Original Paper

Dickkopf-Related Protein 2 is Epigenetically Inactivated and Suppresses Colorectal Cancer Growth and Tumor Metastasis by Antagonizing Wnt/ β -Catenin Signaling

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Key Words

DKK2 • Colon cancer • Tumor suppressor • Methylation • Wnt/ β -catenin

Abstract

Background/Aims: Aberrant activation of the Wnt/ β -catenin signaling pathway plays a key role in the pathogenesis of multiple tumors including digestive cancers. Recent studies have reported that Dickkopf-related protein 2 (*DKK2*) is epigenetically inactivated in numerous types of cancers and that its gene products exhibit tumor-suppressive properties. However, the biological functions and underlying molecular mechanisms of *DKK2* in colon carcinoma remains obscure. **Methods:** We examined the expression of *DKK2* in colon tumor cell lines by RT-PCR and its promoter methylation status in colon tumor cell lines and primary tumors by methylation-specific PCR (MSP). Ectopic expression of *DKK2* was measured by RT-PCR prior to the other experiments. To investigate the function of *DKK2*, we assayed colony formation and cell proliferation, utilized flow cytometric analyses of the cell cycle and acridine orange/ethidium bromide (AO/EB) fluorescence staining for apoptosis, and examined wound healing, transwell migration and tumor growth *in vivo*. Western blots were used to explore the mechanisms of *DKK2* in epithelial- mesenchymal transition and canonical Wnt/ β -catenin signaling. **Results:** We show here that downregulation or silencing of *DKK2* was closely associated with the hypermethylation status of its promoter and that *DKK2* expression could be restored by demethylation treatment. Methylation of the *DKK2* promoter was detected in nearly all tumors and tumor-adjacent tissues, but not in normal colon tissues. Ectopic expression of *DKK2* in colon cell lines HCT116 and HT-29 inhibited colony formation and cell viability by inducing cell cycle G0/G1 arrest and apoptosis, and growth of stable *DKK2*-infected HCT116 cells in nude mice was decreased compared to controls. Furthermore, *DKK2* restrained cell migration through partial reversal of epithelial-to- mesenchymal transition and also by

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downregulating several stem cell markers. Our data further showed that restoration of *DKK2* expression resulted in downregulation of active β -catenin and its downstream target genes.

Conclusion: *DKK2* appears to be a functional tumor suppressor regulating tumorigenesis of colorectal cancer by antagonizing Wnt/ β -catenin signaling.

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Introduction

Colorectal cancer (CRC) is the leading cause of death among digestive tumors worldwide. According to the latest cancer statistics from the American Cancer Society, CRC accounted for 16% (134,490) of all cancer cases and 16% (49,190) of cancer deaths in 2016 [1]. However, physical and biochemical technology for early diagnosis of colon cancer is still limited, underlining the importance of finding a reliable biomarker for the early detection of this disease. Although the specific mechanism of CRC tumorigenesis remains unclear, epigenetic inactivation of tumor suppressor genes by promoter methylation plays an important role in this cancer [2]. A previous study has demonstrated promoter methylation of tumor-related genes and expression of tumor suppressor genes (TSG) in gastrointestinal cancers and precancerous lesions, suggesting their involvement in the multistep progression of colorectal carcinogenesis. Thus, these epigenetically-modified genes could be potential biomarkers for colon cancer.

The Wnt signaling pathway is involved in tumorigenesis of various malignancies and consists of canonical Wnt/ β -catenin signaling and non-canonical Wnt signaling independent of β -catenin [3-5]. Some of the Wnt signaling components are broadly involved in carcinogenesis and pathogenesis. For example, epigenetic and genetic alterations of β -catenin and adenomatous polyposis coli (APC) regulators in the Wnt signaling pathway have been demonstrated in many human tumors, including liver cancer [6], gastric cancer [7, 8] and colon cancer [9, 10]. Therefore, aberrant activation of WNT signaling is a hallmark of carcinogenesis [11, 12], and epigenetic modifications are an important mechanism for regulating this pathway [13].

Researchers have found that aberrant DNA methylation is related to the genesis of early CRC lesions, indicating that epigenetic alterations are involved in the initiation of CRC [14]. Thus, epigenetic changes, especially the methylation of CpG islands in the promoters of tumor suppressor genes, may cause gene silencing and the onset of tumors [15, 16].

The Dickkopf (*DKK*)-related protein family is composed of four secreted glycoproteins, *DKK1* through *DKK4*. The *DKK2* gene is located at 4q25, a commonly-deleted tumor suppressive locus in multiple tumors. Although epigenetic silencing of *DKK2* by promoter methylation has been observed in several tumors [17, 18], its biological functions and related molecular mechanisms in CRC have not been elucidated. In this study, we investigated *DKK2* expression and promoter methylation status in CRC. We also analyzed the tumor-suppressive functions and mechanisms of *DKK2* in CRC. Our findings suggest that *DKK2* functions as a tumor suppressor and regulator of the Wnt/ β -catenin pathway in CRC.

