third leading cause of cancer-associated mortality (1). Alterations in lifestyle, dietary and environmental factors in China have led to the increased incidence of CRC in the Chinese population. The incidence of CRC increased from 12.8 per 100,000 people in 2003 to 16.8 per 100,000 in 2011, while the mortality rose from 5.9 to 7.8 per100,000 (2,3). As a result, early diagnosis, treatment and survival prolongation of patients with CRC have become increasingly important areas of research.

In recent years, with the development of novel molecular biology techniques, researchers are gaining an improved understanding of the underlying mechanisms involved in CRC. The occurrence and development of CRC is a complex process regulated by a number of genes and regulatory pathways, which lead to dysfunction of cell proliferation and apoptosis (4). During this process, cancer gene activation, anti-cancer gene inactivation, mismatch of repair genes and alterations to a number of genes have been observed (4,5). Previous studies have demonstrated that >90% of CRC cases are positive for Wnt signaling pathway activation, which leads to a subsequent accumulation of β-catenin in the cells (6,7). Abnormal alterations to key molecules in the Wnt/β-catenin signaling pathway, including adenomatous polyposis coli (APC), Axin and β-catenin, induce Wnt signaling pathway hyperactivation. The APC anti-cancer gene is considered to be a key molecule involved in the pathogenesis of CRC (7). APC is composed of multiple functional domains, with three 15-aa tandem sequences that bind to β-catenin (aa, 1,020-1,169) (8), and seven domains consisting of 20-aa tandem sequences, which bind to glycogen synthase kinase (GSK)-3β via phosphorylation (aa, 1,262-2,033) (9). In addition, this region contains three Axin-binding extension structures, known as Ser-Ala-Met-Pro (SAMP) repeats (10). Axin functions as a scaffold protein in Wnt signaling and forms a 'destruction complex' with APC, GSK-3β, β-catenin and casein kinase I (CKI) to regulate β-catenin stability (9). In the absence of Wnt signal stimulation, the 'destruction complex' induces phosphorylation of β-catenin at Ser41, Thr33 and Thr3 (11,12) and subsequent degradation via the proteasome pathway (13). This ensures that cellular β-catenin is maintained at a relatively

low level. Upon stimulation of Wnt signaling or in the event of a mutation in APC, Axin or β-catenin, the 'destruction complex' accumulates, thus preventing β-catenin phosphorylation by GSK-3β and subsequent degradation (14). As a result, β-catenin remains stable in the cytosol and translocates into the nucleus to form a protein complex with the T-cell factor (TCF)/lymphocyte enhancing factor (LEF) transcription factor. β-catenin facilitates TCF/LEF binding to the promoter region of target genes, including c-myc, survivin, cyclinD1, vascular endothelial growth factor, matrix metalloproteinase-7, cluster of differentiation 44 and cyclooxygenase-2, which induces their transcription (15). Overexpression of these target genes promotes cancer.

Exogenous induction of APC or Axin alone in CRC cells has been demonstrated to partially inhibit tumor cell growth and promote apoptosis (16,17). However, whether co-transfection of APC and Axin would result in increased anti-cancer effects requires further investigation. In a previous study (15), five functional APC domains were cloned, and it was demonstrated that a particular APC gene fragment (aa, 1,020-1,698) was the smallest functional unit that effectively reduced β-catenin expression. This fragment contained the tandem 15-aa sequence, the tandem 20 a sequence and the SAMP sequence, thus retaining its β-catenin, GSK-3β and Axin binding sites.

To investigate whether the APC5 gene fragment and Axin synergistically inhibit cancer cell growth *in vitro*, the pEGFP-N_3-APC5 recombinant plasmid containing APC5 functional fragments was constructed and used to transfect SW480 CRC cells. Stable transfection pools were then selected and transfected with the pCS2-MT-Axin recombinant plasmid to generate SW480 cells that stably and concurrently express APC5 and Axin. The results provide an experimental basis for the use of combined gene therapy in CRC.

Materials and methods

Plasmids and the CRC cell line. Human SW480 CRC cells were purchased from the central lab cell bank of Zhongnan University Xiangya Medical Center (Xiangya, China). APC5 is the fragment of APC containing gene fragment1020-1698 (aa 1,020-1,698), which was generated in a previous study (18). The pEGFP-N_3-APC5 recombinant plasmid was prepared as previously described (18). The pCS2-MT plasmid and pCS2-MT plasmid containing full-length Axin were kindly provided by Professor Peter Klein of The University of Pennsylvania (Philadelphia, PA, USA).

Experimental groups. Cell groups were defined as follows: SW480, untransfected SW480 cells; SW480/vector-vector, SW480 co-transfected with pEGFP-N_3 and pCS2-MT; SW480/APC5, SW480 transfected with pEGFP-N_3-APC5; and SW480/APC5-Axin, SW480 co-transfected with pEGFP-N_3-APC5 and pCS2-MT-Axin.

