

shRNA-induced silencing of Ras-related C3 botulinum toxin substrate 1 inhibits the proliferation of colon cancer cells through upregulation of BAD and downregulation of cyclin D1

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Abstract. Ras-related C3 botulinum toxin substrate 1 (RAC1) is a member of the Rho family of small GTPases. Recent studies have reported that RAC1 serves an important role in colon cancer cell proliferation. The present study aimed to investigate the effects of *RAC1* knockdown on cell proliferation, cell cycle progression and apoptosis of colon cancer cells. Lentivirus-mediated short hairpin RNA (shRNA) was used to knockdown *RAC1* expression in colon cancer cell lines, and cell proliferation, apoptosis, cell cycle progression were evaluated by MTT assays and flow cytometry. The differences in mRNAs expression were identified between *RAC1*-knockdown cells and control cells using a mRNA microarray, following which quantitative PCR (qPCR) and western blot were employed to confirm the results of the mRNA microarray. The proliferative ability of colon cancer cells was significantly decreased following *RAC1* knockdown, and *RAC1* knockdown increased the apoptotic rate and enhanced cell cycle arrest at G₁ phase in colon cancer cells. In addition, >1,200 known genes were demonstrated to be involved in *RAC1*-associated tumorigenic functions in SW620 colon cancer cells, as determined by gene chip analysis; these genes were associated with cell proliferation, cell cycle, apoptosis and metastasis. Furthermore, western blot analysis indicated that cyclin D1 was downregulated, whereas B-cell lymphoma 2-associated agonist of cell death (BAD) was upregulated following *RAC1* knockdown in colon cancer cells. In conclusion, *RAC1* silencing may suppress the proliferation of colon cancer cells by inducing apoptosis and cell cycle arrest. In addition, a large number of genes were revealed to be involved in the process, including *BAD*, which was upregulated and cyclin D1, which was downregulated.

Further studies on these differentially expressed genes may provide a better understanding of the potential roles of RAC1 in colon carcinogenesis.

Introduction

Colon cancer is one of the most common malignant tumors worldwide. In China, colon cancer is the fifth most common type of cancer, and metastasis and recurrence of colon cancer are the leading causes of mortality in most patients (1). At present, surgical resection is the first-line treatment for patients with colon cancer (1,2); however, surgical treatment is difficult to perform in patients with recurrent cancer and metastasis (2). Numerous studies have reported that molecular targeted therapy may further improve the survival of patients with metastatic colon cancer (2-4). For example, treatment with cetuximab, an anti-vascular endothelial growth factor receptor antibody, alongside standard chemotherapy is approved for first-line therapy in patients with K-ras mutations, and for second-line therapy in patients with metastatic colon cancer harboring wild-type K-ras (3,5). Although numerous molecular targets have been identified for individual-specific, targeted therapies in patients with colon cancer, the mechanisms underlying these therapies remain unclear, few agents are available, and the therapeutic efficacy is still not satisfactory (5). Therefore, more studies are required to fully elucidate the pathogenesis of colon cancer and to identify potential therapeutic targets for this disease.

Ras-related C3 botulinum toxin substrate 1 (RAC1) is an important member of the Rho family of GTPases [Rac, Rho and cell division cycle (CDC)42], which are molecular switches that regulate key cellular activities, including cell proliferation, apoptosis, gene expression and directional movement by cytoskeleton remodeling (6-9). Numerous studies have demonstrated that abnormal RAC1 signaling is associated with various human diseases, including cancer, inflammation, neurodegenerative disorders, kidney disorders and cardiovascular diseases (10). In addition, dysregulated RAC1 expression and activity has been detected in various types of cancer cell, including colon cancer (11), gastric cancer (12), lung cancer (13) and breast cancer cells (14), and it has been reported to modulate cancer cell proliferation, inva-

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sion, metastasis and epithelial mesenchymal transition (EMT) by regulating several cancer-associated signaling pathways, including p21-activated kinases (PAK1-3), actin-binding LIM kinases (LIMK1 and LIMK2), Wnt, phosphoinositol 3-kinase and nuclear factor (NF)- κ B (10,15-18). Therefore, it has been hypothesized that RAC1 represents an attractive therapeutic target, due to its role in promoting cancer initiation (10,15-18).

RAC1 overexpression can initiate intestinal stem cell proliferation and regeneration (19), thus resulting in accelerated tumorigenic processes, reduced survival times (20), and disruption of RAC1-mediated immune responses governing neutrophil chemotaxis and apoptosis-associated carcinogenesis in ulcerative colitis (21). Overexpression of the RAC1 splice variant RAC1b is also correlated with poor prognosis in patients with metastatic colorectal cancer (11). Furthermore, increased RAC1 or RAC1b expression can promote cellular transformation, thereby enhancing colorectal cancer cell survival (22); conversely, silencing of RAC1 expression can induce apoptosis and cell cycle arrest, and inhibit proliferation of colon cancer cells. Although previous studies have demonstrated that abnormal RAC1 signaling is associated with the pathogenesis of colon cancer, it remains unclear as to whether RAC1 signaling mechanisms regulate colon cancer.

In our previous study, *RAC1* mRNA expression was downregulated in HT-29 colon cancer cells following treatment with the anticancer agent diallyl disulfide (DADS) (23,24). An additional study indicated that DADS may suppress SW480 cell migration and invasion by downregulating the RAC1-Rho-associated protein kinase 1 (ROCK1)/PAK1-LIMK1-actin-depolymerizing factor/cofilin signaling pathway (24).

Accordingly, the present study used RNA interference (RNAi) technology to silence *RAC1* gene expression in colon cancer cells. Subsequently, cell proliferation, apoptosis and cell cycle distribution were evaluated, in order to determine the role of RAC1 in colon cancer cells. Gene expression profiles were analyzed and bioinformatics analysis was performed to determine the possible molecular mechanisms through which short hairpin (sh)RNA-induced silencing of *RAC1* modulated cell proliferation in colon cancer.