Materials and Methods

Cell lines and tumor samples

Human colon cancer cell lines (HT-29, HCT116, HCT15, LoVo, SW480, RKO and Caco2) were obtained from American type culture collection (Manassas, VA, USA). Cell lines were maintained in RPMI-1640 with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 5% CO₂ as previously reported [19].

All colon tissues were collected from surgical patients enrolled in the study as previously described [19]. Fresh specimens were obtained during surgery without any other therapeutic intervention from the Department of Gastrointestinal Surgery (the First Affiliated Hospital of Chongqing Medical University,

Chongqing, China). The samples were immediately snap-frozen in liquid nitrogen and then stored at -80°C until analysis. Pathological diagnosis was performed and verified at the Pathology Department, Chongqing Medical University. The study protocol was authorized under guidelines of the ethics committee of Chongqing Medical University.

Pharmacologic demethylation

CRC cell lines were diluted at a density of 1×10^6 cells/ml and cultured for 24 h. Cells were treated with demethylating agent 5-aza-2'-deoxycytidine (Aza; Sigma-Aldrich, St. Louis, MO, USA) alone for 3 days and subsequently with the histone deacetylase inhibitor trichostatin A (TSA; Cayman Chemical Co., Ann Arbor, MI, USA) for 24 h as described [20]. Cells were then collected for RNA and DNA extraction.

Nucleic acid extraction

Total DNA and RNA were isolated as previously described [21]. RNA was extracted from cell lines using TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA). DNA was extracted from cell lines and tumor tissues with Qiagen DNeasy Tissue kit (Qiagen, Inc., Valencia, CA, USA). Extracted samples were quantified using spectrophotometry and subsequently stored at -80°C until use.

Semiquantitative PCR and quantitative RT-PCR (qRT-PCR) analysis

Reverse transcription PCR (RT-PCR) was performed as previously described using Go-Taq (Promega, Madison, WI, USA), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control [22]. RT-PCR was carried out in the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and performed with 32 cycles for *DKK2* and 23 cycles for GAPDH. The PCR program began with an initial denaturation at 95°C for 2 min, followed by the amplification reaction cycles (95°C for 30 s, 55°C for 30 s and 72°C for 30 s), with a final extension at 72°C for 3 min. The primer sequences are shown in Table 1. qRT-PCR was carried out in the HT7500 system (Applied Biosystems), using SYBR® Green PCR Master Mix (Thermo Fisher Scientific, Hong Kong, China) with β -actin as a control [23]. The primer sequences are shown in Table 2.

Bisulfite treatment and methylation analysis

Bisulfite modification of DNA and methylation-specific PCR (MSP) were carried out according to our earlier reports [24]. Bisulfite-modified DNA was amplified with either promoter-methylated (m) or unmethylated (u) primer sequences (methylation-specific primers are shown in Table 1). MSP for the *DKK2* promoter was performed using AmpliTaq-Gold DNA Polymerase (Applied Biosystems), with the reaction conditions as follows: initial denaturation for 10 min at 95°C , then 40 cycles consisting of 95°C for 30 s, 60°C (methylated reactions) or 58°C (unmethylated reactions) for 30 s, 72°C for 30 s, and a final extension for 5 min at 72°C . Methylated and unmethylated human DNA were used as positive and negative controls, respectively.

DKK2 overexpression in HCT116 and HT-29 cells

For *DKK2* ectopic expression, cDNA encoding *DKK2* was subcloned

Table 1. List of primers used in this study. F, forward; R, reverse

PCR	Primer	Sequence (5'-3')	Product size (bp)	PCR cycles	Annealing temperature ($^{\circ}\text{C}$)
MSP	DKK2 m1	AGAGTTAAATCGTCGAGATTTC	146	41	60
	DKK2 m2	CTAAAAACAATCAAATACGAAACG			
RT-PCR	DKK2u1	GGAGAGTTAAATTGTTGAGATTTT	149	41	60
	DKK2u2	ACTAAAAACAATCAAATACAAAACA			
	β -actin-F	TCCTGTGGCATCCAGAAACT	315	23	55
	β -actin-R	GAAGCATTTGCGGTGGACGAT			
	DKK2-F	GTACCAAGGACTGGCATTTCG	169	32	55
	DKK2-R	ATCTCGGTGGCAGCGCTTCT			
	STAT3-F	CCAATGGAATCAGCTACAGC	236	32	55
	STAT3-R	GCTGATAGAGAACATTGCACTC			
	BMI-F	GATCACTGAGCTAAATCCCC	287	32	55
	BMI-R	CAGAAGGATGAGCTGCATAAA			
	ALDH1-F	GAAATGTCATCTCAGGCAC	171	32	55
	ALDH1-R	ATCTCCTTCTTCTACCTGGC			
	KLF4-F	TCCATCTTTCTCCACGTTT	262	32	55
	KLF4-R	TCCAGGAGATCGTTGAACCTC			
	SOX2-F	AGCAACGGCAGCTACAGCA	281	32	55
	SOX2-R	TGGGAGGAAGAGGTAACACAG			
MSP	MAD2-F	AAATCGTGGCCGAGTTCTTC	244	32	55
	MAD2-R	CTTTCCAGGACCTCACCACCT			
	ABCG2-F	CAGTGTCAAGGAAACACC	194	32	55
	ABCG2-R	GAGACCAGGTTTCATGATCC			
	CD44-F	TGGACAAGTTTGGTGGCAC	176	32	55
	CD44-R	GGTGCTATTGAAAGCCTTGC			
	CD133-F	CCACAGATGCTCCTAAGGC	187	32	55
	CD133-R	TTGGATTCATATGCCTTCTG			

into a lentiviral vector (MacroGen, Seoul, South Korea). Then, vectors with and without *DKK2* were packaged in the HEK293T cell line. After 24 h, viruses were added to HCT116 and HT-29 cells. The next day, the medium was replaced with fresh medium containing 2 μ g/ml puromycin, and cells remained in medium containing puromycin during selection. Puromycin-resistant clones were picked after 1 week.