Cell culture. SW480 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in a humidified environment with 5% CO_2. At 90 to 100% confluence, cells were passaged following trypsinization, every 2 to 3 days.

Cell transfection. SW480 cells were transfected at 80 to 90% confluence following overnight culture using Lipofectamine™ 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, $1x10^6$ cells were treated with a DNA-Lipofectamine mixture for 4 to 6 h and cultured in serum-containing medium. Green fluorescent protein (GFP) expression was assessed using an Olympus IX70-141 converted fluorescence microscope (Olympus Corporation, Tokyo, Japan). pCS2-MT-Axin was transfected at 48 h following pEGFP-N_3-APC5 transfection. mRNA or protein expression were evaluated at 48 h following co-transfection.

Western blotting. Transfected cells were washed twice with ice-cold phosphate-buffered saline (PBS), followed by treatment with 150 ml cell lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Cell lysates were centrifuged at 3,000 x g for 5 min at 4˚C. The supernatants were then collected, and 40 μg total protein were resolved on a 12% SDS-PAGE gel. The separated proteins were transferred onto a polyvinylidene difluoride membrane (Amersham; GE Healthcare Life Sciences, Shanghai, China), which was blocked for 1 h at room temperature with 5% fat-free milk. After 3 washes with Tris-buffered saline containing 0.1% Tween-20, membranes were incubated with a mouse anti-GFP monoclonal antibody (catalog no. MAB2510; dilution 1:500; EMD Millipore, Billerica, MA, USA) and rabbit anti-myc polyclonal antibody (catalog no. 06-549; dilution1:500; EMD Millipore), mouse anti-β-catenin antibody (catalog no. sc-7963; dilution 1:500; SantaCruz Biotechnology, Inc., Dallas, TX, USA), mouse anti-c-Myc antibody (catalog no. MAB8864; dilution1:500; EMD Millipore) and rabbit anti-survivin antibody (catalog no. sc-10811, dilution 1:500; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. After washing three times, the membrane was incubated with horseradish peroxidase-labeled goat anti-mouse IgG (catalog no. ab6789; dilution 1:5,000; Abcam, Cambridge, UK) and a goat anti-rabbit IgG secondary antibody (catalog no. ab6721, dilution 1:5,000; Abcam) as appropriate, for 1 h at room temperature. Detection was performed using an Enhanced Chemiluminescence system (Amersham; GE Healthcare Life Sciences) according to the manufacturer's protocol.

MTT assay. Cells in the exponential phase of growth were seeded at $5x10^4$ cells/ml in 96 well plates (100 μl/well) and cultured for 44 h. Cells were then treated with 20 μl MTT solution (5 mg/ml; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) at 37˚C for 4 h. Following careful removal of the culture medium, 150 μl dimethyl sulphoxide was added to each well and mixed for 10 min. The absorbance at 490 nm was measured using a spectrometer (model DU530; Beckman Coulter, Inc., Brea, CA, USA), and cell growth inhibition rate was calculated according to the following equation: Growth inhibition rate (%)=$[(1-OD_{transfected\ cells})/OD_{blank\ cells}]$ x100.

Flow cytometry. A total of $1x10^6$ cultured cells were seeded in 60-mm culture dishes and transfected with pCS2-MT, pEGFP-N3, pCS2-MT/Axin or pEGFP-N3/APC5 plasmids using Lipofectamine 2000. At 72 h following transfection, cells were washed twice with ice-cold PBS, resuspended in1 ml pre-cooled 70% ethanol and fixed at 4˚C for 24 h. Following

Table I. Primer sequence information.

Primer (NCBI accession number)	Sequence (5'-3')	Size (bp)
β-catenin (NM_001012329)	F: GGGCGGCACCTTCCTACTTC R: GACCTGGAAAACGCCATCAC	331
c-myc (NM_002467)	F: GATTCTCTGCTCTCCTCGAC R: ACCCTCTTGGCAGCAGGATA	314
Survivin (NM_001012271)	F: ACCGCATCTCTACATTCAAG R: CTTTCTTCGCAGTTTCCTC	342
β-actin (NM_001101)	F: CGCGAGAAGATGACCCAGAT R: GCACTGTGTTGGCGTACAGG	550
APC5	F: TGGGAACCAAGGTGGAAATG R: CTGGCCCGAGCCTCTTTACT	
Axin	F: GACAGGGAAGGGCATATCTGG R: GCATAGCCGGCATTGACATAA	

NCBI, National Center for Biotechnology Information; F, forward primer; R, reverse primer.