Materials and methods

Cell lines and culture. The human colon cancer cell lines used in the present study (i.e., HT-29, SW620 and HCT116 cells) and 293T cells were purchased from China Typical Culture Center (Wuhan, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 ml/l fetal bovine serum (FBS) (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

Design and lentiviral packaging of *RAC1* shRNA. Three pairs of shRNA sequences targeting the human *RAC1* gene were designed using the latest version of the online RNAi design web tool (<http://jura.wi.mit.edu/bioc/siRNA>), as listed in Table I. The negative control duplexes of shRNA (shRNA-NC) were random sequences (TTCTCCGAACGTGTCACGT), which did not target any known mammalian gene, using

the Blast website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The shRNA sequences were then cloned into the lentiviral vector GV248 (hU6-MCS-Ubi-EGFP-IRES-Puro; Shanghai GeneChem Co., Ltd., Shanghai, China). Lentivirus (LV) (LV-shRNA-RAC1 and LV-shRNA-NC) amplification and packaging was conducted according to the lentiviral packaging protocol (Shanghai GeneChem Co., Ltd.). Briefly, the 293T packaging cell line was cotransfected with GV248 carrying shRNA (LV-shRNA-RAC1 and LV-shRNA-NC) and pHelper plasmids. The next day, medium was replaced with fresh DMEM and culture was continued for 24 h at 37°C. The viral supernatant was then collected, filtered, concentrated and stored in small aliquots at -80°C for titration and cell infection.

Infection of colon cancer cells with LV-shRNA. Colon cancer cells were seeded in 6-well plates (6x10⁵ cells/well) and were incubated for 24 h in a humidified atmosphere. The cells were divided into three groups: KD group, NC group and control group (untreated colon cancer cells). Cells in the KD and NC groups were infected with LV-shRNA-RAC1 or LV-shRNA-NC, respectively, at a multiplicity of infection of 10, according to the manufacturer's protocol (Shanghai GeneChem Co., Ltd.). After 24 h at 37°C, the medium was replaced with fresh DMEM and cells were cultured for a further 48 h. The lentivirus contained the green fluorescent protein (GFP), and the number of GFP positive cells were counted by inverted fluorescence microscopy (Olympus IX53; Olympus Co., Ltd., Shanghai, China). Finally, the cells were harvested and prepared for subsequent analysis.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from cells using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RT-qPCR was then performed. The RT-qPCR primers for *RAC1* and *GAPDH* (internal control) were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). RT was performed using a FastQuant RT kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. The PCR primers for *RAC1* and *GAPDH* were as follows: *RAC1*, forward 5'-ATGTCCGTGCA AAGTGGTATC-3', reverse 5'-CTCGGATCGCTTC GTCAAACA-3'; and *GAPDH*, forward 5'-GCCAAAAGGGTC ATCATCTC-3' and reverse 5'-GTAGAGGCAGGGATG ATGTTTC-3'. qPCR was performed using Taq PCR MasterMix (Tiangen Biotech Co., Ltd.) and a ViiA[™] system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermal cycling conditions were as follows: Initial denaturation at 95°C for 60 sec, 40 cycles of amplification at 95°C for 20 sec, annealing and extension at 60°C for 30 sec. *GAPDH* was used as an internal control for PCR amplification. The data were analyzed using the 2^{- $\Delta\Delta$ C_t} method (25).

Proliferation assays. Cells were trypsinized 72 h postinfection and were seeded in 96-well plates in triplicate (2x10³ cells/well). Following adherence, the cells were exposed to DMEM containing 0.5% FBS for 0, 12, 24, 48 or 72 h at 37°C, and the effects of *RAC1* knockdown on colon cancer cell proliferation were evaluated by MTT colorimetric assays. Briefly, the medium was removed and replaced with medium containing 5 mg/ml MTT. The cells were then incubated for 4 h at 37°C, after which 100 μ l dimethyl sulfoxide solution was

Table I. shRNA sequences targeting RAC1.

ID	Target	Sense	Antisense
RAC1-shRNA-1	5'-TTCTTAACATCACTGTCTT-3'	5'-ccTTCTTAACATCACTGTCTT-3'	5'-AAGACAGTGATGTTAAGAAgg-3'
RAC1-shRNA-2	5'-CAAACAGATGTGTTCTTAA-3'	5'-cgCAAACAGATGTGTTCTTAA-3'	5'-TTAAGAACACATCTGTTTgcg-3'
RAC1-shRNA-3	5'-TGAAGAAGAGGAAGAGAAA-3'	5'-cgTGAAGAAGAGGAAGAGAAA-3'	5'-TTTCTTCTCTTCTTCAcg-3'

RAC1, Ras-related C3 botulinum toxin substrate 1; shRNA, short hairpin RNA.

Table II. List of primary antibodies used for western blotting.

Protein	Description	Vendor	Product code	Dilution	Molecular weight
Cyclin D1	Rabbit	Abcam	ab16663	1:100	33 kDa
BAD	Rabbit	Abcam	ab32445	1:2,000	23 kDa
RAC1	Rabbit	Abcam	ab97568	1:200	21 kDa
GAPDH	Rabbit	Abcam	ab9485	1:2,000	37 kDa

BAD, B-cell lymphoma 2-associated agonist of cell death; RAC1, Ras-related C3 botulinum toxin substrate 1.

added. A microplate reader was used to measure absorbance at 570 nm for each well. The growth inhibition rate was calculated as follows: Growth inhibition rate = $1 - A_{570 \text{ nm}}$ of treated cells/ $A_{570 \text{ nm}}$.

Colony formation assay. Colon cancer cells from each group were plated in 6-well plates (1,000 cells/well) and were incubated for 15 days at 37°C in a humidified atmosphere; the medium was replaced every 3 days. On day 15, the cells were washed with PBS, fixed with 4% paraformaldehyde for 30 min at 25°C and stained with Giemsa (Tiangen Biotech Co., Ltd.) for 15 min. The number of colonies containing >50 cells was counted under an inverted microscope (Olympus Co., Ltd.).

Affymetrix microarray analysis. Gene chip assays and data analysis were performed by Shanghai GeneChem Co., Ltd. using the GeneChip PrimeView Human Gene Expression Array (cat. no. 901838; Affymetrix; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Briefly, three biological replicates collected from each group were subjected to microarray analysis. Total RNA was extracted from RAC1-knockdown cells and NC cells, and RNA quality and quantity were measured using a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). RNA integrity was determined by gel electrophoresis, and purified total RNA was subsequently subjected to DNase I treatment. For microarray analysis, total RNA (300 ng) was first reverse transcribed into a double-stranded cDNA template. Biotin-labeled complementary RNA (cRNA) was then synthesized, amplified and purified by *in vitro* transcription of the double-stranded cDNA template using T7 RNA polymerase. The purified cRNA was fragmented and prepared for hybridization onto the GeneChip cartridge arrays. Hybridization, washing and staining were performed using a GeneChip Hybridization Wash and Stain

kit (Affymetrix; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Scanning of hybridized arrays was performed using a GeneChip Scanner 3000 (Affymetrix; Thermo Fisher Scientific, Inc.). The data were analyzed with Microarray Suite version 5.0 (MAS 5.0) using Partek Genomics Suite software (Affymetrix; Thermo Fisher Scientific, Inc.). Expression values underwent Robust Multiarray Averaging normalization and fold-change values were then calculated using the least-squares mean between samples. The significance of differences in gene expression in the different groups (P-value) was estimated using Student's t-test. Genes with changes in expression ≥ 2 -fold (P<0.05) were regarded as differentially expressed.