Table 2. List of qRT-PCR primers used in this study. F, forward; R, reverse

Primer	Sequence (5'-3')	Product size (bp)	PCR cycles	Annealing temperature (°C)
Ecad-F	TACACTGCCCAGGAGCCAGA	103	40	60
Ecad-R	TGGCACCAGTGTCCGGATTA			
Vimentin-F	GACCAGCTAACCAACGACAA	150	40	60
Vimentin-R	GTCAACATCCTGTCTGAAAGAT			
Ncad-F	CGAATGGATGAAAGACCCATCC	174	40	60
Ncad-R	GGAGCCACTGCCTTCATAGTCAA			
Twist-F	GGAGTCCGAGTCTTACGAG	201	40	60
Twist-R	TCTGGAGGACCTGGTAGAGG			
cyclinD1-F	CTAGCAAGCTGCCGAACC	90	40	60
cyclinD1-R	TCCGAGCACAGGATGACC			
p21-F	TGGAGACTCTCAGGGTCGAAA	65	40	60
p21-R	GGCGTTTGGAGTGGTAGAAATC			
GAPDH-F	GGAGTCAACGGATTGGT	206	40	60
GAPDH-R	GTGATGGGATTCCATTGAT			

Colony formation assay

For monolayer culture colony formation assays, stably-expressing *DKK2* and empty vector control cells (HCT116 and HT-29) were plated in six-well plates and selected for 2 weeks in complete medium containing 2 μ g/ml puromycin. Surviving colonies (≥ 50 cells/colony) were fixed with 4% paraformaldehyde, stained with Giemsa's solution and visible colonies were counted. The experiments were repeated three times.

Cell proliferation assay

Cell proliferation was assessed by MTS assay. Stable *DKK2*-expressing and empty vector cells (HCT116 and HT-29) were seeded in 96-well plates (3000 cells/well) with 100 μ L of complete culture medium. Cells were incubated for 24, 48 or 72 h, followed by the addition of 100 μ L/well of medium containing 20 μ L MTS reagent and subsequent incubation at 37°C for 2 h. Absorbance was measured at 490 nm using a microplate reader (Multiskan MK3; Thermo Fisher Scientific). Data were obtained from three independent cultures and each experiment was repeated three times.

Flow cytometry analysis of cell cycle and apoptosis

For cell cycle assays, HCT116 and HT-29 cells were cultured in six-well plates at a density of 2×10^5 cells/well and grown overnight. Cultures were then transiently infected with LV-*DKK2* or LV-control according to the manufacturer's instructions. After 48 h, cells were collected, digested and centrifuged at 1000 rpm for 5 min. Cells were washed twice with phosphate buffer saline (PBS), fixed in ice-cold 70% ethanol overnight and treated with 50 μ g/l propidium iodide (PI; BD Pharmingen, San Jose, CA, USA) for 30 min at 4°C in the dark. The cells were sorted using a FACSCalibur machine (BD Biosciences, Franklin Lakes, NJ, USA) and data were analyzed by ModFit 3.0 software (Verity Software House, Topsham, ME, USA).

Apoptosis was measured using annexin V-fluorescein isothiocyanate (FITC; BD Pharmingen) and PI according to the manufacturer's protocol. Data were analyzed using CellQuest™ Pro (BD Biosciences). Acridine orange/ethidium bromide (AO/EB) fluorescence staining was also used to examine cell apoptosis as described [19]. After infection for 48 h, cells were washed three times with PBS and visualized immediately under a fluorescence microscope.

Wound healing and transwell assays

Stably-infected cells were plated in six-well plates (5×10^5 cells/well) until 90% confluent. Cell layers were scratched using sterile P-20 pipette tips, then washed twice with PBS to carefully remove cell debris and incubated in serum-free media. The cell-free wound area was photographed every 12 hours at 100 $\times$ magnification. The experiments were performed in triplicate.

Transwell chambers (8- μ m pore size; Corning, Corning, NY, USA) were also used to assess cell migration ability as described previously [25]. Cells were collected and washed twice in serum-free medium, then added to the upper chamber (5×10^4 cells/chamber), with 700 μ L migration induction medium containing 10% FBS in the lower chamber. After incubation for 48 h, the lower side of the inserts was fixed with

methanol and stained with Giemsa's solution. Non-migratory cells on the upper side were wiped off using a Q-tip. Migrating cells were counted under microscopy at 400 $\times$ magnification.