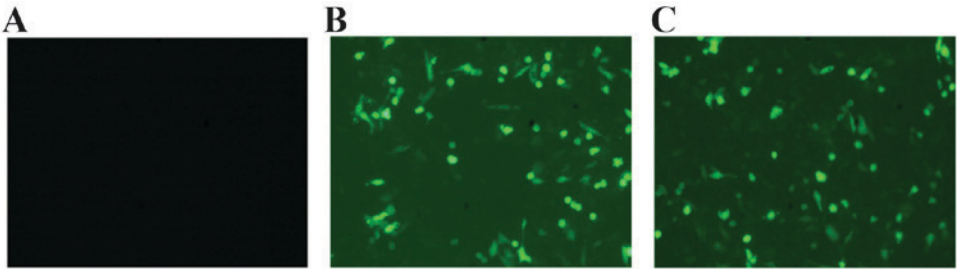

Figure 1. GFP expression in transfected cells. GFP expression was assessed in SW480 cells transfected with different plasmids using an Olympus IX70-141 converted fluorescence microscope (magnification, x400). (A) Untransfected SW480 cells. (B) SW480/vector-vector. (C) SW480/APC5. GFP, green fluorescence protein; APC5, adenomatous polyposis coli 5; SW480/vector-vector, SW480 co-transfected with pEGFP-N_3 and pCS2-MT; SW480/APC5, SW480 transfected with pEGFP-N_3-APC5.

fixation, cells were resuspended in 0.5 ml PBS. Propidium iodide and RNaseA (Sigma-Aldrich; Merck Millipore) were added to the cell suspension at a final concentration of 50 μg/ml, and incubated at 37˚C for 30 min. Cell cycle distribution was assessed using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) by measuring red fluorescence at the excitation wavelength of 488 nm, with Flow Jo software version 7.0 (Tree Star, Inc., Ashland, OR, USA).

Reverse transcription-quantitative polymerase chain reaction. Total RNA was isolated from cell cultures using RNAprep (Thermo Fisher Scientific Inc.). cDNAs were synthesized using RevertAid™ First Strand cDNA Synthesis kit with total RNA as templates. PCR amplification was performed with Taq DNA polymerase (Promega Corporation, Madison, WI, USA) with following primers: Forward, 5'-GGGCGGCACCTTCCTACT TCR-3' and reverse, 5'-GACCTGGAAAACGCCATCAC-3' for β-catenin; forward, 5'-GATTCTCTGCTCTCCTCGACR-3' and reverse, 5'-ACCCTCTTGGCAGCAGGATA-3' for c-myc; forward, 5'-ACCGCATCTCTACATTCAAG-3' and reverse, 5'-CTTTCTTCGCAGTTTCCTC-3' for survivin. Thermal cycling parameters were selected as follows: Predenaturation at 95˚C for 5 min, followed by 30 PCR cycles of denaturation at 95˚C for 30 sec, annealing at 58˚C for 30 sec, extension at 72˚C for 60 sec and then a further extension at 72˚C for 10 min. The mRNA level of target genes was derived as the ratio of its expression relative to β-actin. The PCR reaction was performed with SYBR Green Super mix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) as previously described (19,20) on a ABI StepOne Plus Real Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Each sample was analyzed in triplicate with β-actin as the inner control. The data were analyzed using the $2^{-\Delta\Delta Cq}$ method (21). Primer sequences are listed in Table I.

Statistical analysis. Statistical analysis was performed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation. Differences among experimental groups were analyzed using a one-way analysis of variance followed by the Student-Newman-Keuls post hoc test. Differences between 2 groups were assessed using a Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Successful expression of APC5 and Axin in SW480 cells. At 48 h following transfection, SW480, SW480/vector-vector and SW480/APC5 cells exhibited specific green fluorescence, as determined by fluorescence microscopy (Fig. 1). These results

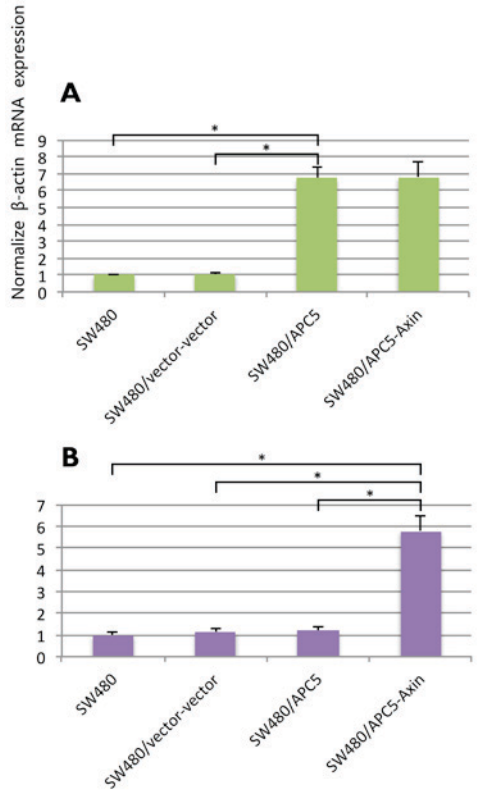

Figure 2. Expression levels of (A) APC5 and (B) Axin mRNA in SW480 cells following co-transfection with pCS2-MT-Axin and pEGFP-N3-APC5 plasmids. APC, adenomatous polyposis coli. Data are presented as the mean ± standard deviation (n=3). *P<0.05.