Cell cycle and apoptosis analysis. Colon cancer cells were harvested and fixed in 70% ethanol at 4°C for 24 h after cells were grown to 80% confluence. Fixed cells were washed with PBS and suspended in 1 ml propidium iodide (PI) staining reagent (20 mg/l RNase A and 50 mg/l PI). Samples were then incubated in the dark for 30 min at 25°C prior to cell cycle analysis. Cell cycle distribution was determined and analyzed using flow cytometry (FACSCalibur; Becton-Dickinson, San Jose, CA, USA).

The apoptotic rate was determined using an Annexin V-fluorescein isothiocyanate (FITC) detection kit (cat. no. 88-8007; eBioscience; Thermo Fisher Scientific, Inc.). Specific binding of Annexin V-FITC was performed by incubating the cells for 15 min at room temperature in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4) containing a saturating concentration of Annexin V-FITC. After incubation, 1×10^6 colon cancer cells of each group were harvested and fixed in 70% ethanol at 4°C for 24 h after cells were grown to 80% confluence. Cells were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Western blot analysis. Western blot analyses were performed as previously described (8). Briefly, colon cancer cells were harvested, rinsed twice with cold PBS and incubated in lysis buffer (cat. no. P0013; Beyotime; Jiangsu, China). Following centrifugation at 12,000 x g for 30 min at 4°C, the amount of protein in the supernatant was determined using bicinchoninic acid protein assay reagent. Equal amounts of protein (30 μ g) were completely vortexed with 2X SDS-gel buffer and boiled for 5 min at 100°C to dissolve bound proteins. Whole-cell lysates were then separated by 10% SDS-PAGE and were transferred to polyvinylidene difluoride membranes, which were blocked with 50 g/l nonfat milk at 25°C for 1.5 h. The association between RAC1 and differential expression genes involved in cancer-related pathways was investigated with

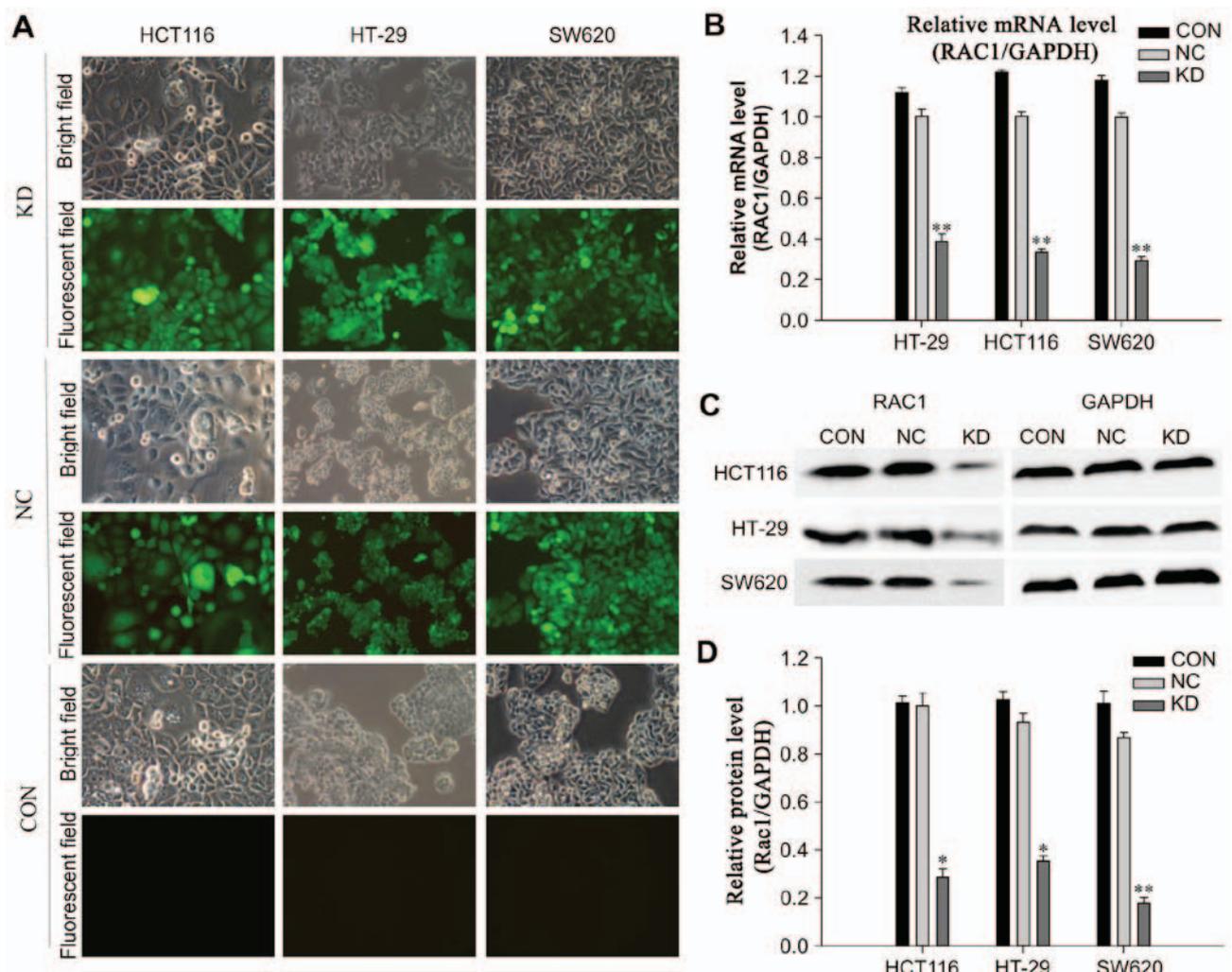


Figure 1. Effects of shRNA on RAC1 expression in colon cancer cells. (A) Representative images of colon cancer cells transfected with LV-shRNA-RAC1 or LV-NC for 48 h and of untreated cells (CON) (magnification, $\times 200$). Green fluorescent protein expression indicated that the cells had been successfully infected with the shRNA. Quantitative polymerase chain reaction analysis of *RAC1* (B) mRNA and (D) protein expression. (C) Western blot analysis of RAC1 protein expression. GAPDH was used as an internal control. Data are presented as the means \pm standard deviation. * $P < 0.05$ and ** $P < 0.01$ vs. NC. CON, control; KD, knockdown; LV, lentivirus; NC, negative control; RAC1, Ras-related C3 botulinum toxin substrate 1; shRNA, short hairpin RNA.