In vivo tumorigenicity

HCT116 cells stably infected with LV-*DKK2* or empty LV-control (5×10^6 cells in 0.2 ml serum-free medium) were injected subcutaneously into female BALB/c nude mice (4 weeks old, five mice/group). Tumor volume (mm^3) was calculated using the following equation: $\text{volume} = 0.5 \times \text{length} \times \text{width}^2$. When the length of the tumor reached 1 cm, the mice were euthanatized and xenograft tumors were removed. The tumors were immediately weighed, then fixed, dehydrated and embedded in paraffin for immunohistochemistry and hematoxylin/eosin (HE) staining. The animal experiment was authorized by the Committee on Ethical Use of Animals of Chongqing Medical University.

Western blot analysis

Cells were collected 48 h post-infection for protein extraction, using protein extraction reagent (Thermo Scientific, Rockford, IL, USA) containing a phosphatase inhibitor cocktail (Sigma-Aldrich) and the protease inhibitor phenylmethanesulfonyl fluoride. Proteins (40 μg /lane of total extract) were subjected to western blotting as previously described [25]. The primary antibodies used were as follows: *DKK2* (1:1000, ab38594; Abcam, Cambridge, UK), β -catenin (1:1000, #2677; Cell Signaling, Danvers, MA, USA), active- β -catenin (1:1000, #4270; Cell Signaling), phosphor-Gsk-3 β (1:1000, #9322; Cell Signaling), cyclinD1 (1:1000, #2261; Epitomics, Burlingame, CA, USA), c-Myc (1:1000, #1472-1; Epitomics), p21 (1:1000, sc-126; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p27 (1:1000, sc-393380; Santa Cruz), E-Cadherin (1:1000, #14472; Cell Signaling), vimentin (1:1000, sc-59533; Santa Cruz), N-Cadherin (1:1000, #14215; Cell Signaling), CD44 (1:1000, #4041; Cell Signaling), STAT3 (1:1000, #8204; Cell Signaling), MMP-7 (1:1000, ab207299; Abcam), MMP-11 (1:2000, ab52904; Abcam), MMP-14 (1:2000, ab51074; Abcam), cleaved-caspase 3 (1:1000, #9665; Cell Signaling), cleaved-caspase 7 (1:1000, #9491; Cell Signaling), cleaved-PARP (1:1000, #7851; Cell Signaling) and β -actin (1:1000, LK-ab008-100; Liankebio, China) as a control. Protein blots were analyzed using an enhanced chemiluminescence kit (ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA) and visualized with a Las-4000 Imaging System (Medical Systems, Fujifilm Global, Tokyo, Japan).

Immunohistochemistry staining

Immunohistochemistry was performed on paraffin sections of transplanted tumor tissues from nude mice, using anti-*DKK2* (1:200, ab38594; Abcam) and anti-Ki67 (1:200, ab15580; Abcam) antibodies. In brief, the tissue samples were sectioned at 4 μm and then dewaxed, rinsed in absolute xylene and rehydrated through graded alcohol. Antigen retrieval was performed as previously described [25]. The section samples were incubated in 3% hydrogen peroxide to block endogenous peroxidase activity, washed three times with PBS and then blocked with 5% FBS-PBS solution at room temperature for 15 min. The sections were incubated with the primary antibody at 4°C overnight. The next day, the sections were incubated with secondary antibody (1:1000 dilution) for 60 min at room temperature, followed by color development with diaminobenzidine and counterstaining with hematoxylin (HE). Images were captured under a microscope at 400 $\times$ magnification.

Statistical analysis

SPSS 16.0 statistical software (SPSS Inc. Chicago, IL, USA) was employed for statistical analyses. All data were expressed as means $\pm$ standard deviation (SD). Data were analyzed using Student's t-test and the chi-square test as appropriate. Differences were considered statistically significant when $p < 0.05$.

Results

DKK2 is frequently downregulated in CRC tumor tissues and cell lines

To examine the relative expression levels of *DKK2* in CRC specimens, analysis of *DKK2* mRNA expression was performed using an online cancer microarray database. Data from Oncomine (Compendia Bioscience, Ann Arbor, MI) and TCGA colorectal statistics (<https://>

Fig. 1. Expression and methylation status of *DKK2* in CRC. (A) *DKK2* expression (median expression intensity) in CRC from the Oncomine database (<https://www.oncomine.org/>). CRC: Colon Adenocarcinoma. (B) *DKK2* gene copy number (median expression intensity) in CRC from the Oncomine database. MCC: Colon Mucinous Adenocarcinoma. (C) *DKK2* expression by semi-quantitative PCR and methylation status of the *DKK2* promoter by MSP in a panel of human colon cancer cell lines and normal colon tissue. GAPDH was used as control. -ve: negative control; M: methylated; U: unmethylated. (D) Pharmacological demethylation restored expression of *DKK2*. HCT116, HT-29 and SW480 cells were treated with Aza combined with TSA (A+T). *DKK2* expression by semi-quantitative PCR and methylation status of the *DKK2* promoter by MSP in HCT116 and HT-29 cell lines after A+T treatment. GAPDH was used as control.