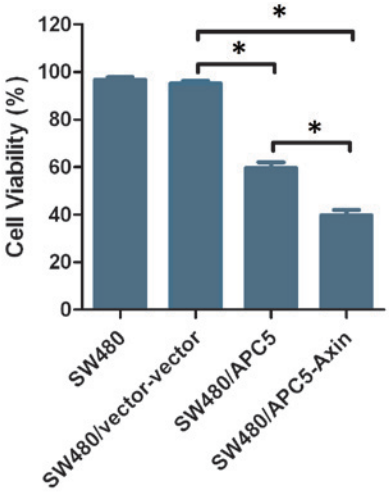

Figure 3. MTT assay results of transfected SW480 cells. SW480 cells in the exponential growth phase were cultured for 44 h and treated with MTT solution for 4 h. Following solubilization of the formazan crystals with dimethylsulphoxide, the absorbance was read at 490 nm. Data are presented as the mean ± standard deviation (n=3). *P<0.05. SW480, untransfected SW480 cells; SW480/vector-vector, SW480 co-transfected with pEGFP-N_3 and pCS2-MT; SW480/APC5, SW480 transfected with pEGFP-N_3-APC5; SW480/APC5-Axin, SW480 co-transfected with pEGFP-N_3-APC5 and pCS2-MT-Axin.

demonstrated the successful transfection ofAPC5 in SW480 cells. As expected, untransfected SW480 cells exhibited no detectable GFP expression (Fig. 1A). As shown in Fig. 2, co-transfection of SW480 cells with APC5 and Axin resulted in overexpression of the two genes, when compared with empty vector controls.

Effect of recombinant APC5 and Axin expression on SW480 cell proliferation. Cell proliferation was assessed by MTT assay. As shown in Fig. 3, inhibition rates of 40.33% and 61.27% were observed in the SW480/APC5 and SW480/APC5-Axin cell groups, respectively, when compared with the control SW480 group. The results demonstrated that APC5-Axin co-transfected cells exhibited a significantly higher cell growth inhibition rate when compared with the SW480/APC5 group (P<0.05; Fig. 3). Notably, the SW480/vector-vector group did not exhibit significant alterations in cell viability when compared with the untransfected SW480 group (Fig. 3). This suggests that Axin enhanced the inhibitory effects of APC5 on SW480 cell growth.

The cell cycle distribution was then analyzed by flow cytometry. As expected, untransfected and empty vector-transfected cells demonstrated similar cell distribution patterns (Fig. 4). However, cells overexpressing APC5 and APC5-Axin exhibited different distribution patterns when compared to the untransfected SW480 control group, with a marked increase in the number of cells in G1/G0 phases. The percentage of cells in G1/G0 phases in the SW480/APC5 and SW480/APC5-Axin groups were 46.2 and 53.3%, respectively. These rates were significantly higher when compared with the SW480/vector-vector cells (G1/G0, 37.5%; P<0.05) and untransfected controls (G1/G0, 36.3%; P<0.05). A significantly higher number of APC5-Axin co-transfected were in G1/G0 phase when compared with the SW480/APC5 group (P<0.05). These results indicated that APC5 may induce G1/S arrest in SW480 cells, with Axin enhancing this effect.

APC5 and Axin affect SW480 cells through the Wnt signaling pathway. As shown in Fig. 5A, the SW480/vector-vector and SW480 groups exhibited high β-catenin mRNA levels. SW480/APC5 and SW480/APC5-Axin cells demonstrated lower β-catenin mRNA levels relative to SW480 and SW480/vector-vector groups, and a significant difference was observed between SW480/APC5 and SW480/APC5-Axin groups (P<0.05; Fig. 5A). In addition, c-myc mRNA levels in SW480 and SW480/vector-vector cells were similar, indicating that vector transfection served no role in c-myc expression (Fig. 5B). However, lower c-myc mRNA levels were observed in the SW480/APC5 and SW480/APC5-Axin groups, with APC5 and Axin co-transfection associated with a significant reduction in c-myc mRNA expression compared with cells overexpressing APC5 alone (P<0.05; Fig. 5B). A similar expression pattern of survivin mRNA among all experimental groups was observed (Fig. 5C). Survivin mRNA levels in SW480 and SW480/vector-vector cells were high, with no statistically significant difference observed between the two groups (P>0.05; Fig. 5C). Lower survivin mRNA levels were observed in the SW480/APC5 and SW480/APC5-Axin groups, with co-transfection of Axin and APC5 resulting in significantly reduced gene expression when compared with APC transfection alone (P<0.05; Fig. 5C).