Ingenuity Pathway Analysis (IPA), a web delivered tool (www.ingenuity.com). Proteins were detected following incubation of membranes with specific primary antibodies (Table II) at 4°C overnight, and horseradish peroxidase (HRP)-conjugated secondary antibodies (ab6721; dilution 1:2,000; Abcam, Shanghai, China) for 1 h at 25°C. The membranes were then incubated in SuperSignal enhanced chemiluminescence-HRP detection reagent (cat. no. P1108; Beyotime) for 1 min, and semi-quantitative data were obtained using a computing densitometer with a scientific imaging system (Tanon 5500; Shanghai Tian Neng Technology Co., Ltd., Shanghai, China). GAPDH was used as a loading control for western blot analysis.

Statistical analysis. Data were analyzed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). Measurement data (mRNA/protein levels, cell cycle, cell apoptosis, colony formation and proliferation) are presented as the means \pm standard deviation from at least three independent experiments. Data were analyzed by Student's t-test or one-way analysis of variance followed by Fisher's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of shRNA on RAC1 expression in colon cancer cells.

The expression levels of RAC1 were detected in three colon cancer cell lines (HT-29, HCT116 and SW620). There were no significant differences in the expression of RAC1, and all three cell lines exhibited high levels of RAC1 mRNA and protein (Fig. 1). Conversely, the expression levels of RAC1 were stably reduced using LV-mediated RNAi, and infection efficiency of LV-shRNA-RAC1 and LV-NC was determined using fluorescence microscopy. The results revealed that the majority of cells (>95%) were successfully infected with shRNA (Fig. 1A). The interference efficiency was observed by RT-qPCR and western blotting 72 h postinfection. The expression levels of RAC1 mRNA and protein were significantly decreased in cells in the KD group compared with in the NC and control groups ($P < 0.01$). In the KD group of HCT116, HT-29 and SW620 cells, RAC1 protein levels were reduced by 71.4, 64.6 and 82.2%, respectively, when compared with the NC group (Fig. 1B-D). These results indicated that LV-mediated *RAC1* shRNA was able to efficiently downregulate RAC1 expression in colon cancer cells.

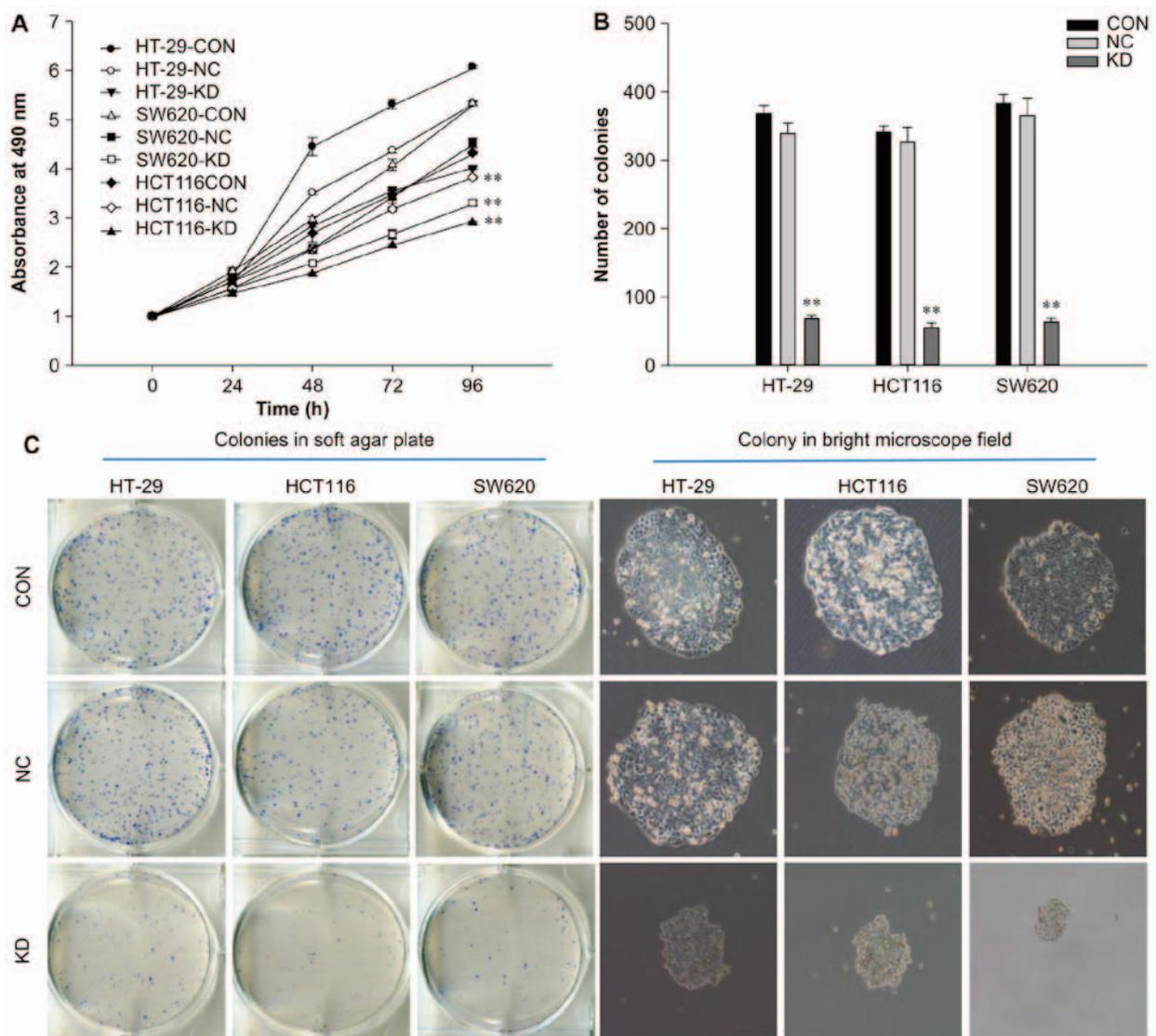


Figure 2. Effects of *RAC1* silencing on colon cancer cell proliferation. (A) MTT assays were used to measure cell proliferation in LV-infected and untreated colon cancer cells. (B) Average number of colonies in colon cancer cells infected with LV-shRNA-*RAC1* or LV-NC and in untreated cells. (C) Representative images of colony formation assays for colon cancer cells in 6-well soft agar plates. The size of each colony was observed using a bright-field microscope (magnification, x200). Data are presented as the means \pm standard deviation. ** $P < 0.01$ vs. NC. CON, control; KD, knockdown; LV, lentivirus; NC, negative control; *RAC1*, Ras-related C3 botulinum toxin substrate 1; shRNA, short hairpin RNA.