Fig. 2. *DKK2* is frequently methylated in primary colon tumors. (A) Promoter methylation of paired colon tumor and surgical margin tissues was detected by MSP. -ve: negative control; +ve: positive control. T: Tumor tissue; P: Paired normal tumor-adjacent tissue. M: methylated; U: unmethylated. (B) Representative analysis of methylation of the *DKK2* promoter in colon tissues, with HK1 and HONE1 as positive controls. M, methylated; U, unmethylated.

tcga-data.nci.nih.gov/tcga/) showed that *DKK2* expression was downregulated in CRC samples compared to normal colon tissues (Fig. 1A). Gene copy number also decreased in colon adenocarcinoma and in colon mucinous adenocarcinoma (MCC; Fig. 1B). In addition,

Fig. 3. Ectopic expression of *DKK2* inhibited cell proliferation in HCT116 and HT-29 cell lines. (A) Expression of *DKK2* in vector- and *DKK2*-infected cells by RT-PCR. (B) MTS assay for cellular proliferation of vector- and *DKK2*-infected tumor cell lines. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. (C) Representative colony formation assay of *DKK2*-expressing HCT116 and HT-29 cell lines. (D) Quantitative analysis of colonies; the number of colonies in control vector samples was set to 100%. (E) qRT-PCR analysis of p21 and *CCND1* mRNA expression. * $p < 0.05$, *** $p < 0.001$. (F) Western blot analysis of cell cycle-related protein expression showing decreased expression of *CCND1* and upregulation of p21 and p27 proteins, with β -actin as control.

we used RT-PCR to examine mRNA expression of *DDK2* in CRC cell lines and normal colon tissues. *DDK2* was absent or downregulated in nearly all of the CRC cell lines, but was strongly expressed in normal colon tissue (Fig. 1C).

DDK2 downregulation is mediated by promoter methylation in colon cancer

Abnormal promoter methylation is an important mechanism responsible for the silencing of many TSGs in carcinogenesis [26]. Our initial report demonstrated that typical CpG islands were present in the promoter region of *DDK2* using CpG island analysis software (CpGPlot/CpGReport/Isochore, provided by the European EBI) [27]. We used MSP to analyze *DDK2* promoter methylation status in seven colon tumor cell lines. Methylation of the *DDK2* promoter was observed in all of these cell lines, which was consistent with the low expression or silencing of *DDK2* (Fig. 1C). To determine if *DDK2* expression was regulated by promoter methylation, we treated three colon cancer cell lines (HCT116, HT-29 and SW480) with demethylation drugs Aza and TSA and then performed RT-PCR and MSP analysis. Results showed that pharmaceutical demethylation restored *DDK2* expression in all three cell lines, along with an increase in unmethylated promoter alleles in HT-29 cell

Fig. 4. Ectopic expression of *DDK2* induced cell cycle arrest at G1. Left: Representative distribution of cell cycles in HCT116 and HT-29 cell lines. Right: Summary of flow cytometry data. Values are expressed as means $\pm$ SD from three experiments and the asterisks indicate statistical significance compared to controls. *** $p < 0.001$.

lines (Fig. 1D). Thus, silencing of *DDK2* expression in CRC was at least partially caused by methylation of its promoter. Data taken from an online database (<http://methhc.mbc.nctu.edu.tw/php/index.php>) showed that downregulation of *DDK2* expression was inversely correlated with its promoter CpG methylation status (for online suppl. material, see www.karger.com/doi/10.1159/000471861). We also investigated *DDK2* promoter methylation in paired colon tumor tissues. We observed that hypermethylation of the *DDK2* promoter commonly occurred in nearly all primary colon tumors, and also found significantly lower methylation in paired tumor-adjacent tissues, but not in normal colon tissues (0/10; Fig. 2A, 2B). However, there was no correlation between *DDK2* promoter methylation and clinicopathological characteristics (data not shown).

DDK2 suppresses tumor cell growth and viability in colorectal cancer cell lines

To ascertain whether *DDK2* is a functional TSG in CRC, we used MTS and colony formation assays to examine cell viability and proliferation after stable ectopic expression of *DDK2* and empty vector viruses in HCT116 and HT-29 cell lines. Ectopic expression of *DDK2* in HCT116 and HT-29 cell lines was confirmed by RT-PCR (Fig. 3A). MTS cell viability assays showed that ectopic expression of *DDK2* in HCT116 and HT-29 cell lines resulted in a significant decrease in cell viability at 24, 48 and 72 h post-infection ($p < 0.05$, Fig. 3B). Furthermore, in colony formation assays we found that the colonies formed after *DDK2* expression in HCT116 and HT-29 cell lines were significantly fewer and smaller than those formed after infection with the empty vector control (reduced to 40%–80% of empty control, $p < 0.001$, Fig. 3C, 3D), indicating that *DDK2* suppressed the proliferation and tumorigenicity of CRC cell lines. In addition, qRT-PCR showed that mRNA expression of cell cycle-related *CCND1* decreased while p21 expression increased (Fig. 3E). Western blots further verified that overexpression of *DDK2* decreased the expression of *CCND1* and upregulated p21 and p27 proteins (Fig. 3F).