Similar results were obtained at the protein level, with a reduction in β-catenin protein levels in the SW480/APC5 and

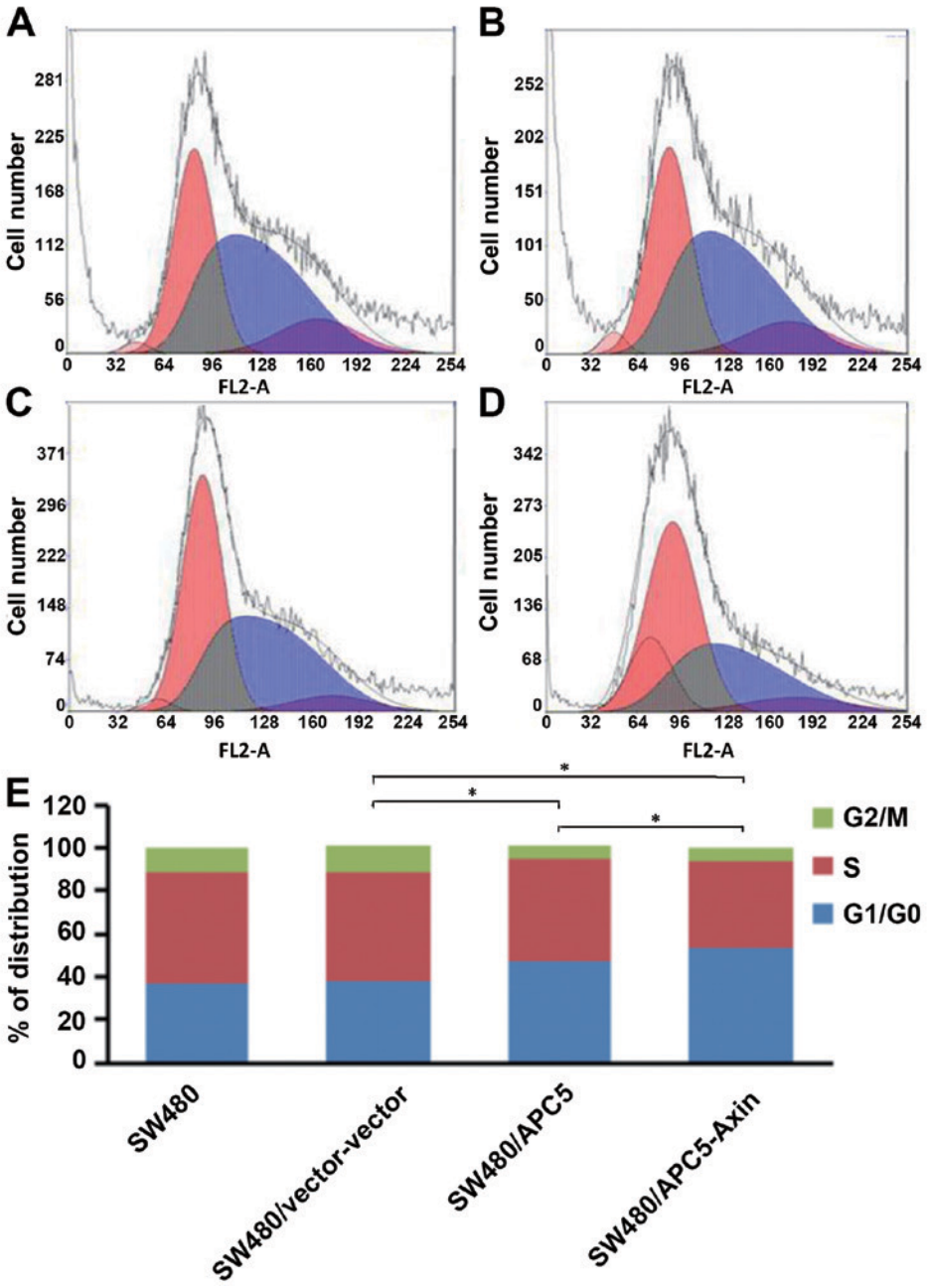

Figure 4. Cell cycle distribution of transfected cells. Cell cycle distribution in SW480cells was assessed by flow cytometry. (A) SW480. (B) SW480/vector-vector. (C) SW480/APC5. (D) SW480/APC5-Axin. (E) The percentage of cells in each cell cycle phase. *P<0.05. APC, adenomatous polyposis coli; SW480, untransfected SW480 cells; SW480/vector-vector, SW480 co-transfected with pEGFP-N_3 and pCS2-MT; SW480/APC5, SW480 transfected with pEGFP-N_3-APC5; SW480/APC5-Axin, SW480 co-transfected with pEGFP-N_3-APC5 and pCS2-MT-Axin.