RAC1 knockdown suppresses the proliferation and colony formation of colon cancer cells. MTT assays were used to determine whether silencing *RAC1* expression inhibited colon cancer cell viability. Time-dependent inhibition of cell growth was determined in the KD group, whereas no significant inhibitory effects were observed in the NC and control groups (Fig. 2A). Cell proliferation was significantly inhibited after 4 days of infection ($P < 0.01$), with proliferation decreased by 24.6, 11.4 and 27.2% in the KD group of HT-29, HCT116 and SW620 cells, respectively, when compared with the NC group. In addition, following *RAC1* knockdown, the colony-forming capacity of colon cancer cells was significantly decreased by 60-80% ($P < 0.01$), and colony size was reduced compared with in the NC and control groups ($P < 0.01$; Fig. 2B and C). These data suggested that *RAC1* may serve a critical role in colon cancer cell proliferation, particularly in SW620 KD group. Therefore, SW620 cells were selected for subsequent gene chip analysis.

RAC1 knockdown induces differential gene expression. To explore the molecular effectors associated with *RAC1* activity in colon cancer, microarray analysis was performed to determine the mRNA profiles of SW620 cells following *RAC1* knockdown (Fig. 3). From this analysis, 1,215 transcripts were identified as having ≥ 1.5 -fold differential expression; 604 genes were upregulated, whereas 611 genes were downregulated after *RAC1* silencing (Fig. 3B). The results of unsupervised hierarchical clustering of gene expression levels demonstrated that SW620 cells with *RAC1* knockdown were easily distinguishable from SW620 cells without *RAC1* knockdown, and six samples were essentially partitioned into two groups: The first containing knockdown cells and the other containing cells without *RAC1* knockdown (Fig. 3A). All differentially expressed genes were divided into groups according to their biological functions using Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (<https://david.ncifcrf.gov/>). The main functional groups and molecular pathways included

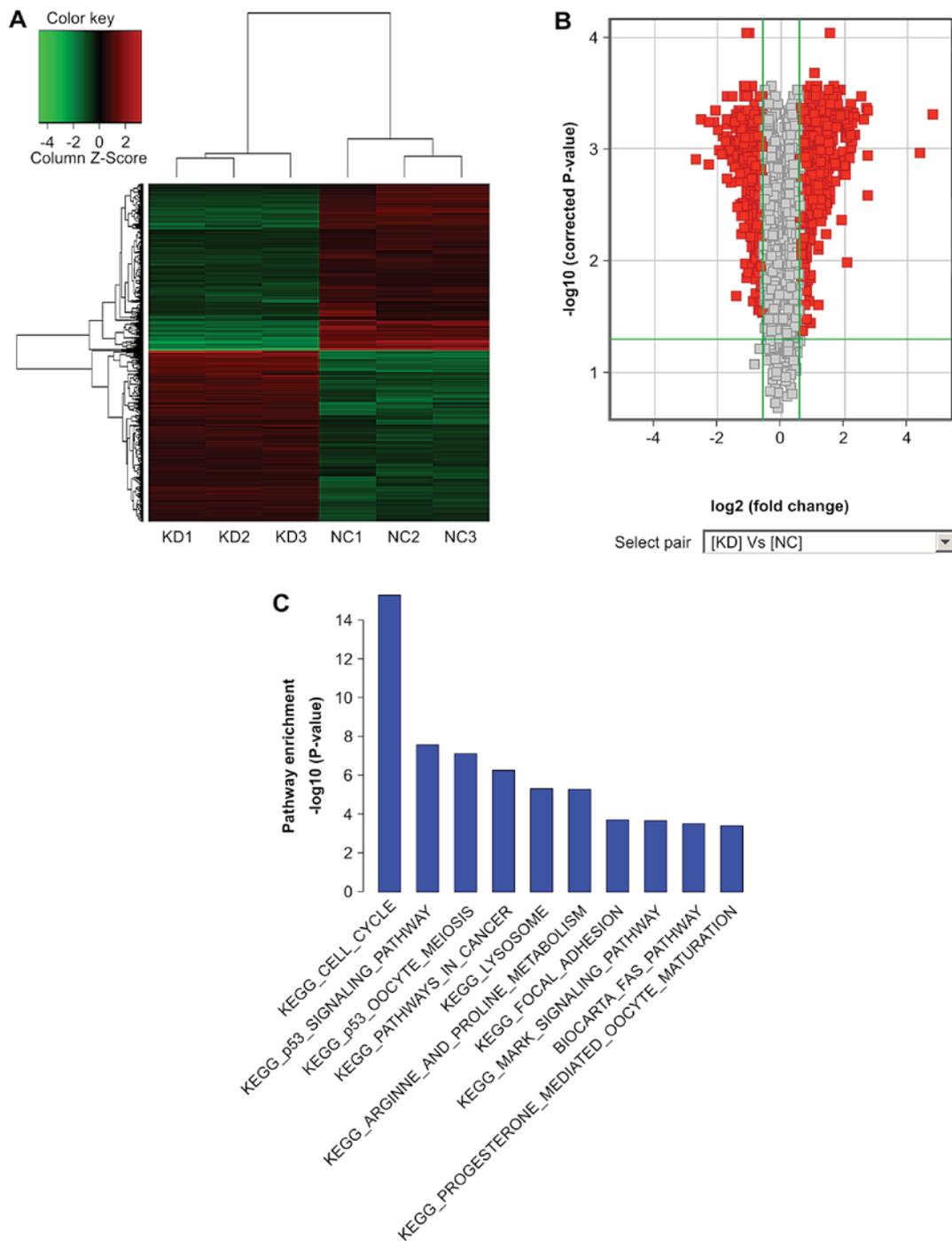


Figure 3. (A) Unsupervised hierarchical clustering based on the mRNA expression levels of genes. Gene microarray expression profiles from three sets of matched SW620 cells transfected with LV-short hairpin RNA-Ras-related C3 botulinum toxin substrate 1 (KD1-KD3) and LV-NC (NC1-NC3). Red indicates upregulated genes, green indicates downregulated genes. (B) Volcano plot analysis based on mRNA expression levels. The vertical green lines correspond to 1.5-fold increases or decreases in expression, and the horizontal green line represents a P-value of 0.05. Therefore, red points in the plot represent differentially expressed genes with statistical significance. (C) Top 10 signal pathways are listed according to their P-values, as determined by KEGG analysis. KD, knock-down; KEGG, Kyoto Encyclopedia of Genes and Genomes; LV, lentivirus; NC, negative control.