DDK2 causes cell cycle arrest in G0/G1 phase and induces apoptosis

Flow cytometric analyses of cell cycle and apoptosis were performed to confirm the mechanisms underlying *DDK2* suppression of cell growth. After 48 h of infection, *DDK2* significantly increased the percentage of HCT116 and HT-29 cells in G0-G1 phase by 10%–20% compared to controls ($p < 0.001$, Fig. 4). These results suggested that *DDK2* inhibition of cell proliferation was likely mediated by inducing cell cycle arrest at G0/G1.

AO/EB staining was performed to detect apoptotic changes and showed an increase in the number of cell nuclei showing concentrated orange fluorescence in cells infected with *DDK2*, indicating late apoptotic cells ($p < 0.01$, Fig. 5A, 5B). Furthermore, we also evaluated the distribution of apoptosis induced by *DDK2* using double staining with Annexin

Fig. 5. Detection of apoptosis induced by *DKK2*. (A) Apoptosis was examined using AO/EB fluorescence staining. (B) Results of AO/EB staining are presented as means $\pm$ SD; statistical significance was assessed by Student's t-test. ** $p < 0.01$. (C) Relative cell apoptosis rate was detected by flow cytometric analysis with AnnexinV-FITC and PI double staining. (D) Quantitative analysis of flow cytometry assays. ** $p < 0.01$. (E) Cleaved-caspase3 and cleaved-caspase7 levels were evaluated in vector- and *DKK2*-infected cells by western blot. β -actin was used as an internal control.

V-FITC/PI and found that 20%–40% of cells stained positive for Annexin V ($p < 0.01$, Fig. 5C, 5D), consistent with the AO/EB staining results. Caspases involved in the proteolytic cascade need to work together to execute apoptosis [28]. Western blots showed that *DKK2* significantly promoted the generation of cleaved-caspase 3 and cleaved-caspase 7 in both cell lines compared with the vector controls (Fig. 5E). Overall, these analyses indicated that the inhibition of cell proliferation by *DKK2* was most likely mediated by inducing G0/G1 cell cycle arrest and apoptosis.

DKK2 inhibits migration of colon tumor cells and epithelial- mesenchymal transition (EMT)

To investigate whether ectopic expression of *DKK2* altered cell migration, we first tested the effects of *DKK2* on CRC cell migration through monolayer wound-healing scratch assays. Results showed that compared with cells infected with the empty control vector, ectopic expression of *DKK2* in cell lines delayed the closure of wound gaps as observed at 12 h intervals ($p < 0.05$, Fig. 6A). Furthermore, to quantitatively assess cell migration, transwell chamber assays showed that the number of migrating cells with ectopic expression of *DKK2* was obviously reduced compared with control cells ($p < 0.01$, Fig. 6B). These results suggested that *DKK2* indeed possesses the ability to inhibit migration of colon cancer cells.

In CRC, the EMT program affects a series of malignant phenotypes related to metastasis, including the generation of colon stem cells (CSCs), circulating tumor cells, tumor budding and drug resistance [29]. In the present study, qRT-PCR demonstrated that the expression

Fig. 6. Ectopic expression of *DKK2* inhibited cell motility in HCT116 and HT-29 cells. (A) Migration of empty vector- or *DKK2*-infected tumor cells in scratch wound healing assays. Left: microscopic observations were recorded 0, 36 and 72 h after scratching the cell surface. Original magnification $\times 100$. Right panel displays quantitative analysis of migration length of vector- and *DKK2*-infected HCT116 and HT-29 cells, shown as means $\pm$ SD. $^{**}p < 0.01$, $^{*}p < 0.05$. (B) Representative images of transwell migration assays. The pictures were taken 48 h after seeding (magnification $\times 200$). The number of cells was counted in five representative high power fields per transwell (left). Right panel shows quantitative analysis of the number of migrating cells. $^{**}p < 0.01$, $^{***}p < 0.001$. (C) *DKK2* regulated partial expression of EMT markers. Western blot shows expression of vimentin and E-cadherin in control vector- and *DKK2*-overexpressing cells; β -actin was used as a loading control. (D) Relative mRNA expression by qRT-PCR of E-cadherin, vimentin, N-cadherin and twist in HCT116 and HT-29 cells stably overexpressing *DKK2* or control vector. Expression levels were normalized to β -actin expression. $^{***}p < 0.001$. (E) RT-PCR showing representative colon stem cell markers in *DKK2*-infected HCT116 and HT-29 cells. *Indicates significantly downregulated bands. (F) Protein expression of STAT3 and CD44 in vector- and *DKK2*-infected HCT116 and HT-29 cells detected by western blot. $^{**}p < 0.01$.

of epithelial marker E-cadherin was upregulated by *DKK2* overexpression, while vimentin, N-cadherin and twist were repressed ($p < 0.001$, Fig. 6C). In addition, western blot analysis indicated that E-cadherin was increased while vimentin and N-cadherin were decreased in *DKK2*-overexpressing cell lines (Fig. 6D), indicating that *DKK2* was a negative regulator of EMT. Since EMT has been implicated as a key factor regulating the generation of CSCs [29, 30], we further examined the expression of stem cell markers in *DKK2*-infected colon cancer cells. *DKK2* downregulated stem cell markers in HCT116 and HT-29 cell lines (Fig. 6E, 6F), and thus may play a role in reversing the stem cell-like phenotype of tumor cells.