SW480/APC5-Axin groups when compared with SW480 and SW480/vector-vector groups (Fig. 6A). The greatest reduction in β-catenin protein expression levels was observed in cells co-transfected with APC5 and Axin (Fig. 6A). A similar trend was observed for c-myc protein expression (Fig. 6B). The SW480/APC5 group demonstrated significantly lower c-myc protein levels compared with the SW480 and SW480/vector-vector cells (P<0.05; Fig. 6B). In addition, the level of c-myc protein in SW480/APC5-Axin cells were significantly lower when compared with the SW480/APC5 group (P<0.05). Survivin protein expression levels were high in the SW480 and SW480/vector-vector cells (Fig. 6C). By contrast, SW480/APC5 cells exhibited significantly lower survivin expression levels when compared with SW480 and SW480/vector-vector groups (P<0.05; Fig. 6C). Notably, survivin protein levels in SW480/APC5-Axin cells were significantly lower when compared with the SW480/APC5 group (P<0.05; Fig. 6C).

Discussion

In the present study, co-transfection of APC5 and Axin inhibited SW480 cell growth and induced cell cycle arrest at the G1 phase more effectively than APC5 transfection alone. Notably,

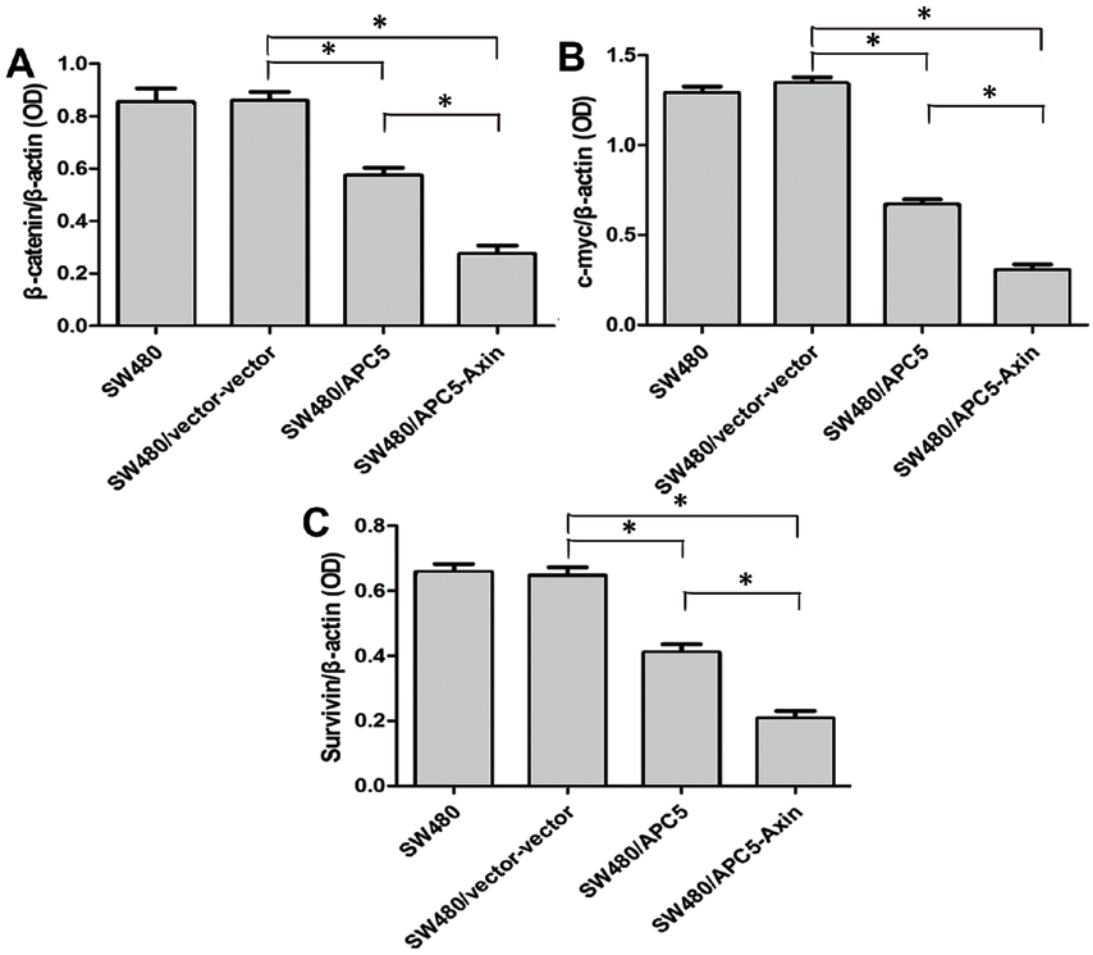

Figure 5. Gene expression levels of Wnt signaling effectors in APC5-Axin SW480 co-transfected cells. The mRNA expression levels (A) β-catenin, (B) c-myc and (C) survivin. Gene expression was assessed by reverse transcription-polymerase chain reaction. Target gene mRNA levels are presented as a ratio of its expression relative to that of β-actin. Data are presented as the mean ± standard deviation (n=3). *P<0.05. APC, adenomatous polyposis coli; OD, optical density; SW480, untransfected SW480 cells; SW480/vector-vector, SW480 co-transfected with pEGFP-N_3 and pCS2-MT; SW480/APC5, SW480 transfected with pEGFP-N_3-APC5; SW480/APC5-Axin, SW480 co-transfected with pEGFP-N_3-APC5 and pCS2-MT-Axin.