cell cycle and apoptosis, cell adhesion, metabolic process, mitosis and cancer-related pathways. Of these, the relationship between cell cycle and *RAC1* silencing was the most significant according to GO analysis ($P=5.19E-16$, $-\log_{10}(5.19E-16)=15.28$); >140 genes, including cyclin D1 (*CCND1*), cyclin-dependent kinase 1 (*CDK1*), cyclin B1 (*CCNB1*), B-cell lymphoma (Bcl)-2-associated agonist of cell death (*BAD*), caspase-8 (*CASP8*) and *MYC* proto-oncogene, bHLH tran-

scription factor (*MYC*), were involved in this process, and the most significant differences in expression were found in cell adhesion-related genes, some of which are listed in Table III. Through KEGG analysis, it was demonstrated that silencing *RAC1* expression inhibited the proliferation of colon cancer cells, potentially via p53 signaling, mitogen-activated protein kinase (MAPK) signaling or cancer-related pathways. The top 10 pathways are listed in Fig. 3C according to their P-values.

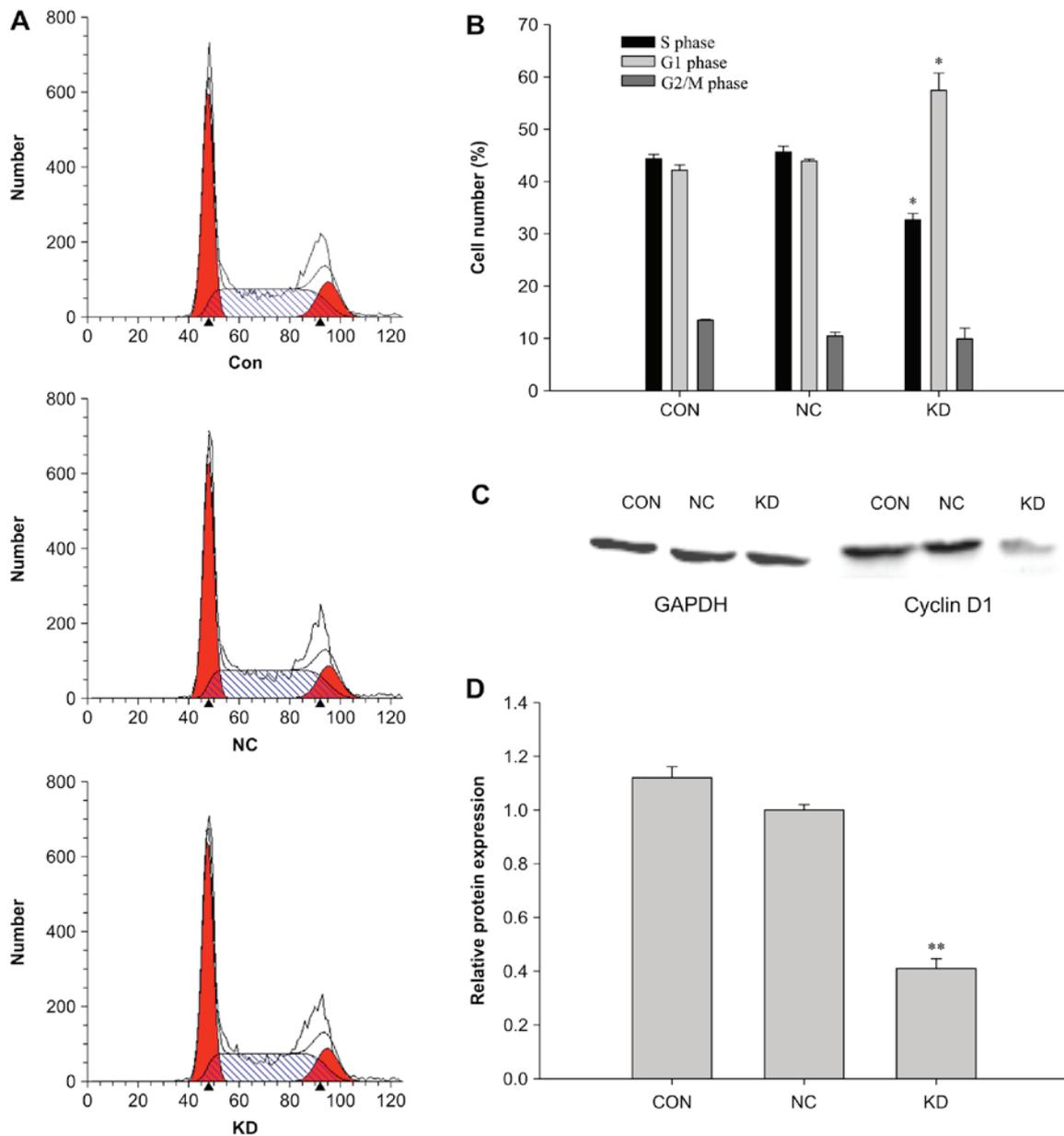


Figure 5. Effects of *RAC1* silencing on cell cycle progression of SW620 cells. (A) Representative results of cell cycle analysis, as determined by flow cytometry. (B) Cell cycle distribution of SW620 cells in each group. (C) Representative results of western blot analysis of cyclin D1 protein expression. (D) Relative protein expression levels of cyclin D1. Data are presented as the means \pm standard deviation. * $P < 0.05$ and ** $P < 0.01$ vs. NC. CON, control; KD, knockdown; NC, negative control; *RAC1*, Ras-related C3 botulinum toxin substrate 1.

however, additional studies are required for the identification of additional potential therapeutic targets, and the development of targeted agents with high efficacy and low toxicity (27,28). Numerous studies have reported that abnormal *RAC1* signaling is associated with various human diseases, and that *RAC1* expression and activity are dysregulated in numerous types of cancer, including colon cancer (10,15). Overexpression of *RAC1* triggers tumor initiation and is associated with the metastasis and invasion of cancer cells; therefore, *RAC1* may be a potent therapeutic target for patients with malignant tumors (10,15).

Downregulation of *RAC1* can inhibit the proliferation of various cancer cells, including gastric cancer (12), colon cancer (20) and breast cancer cells (29), among others (10,13,30). In the present study, the expression of *RAC1* was silenced by LV-mediated RNAi. Subsequently, the proliferation and

colony-forming abilities of HT-29, HCT116 and SW620 colon cancer cells were significantly inhibited, which is consistent with previous data. These findings indicated that *RAC1* may serve an important role in maintaining the growth of colon cancer cells.