DKK2 inhibits xenograft tumor growth in nude mice

Given its anti-proliferative activity and ability to inhibit EMT *in vitro*, we further explored whether *DKK2* could alter growth and EMT of CRC cells in nude mice *in vivo*. Tumors were excised from nude mice 23 days after injection, as shown in Figure 7. Tumor size and mean tumor weight was significantly lower in test mice compared with control (Fig. 7A, 7B, 7C). Immunohistochemistry was performed to analyze the expression of *DKK2* protein, as well as cell proliferation-associated protein Ki-67 expression (Fig. 7D). Ki-67 expression was significantly reduced in tumors expressing *DKK2* compared with control (Fig. 7D). In addition, the proliferation of *DKK2*-infected xenografts was suppressed due to escalating apoptosis, as the levels of cleaved poly ADP-ribose polymerase (PARP) and cleaved caspase-3

Fig. 7. DKK2 inhibited the tumorigenicity of colon cancer *in vivo*. (A) Tumors derived from vector- and DKK2-expressing HCT116 cells in nude mice. (B) Tumor weight of DKK2-expressing cells in nude mice compared with control tumors. * $p < 0.05$. (C) Tumor growth curve for DKK2-expressing tumors compared to vector control tumors in nude mice. (D) Representative images of HE staining and immunohistochemical analysis of the expression of DKK2 and Ki67 in tumors from nude mice (magnification $\times 400$). (E) Detection of apoptosis and EMT-related markers in vector- and DKK2-infected xenografts. (F) Relative protein levels of apoptosis and EMT-related markers in vector- and DKK2-infected xenografts. *** $p < 0.001$.

were significantly higher than the control group (Fig. 7E, 7F), indicating that DKK2 acted as a TSG in CRC tumorigenesis *in vivo*. Meanwhile, western blot analysis showed that E-cadherin was increased, while vimentin and N-cadherin expression decreased in DKK2-infected xenografts (Figure 7E, 7F), suggesting that DKK2 was a negative regulator of EMT *in vivo*.

DKK2 antagonizes the canonical Wnt/ β -catenin signaling pathway

We further analyzed whether the mechanism underlying the tumor-suppressive function of DKK2 was related to Wnt signaling, since Wnt/ β -catenin signaling plays an important role in regulating proliferation and EMT in cancer [31, 32]. Western blots were performed to determine if DKK2 neutralized Wnt/ β -catenin signaling. We found that in HCT116 and HT-29 cell lines, DKK2 expression significantly inhibited the expression of active β -catenin without change in total β -catenin (Fig. 8). β -catenin plays a key role in activating the transcription factor T-cell factor/lymphocyte enhancer factor (TCF/LEF) [33]. However, in our study, the

Fig. 8. Ectopic expression of *DKK2* disrupted Wnt signaling in HCT116 and HT-29 cell lines. Western blot was performed using antibodies against total β -catenin, active β -catenin and its downstream targets; β -actin was used as a control.

levels of active β -catenin as well as GSK-3 β phosphorylation were clearly reduced in *DKK2*-expressing HCT116 and HT-29 cell lines compared with control cells. As a result, the β -catenin downstream target genes c-Myc, CCND1, MMP7, MMP11 and MMP14 were also downregulated by *DKK2* expression (Fig. 8). In summary, these findings suggested that *DKK2* may regulate CRC progression, at least partly, by antagonizing Wnt/ β -catenin signaling through reduction of β -catenin/TCF-mediated transcriptional activity.

Discussion

Previous studies identified *DKK2* as a functional tumor suppressor in multiple cancers which is silenced by promoter methylation [17, 18]. The direct relationship between the function of *DKK2* and its related mechanisms in CRC remains unclear. In this study, we found that *DKK2* was expressed in normal colon tissues, but was frequently absent or downregulated in colon cancer cell lines. The reduced expression was associated with promoter methylation, and demethylation agents were able to restore *DKK2* expression in epigenetically-silenced colon cancer cells. Other studies have confirmed that hypermethylation of *DKK2* is a frequent event in CRC [34, 35]. Our results suggest that *DKK2* expression is epigenetically inactivated through promoter-specific hypermethylation in colon cancer. Data from multiple online databases have also shown that expression of *DKK2* is inversely correlated with its promoter CpG methylation (<http://methhc.mbc.nctu.edu.tw>). In addition, we found that expression of *DKK2* in most colon cancer tissues is downregulated by analyzing the online Skrzypczak colorectal database, and further observed reduced *DKK2* gene copy numbers in the Kurashina Colon and TCGA Colorectal datasets. A DNA copy number variant is defined as a DNA segment that is >1 kb in size and is present at a variable copy number in comparison with a reference genome. Copy number variants can impact the organization of chromatin and gene expression. Gene copy number was also decreased in colon adenocarcinoma and MCC. Moreover, in a study of *DKK2* protein expression, Tadateru found that *DKK2* was reduced in a certain subset of cancer tissues compared to normal colon tissues, with weak or negative staining in 27/60 (45.0%) of CRC tissue sections. Taken together, the above results indicate that *DKK2* is downregulated in CRC.