APC5 overexpression in SW480 CRC cells downregulated β-catenin expression, a key effector of Wnt signaling, as well as its downstream effectors c-myc, survivin, at the gene and protein levels. In addition, overexpression of Axin and APC5 cells further reduced the expression levels of these effectors, indicating that Axin enhanced the APC5-induced negative regulation of Wnt signaling in SW480 cells. Stable expression and nuclear accumulation of β-catenin are observed during Wnt signaling pathway activation. APC is a multi-functional protein, which is involved in Wnt signaling and regulating β-catenin degradation, as well as mediating cytoskeleton movement and affecting cell migration, adhesion and division (6,7). In addition, APC serves a role in apoptosis (22) and cell cycle regulation, which subsequently affects cell growth. It has been suggested that overexpression of the APC gene induces cell cycle arrest at G0/G1-S and G2/M phases, thus inhibiting cell proliferation (23,24). Polakis *et al* (16) reported that transfection of the wild-type APC gene in CRC cells expressing truncated or inactive APC protein induces degradation of β-catenin, thus reducing β-catenin levels. The present study identified APC5 as a negative regulator of Wnt signaling effectors and cell proliferation, indicating that APC5 may, to some degree, retain the anti-tumor function of the wild-type APC gene, which is in agreement with the results presented by Heinen *et al* (23).

Axin, a negative regulator of Wnt signaling, primarily downregulates β-catenin, however, it is additionally involved in cell apoptosis (17,25). Hsu *et al* (17) demonstrated that overexpression of Axin induces apoptosis. In addition, Satoh *et al* (25) demonstrated that deletion of the Axin mutant stabilizes and facilitates the accumulation of β-catenin. Wild-type Axin transduced into Axin- or APC-mutant liver or CRC cells resulted in induced β-catenin degradation and tumor cell apoptosis (25). Axin functions as a scaffold protein to form a 'destruction complex' with APC, GSK-3β, β-catenin and CKI, which regulates β-catenin stability. Mutations in APC, Axin, GSK-3β, β-catenin and TCF may trigger Wnt signaling activation and β-catenin upregulation, thus promoting CRC.

Previous studies have revealed that APC binds Axin and β-catenin to effectively degrade β-catenin (26-29). However, additional reports have indicated that APC-Axin binding may induce β-catenin degradation more effectively than Axin alone (30,31). In order to investigate the possible synergistic effect of Axin and APC5, the present study performed co-transfection of wild-type Axin into SW480/APC5 cells, and assessed cell proliferation and cell cycle distribution. In