To improve understanding of the molecular mechanisms through which shRNA-induced silencing of *RAC1* inhibits proliferation of colon cancer cells, a gene expression microarray analysis was conducted. The results demonstrated that >1,200 genes involved in *RAC1* silencing were differentially expressed, including 604 upregulated and 611 downregulated genes. These genes were divided into various groups according to their biological function using GO terms and KEGG analysis. The main functional groups and signaling pathways included cell cycle, apoptosis, cell adhesion, metabolic process, mitosis, p53 signaling pathway and cancer-related pathways.

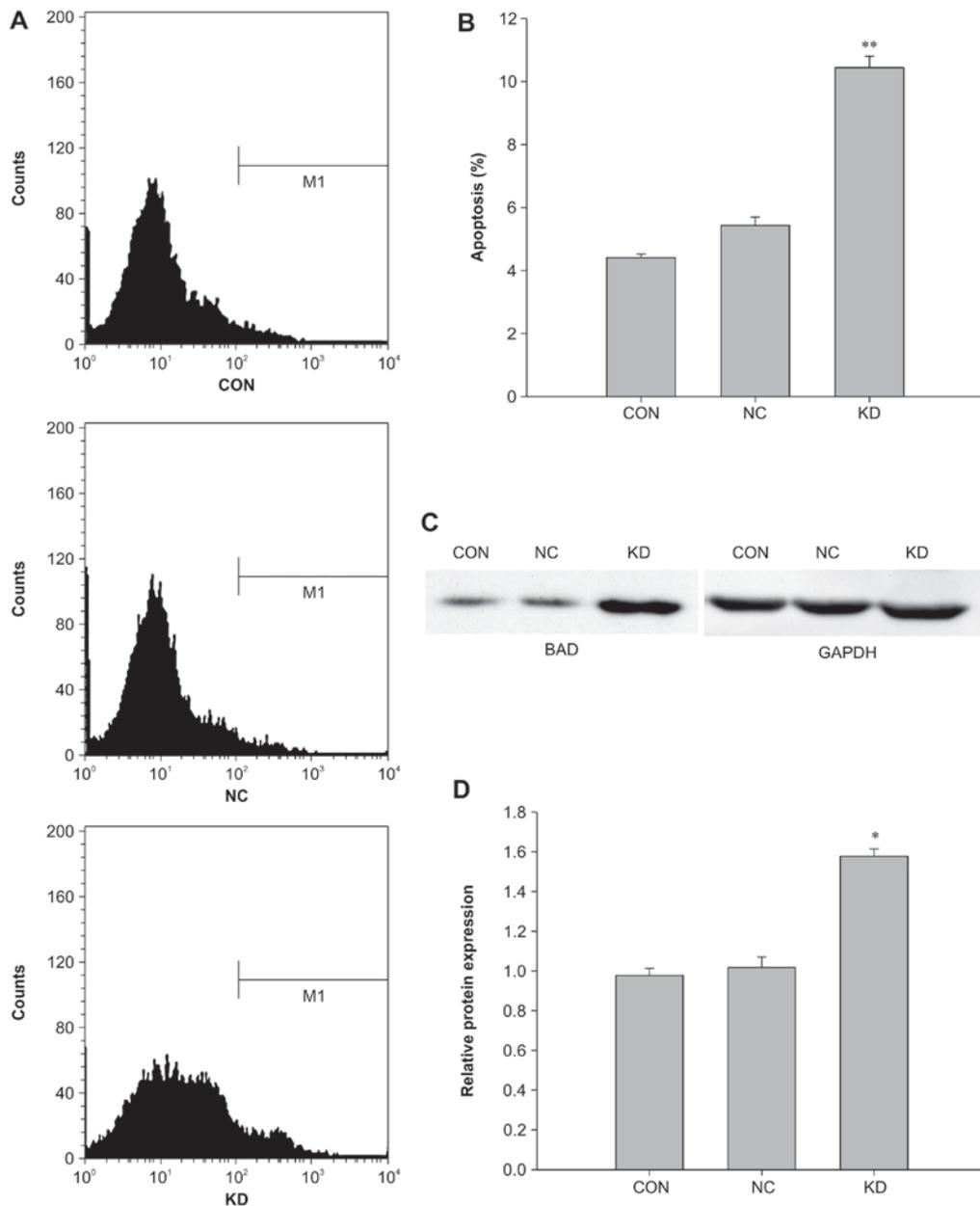


Figure 6. Effects of *RAC1* silencing on apoptosis of SW620 cells. (A) Representative results of apoptosis analysis, as determined by flow cytometry. (B) Apoptotic rate of SW620 cells in each group. (C) Representative results of western blot analysis of BAD protein expression. (D) Relative protein expression levels of BAD. Data are presented as the means \pm standard deviation. * $P < 0.05$ and ** $P < 0.01$ vs. NC. BAD, B-cell lymphoma 2-associated agonist of cell death; CON, control; KD, knockdown; NC, negative control; *RAC1*, Ras-related C3 botulinum toxin substrate 1.

Notably, *RAC1* has been reported to serve an important role in the regulation of various signaling pathways involved in apoptosis and cell cycle progression, and *RAC1* has been revealed to facilitate tumor cell proliferation (31,32). Consistent with these findings, the present results demonstrated that *RAC1* knockdown in SW620 cells induced differential expression of >140 genes associated with the cell cycle and apoptosis, including *CCNB1*, *CCND1*, Fos proto-oncogene, AP-1 transcription factor subunit (*FOS*) and *MYC*, which were downregulated, and *CASP8*, *BAD* and β -catenin, which were upregulated, and genes involved in p53 signaling, MAPK signaling and cancer-related pathways.

Cancer may become autonomous if the genes that drive the cell cycle and apoptosis become dysregulated (33,34). Cyclin/CDK complexes phosphorylate crucial target proteins that drive the cell through the cell cycle (33,35). Cyclin D1,

which is encoded by the *CCND1* gene, forms a complex with CDK4 or CDK6, whose activity is required for the G₁/S transition of the cell cycle (33,35). The *CCNB1* gene encodes the cyclin B1 protein, which has regulatory functions in mitosis. In addition, this protein forms a complex with p34 (CDC2), leading to generation of the maturation-promoting factor, which is expressed predominantly during G₂/M phase (33,35). Increased *MYC* protein expression can activate cyclin/CDK complexes, drive cells into the cell cycle and promote cell proliferation in numerous human cancers (36,37). The tumor suppressor p53 can arrest growth by halting the cell cycle at G₁/S checkpoint and initiate apoptosis by regulating the expression of cyclin D1, cyclin B1 and *MYC* (38). Cyclin D1 and cyclin B1 overexpression, potentially through inactivity of the p53 gene or activity of the *MYC* gene, has been reported

Table III. Differentially expressed genes associated with the cell cycle, apoptosis and cell adhesion following Ras-related C3 botulinum toxin substrate 1 knockdown.