We next investigated the functions of *DKK2* in two colon cancer cell lines. It was reported that the members of the *DKK* family were associated with cancer cell proliferation and other biological functions, including apoptosis and migration/ invasion in multiple tumors [17, 18]. Therefore, we focused primarily on these anti-tumor activities. We found that *DKK2* infection suppressed cell proliferation *in vitro* and increased the percentage of cells in G0/G1 phase. *In vivo*, the diminution of tumor growth by *DKK2* overexpression was also verified by xenograft tumor formation in nude mice. In addition, the number of apoptotic cells was increased after *DKK2* infection, accompanied by changes in apoptotic morphology. It has been reported that ectopic-expression of *DKK2* restrained metastasis in human ovarian carcinoma [17]. We found that migration of HCT116 and HT-29 cells was slightly restrained

after *DKK2* infection, consistent with these previous reports. These results together strongly suggest that *DKK2* functions as a tumor suppressor in CRC cells.

A number of reports have suggested that Wnt/ β -catenin signaling is frequently affected in a broad series of human malignancies. However, most of these studies described strong activation of the Wnt/ β -catenin pathway in colon cancer cell lines, resulting from gain-of-function mutations in β -catenin (HCT116, SW48, Colo205, LS174T) or loss-of-function mutations in the destruction complex component APC (SW480, HT29, DLD1, LoVo, SW620, Caco2, Colo205, SW1116, Colo320) [36, 37]. Increasing evidence has confirmed that these tumor-causative mutations lead to defective stabilization of β -catenin and loss of nuclear β -catenin/TCF-dependent translocation, and further activate downstream target genes related to cell proliferation and transformation [38]. *DKK2*, as a negative regulator of the WNT signaling pathway, can suppress WNT signaling in ovarian and renal carcinoma [17, 18]. Whether or not *DKK2* could inhibit the WNT signaling pathway in CRC was largely unknown. We found that overexpressing *DKK2* led to an obvious downregulation of Wnt/ β -catenin signaling in both HCT116 and HT-29 cell lines. Moreover, to obtain more detailed information about *DKK2* mechanisms, we found that pertinent markers of Wnt/ β -catenin downstream targeted genes, including p-GSK3 β , c-Myc, CCND1 and various MMPs were also significantly downregulated. Thus, *DKK2* antagonized Wnt/ β -catenin signaling. Whether or not *DKK2* could influence mutations of β -catenin and APC need further study.

EMT confers cells with stem-like properties, containing invasiveness with a gain of mesenchymal characteristics and a loss of epithelial characteristics [31]. Two major factors regulating tumor metastasis are tumor cell invasion and degradation of extracellular matrix by specialized proteolytic enzymes. MMPs are an important part of these proteolytic enzymes [39]. They are highly expressed in multiple malignancies, including breast cancer [40], ovarian cancer [41], lung cancer [42] and colon cancer [43]. In addition, it has been reported that blockade of the expression and activity of MMPs could be the major cause of reduced motility and metastasis of cancer cells [44]. Our research indicated that ectopic-expression of *DKK2* downregulated the expression of MMP7, MMP11 and MMP14, suggesting that *DKK2* can suppress tumor metastasis by modulating cancer tissue-remodeling enzymes. We also found that suppression of CD44 is involved in this process. CD44 is a receptor for hyaluronic acid and influences many characteristics of cancer cells, including stemness [45]. This suggests that *DKK2* could possibly act as a suppressor of colon cancer stemness and thus may play a role in the initiation of the disease, which should be further examined. Cumulatively, the aforementioned results provide details on the prospective role of *DKK2* in the regulation of Wnt/ β -catenin signaling, a mechanism that may be responsible for colorectal carcinogenesis.

Recent literature has reported that some Wnt proteins (such as Wnt4, Wnt5A and Wnt11) were not directly involved in β -catenin/TCF-mediated transcriptional regulation, but rather induced Ca²⁺ flux to block downstream signaling by inhibiting TCF-mediated transcription, and thus were actually involved in cancer metastasis [46]. Other *DKK* family members such as *DKK3* can also mediate non-canonical c-Jun N-terminal kinase (JNK) signaling, which regulates apoptotic cell death and has been well-documented [27]. In this study, we found no significant evidence in our western blot results for Wnt/ β -catenin crosstalk with either Notch or the JNK pathway (data not shown). Although we did not examine nuclear factor- κ B and other noncanonical pathways, our research nonetheless suggests that *DKK2* may suppress colon cancer progression primarily through the canonical Wnt pathway.

In summary, our research has shown that *DKK2* expression was epigenetically silenced in CRC, which affected relevant mechanisms governing proliferation, apoptosis and metastasis. These results suggest that *DKK2* functions as a tumor suppressor by antagonizing the Wnt/ β -catenin signaling pathway in colorectal tumorigenesis and progression. Thus we provide a rationale for using *DDK2* along with other TSGs as candidate biomarkers for the early detection and prognosis of colon cancer.

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Disclosure Statement

The authors declare no conflict of interest.

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