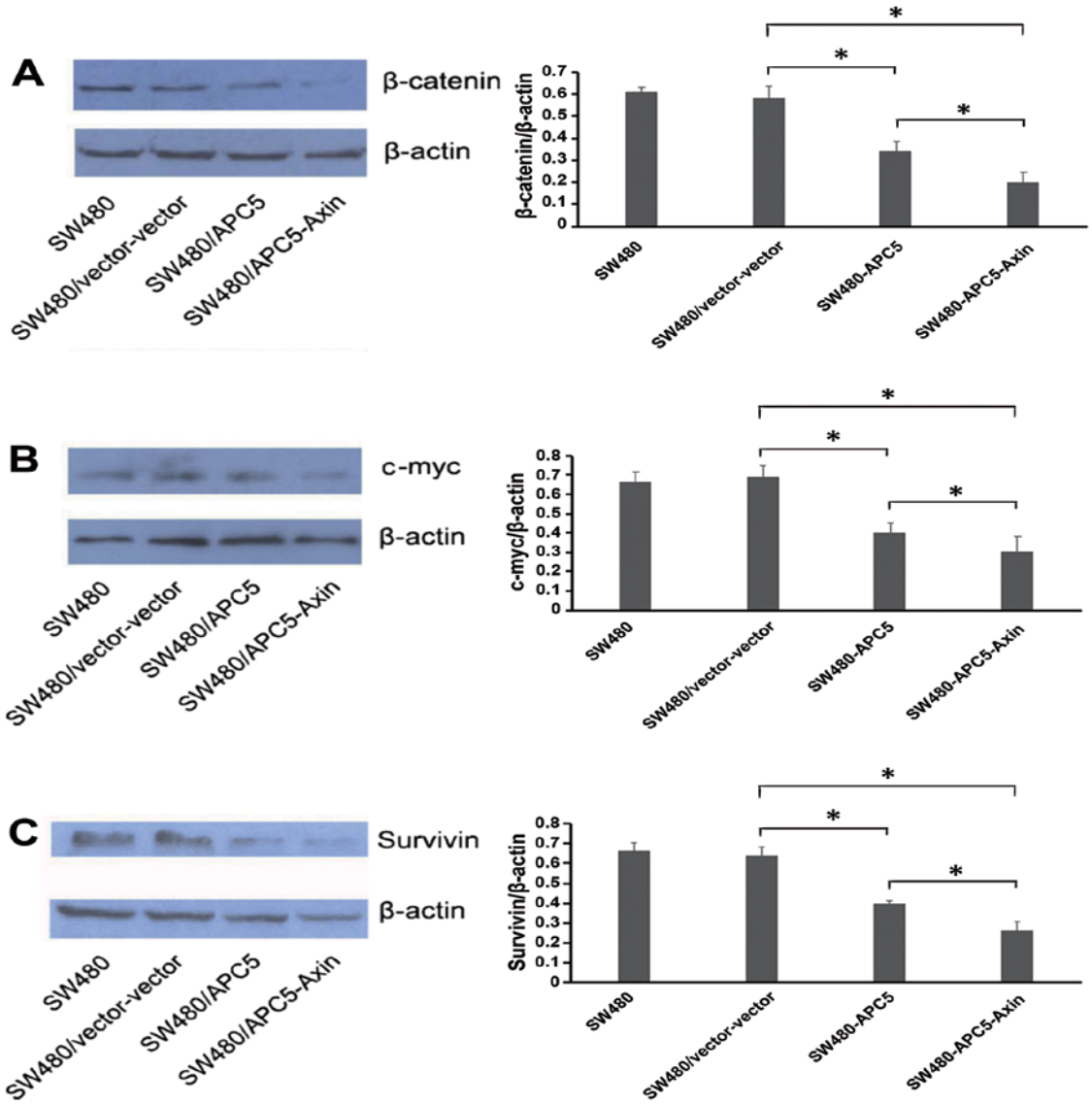

Figure 6. Protein expression levels of Wnt signaling effectors in APC5-Axin SW480 co-transfected cells. Protein expression levels of (A) β-catenin, (B) c-myc and (C) survivin as determined by western blot analysis, with β-actin used as a loading control. Data are presented as the mean ± standard deviation (n=3). *P<0.05. APC, adenomatous polyposis coli; SW480, untransfected SW480 cells; SW480/vector-vector, SW480 co-transfected with pEGFP-N_3 and pCS2-MT; SW480/APC5, SW480 transfected with pEGFP-N_3-APC5; SW480/APC5-Axin, SW480 co-transfected with pEGFP-N_3-APC5 and pCS2-MT-Axin.

the present study, APC5 inhibited SW480 cell proliferation and induced cell cycle arrest in the G1 phase. Co-transfection of APC5 and Axin was associated with a greater reduction in cell viability and cell cycle arrest at G1 phase when compared with APC5 transfection alone. These results indicated that exogenous APC5 and Axin may have synergistic effects in inhibiting tumor cell growth *in vitro*.

To further investigate the molecular mechanism of Axin-assisted APC5 inhibition of tumor cell growth *in vitro*, the effects of Axin-APC5 on Wnt signaling effectors, which are involved in cell growth, were evaluated in the current study. c-myc, survivin and cyclinD1 are involved in cell apoptosis, growth and migration (32). c-myc is a known oncogene, and the first identified target of the β-catenin/TCF complex (32,33); it is overexpressed in various stages of CRC. Overexpression of c-myc activates apoptosis and cell cycle arrest at the G1/S phase (34,35). Pennisi *et al* (36) co-expressed c-myc and a luciferase reporter gene (Luc) in CRC cells, which exhibited positive fluorescent signals. However, when the APC protein was expressed in these cells the fluorescent signal diminished, indicating that APC may mediate c-myc gene suppression. Consistent with these results, the present study demonstrated that APC transfection down-regulated c-myc expression in SW480 cells.

Survivin is an anti-apoptotic gene, which is upregulated in CRC (37). APC and Axin regulate survivin expression by mediating β-catenin phosphorylation and degradation (38). The present study demonstrated that co-transfection of APC and Axin inhibited β-catenin and survivin expression. CyclinD1 is a regulator of cell proliferation and is essential for G1-S phase transition and cell cycle progression (39). The CRC-specific β-catenin mutant triggers high levels of cyclinD1 mRNA expression, whereas wild-type APC and Axin inhibit cyclinD1 activity (40). In the present study, co-transfection of APC5 and Axin downregulated induced cell cycle arrest at the G1 phase.

In conclusion, the present study demonstrated that APC5 reduced the gene and protein expression levels of the Wnt

signaling effectors β-catenin, c-myc, survivin and cyclin D1 in SW480 CRC cells, as well as inhibited tumor cell proliferation and triggered cell cycle arrest. These anti-tumor effects of APC5 were amplified by Axin, thus providing an experimental basis for the potential use of combined gene therapy in CRC.

Acknowledgements

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