Gene name	GenBank no.	Fold-change	Function
<i>CCND1</i>	NM_053056	-2.4881644	Cell cycle
<i>CCNB2</i>	NM_004701	3.625082	Cell cycle
<i>CHEK2</i>	NM_145862	1.6333724	Cell cycle
<i>CDC25C</i>	NM_022809	4.5017295	Cell cycle
<i>BUB1</i>	NM_004336	2.8708594	Cell cycle
<i>SMC1A</i>	NM_006306	-1.6359816	Cell cycle
<i>PTTG1</i>	NM_004219	2.4099076	Cell cycle
<i>ESPL1</i>	NM_012291	2.1459587	Cell cycle
<i>MCM2</i>	NM_004526	-1.723211	Cell cycle
<i>MCM6</i>	NM_005915	-1.8482218	Cell cycle
<i>ORC3</i>	NM_181837	1.6491874	Cell cycle
<i>STAG2</i>	NM_006603	1.7200525	Cell cycle
<i>BUB1B</i>	NM_001211	1.8905722	Cell cycle
<i>TTK</i>	NM_003318	2.1164258	Cell cycle
<i>BUB3</i>	NM_004725	1.5317589	Cell cycle
<i>MYC</i>	NM_002467	-1.549336	Cell cycle, Cell apoptosis
<i>CASP8</i>	NM_033356	2.0469344	Cell apoptosis
<i>ARHGDI3</i>	NM_001175	-1.9277692	Cell apoptosis
<i>CASP7</i>	NM_033338	1.9895566	Cell apoptosis
<i>CASP2</i>	NM_032982	-1.7764742	Cell apoptosis
<i>CASP4</i>	NM_001225	1.9777244	Cell apoptosis
<i>BAD</i>	NM_004322	1.6885499	Cell apoptosis, Focal adhesion
<i>JUN</i>	NM_002228	-1.892828	Focal adhesion
<i>LAMA3</i>	NM_198129	-2.1677043	Focal adhesion
<i>LAMB1</i>	NM_002291	1.8277249	Focal adhesion
<i>LAMB3</i>	NM_000228	-2.2665424	Focal adhesion
<i>PAK2</i>	NM_002577	-1.6881523	Focal adhesion
<i>ITGB7</i>	NM_000889	-2.1417882	Focal adhesion
<i>ITGB8</i>	NM_002214	-6.589815	Focal adhesion
<i>COL6A1</i>	NM_001848	-1.8742688	Focal adhesion
<i>MYL5</i>	NM_002477	-2.0736258	Focal adhesion

to be correlated with tumor progression (39), shorter survival in patients with cancer and increased metastasis (36-39). Previous studies have reported that decreased expression of cyclin D1 (40,41), MYC (42,43) and cyclin B1 (43) using anticancer agents or RNAi may lead to G₁ or G₂/M cell cycle arrest. Activation of the GTPase RAC1 can promote tumor cell proliferation through the abnormal expression of c-Jun, MYC and RAS, and silencing of RAC1 expression leads to cell cycle arrest, potentially through pathways involving PAK1 (44), cyclin D1 (29) and NF- κ B (45) in breast cancer, lung cancer and colon cancer (15). The present findings confirmed these previous results, demonstrating that *RAC1* knockdown induced SW620 cell cycle arrest at G₁ phase, and this process may be associated with the differential expression of numerous genes, including *CCND1*, *CCNB1*, *CDC12* and *MYC*. Notably, the downregulation of cyclin D1 was further validated by western blotting, thus suggesting that silencing of *RAC1* expression may induce G₁ arrest through this pathway.

A large family of genes that regulate apoptosis has been identified, and caspase-8 and BAD are important members of this family (34). Caspase-8 can activate downstream caspases that cause cell death, and BAD proteins are able to promote apoptosis by neutralizing the activity of anti-apoptotic proteins, such as Bcl-2 and Bcl-extra large. MYC protein can either activate CDK and cyclin genes, or suppress the transcription of apoptosis-associated genes (36,46). Suppression of RAC1 expression has been reported to decrease the phosphorylation of MYC and inhibit cell proliferation (47). Furthermore, increased p38 MAPK expression leads to upregulation of FOS activity through a RAC1-dependent and CDC42-independent pathway, and co-expression of the dominant-negative mutants of FOS, p38 and RAC1 blocks MYC-mediated apoptosis (48). The present data demonstrated that knockdown of the *RAC1* gene may induce apoptosis of SW620 colon cancer cells; numerous genes that are potentially involved in this process were identified by gene chip analysis, of which BAD upregulation was further confirmed by western blotting. These results may indicate that silencing *RAC1* expression can induce cell apoptosis through a pathway involving BAD.

Previous studies have reported that RAC1 is involved in cancer invasion and metastasis through controlling cell motility and cell adhesion (10,15). For example, RAC1 hyperactivation or overexpression drives the mesenchymal mode of cell migration by stimulating the formation of actin-rich membrane extensions (49,50); promotes tumor-associated angiogenesis by regulating the balance of inhibitors and stimulators of endothelial cell proliferation, endothelial cell migration and capillary formation molecules (15,51,52); controls the expression and release of matrix metalloproteinases required for proteolytic degradation of the extracellular matrix (ECM) (10,53,54); and induces EMT by mediating cellular plasticity and ECM modulation (10,30,55). In the present study, after silencing *RAC1* expression, some differentially expressed genes involved in cancer-related pathways were detected, including laminin subunit α 3, Jun proto-oncogene, AP-1 transcription factor subunit, *MYC*, laminin subunit β 1, *PAK2* and *ROCK2*, which were downregulated, and thrombospondin 1 and *BAD*, which were upregulated; these genes are involved in cell motility and adhesion. Therefore, the present findings further supported that RAC1 promoted tumor invasion and metastasis through regulating cell motility and adhesion.

In conclusion, silencing *RAC1* expression inhibited colon cancer cell growth, promoted cell cycle arrest and enhanced apoptosis; these functions may be associated with the differential expression of numerous genes involved in various biological functions, including downregulation of *CCND1* and upregulation of *BAD*. Further studies regarding these differentially expressed genes may allow a better understanding of the effects of RAC1 signaling dysregulation on the promotion of colon carcinogenesis.

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