

Transcription factor forkhead box K1 regulates miR-32 expression and enhances cell proliferation in colorectal cancer

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Abstract. Increased microRNA (miR)-32 expression in colorectal cancer (CRC) tissues enhances CRC cell proliferation, migration, invasion and attenuates CRC cell apoptosis by repressing the expression of phosphatase and tensin homolog (PTEN). Forkhead box K1 (FOKK1) was identified as a potential interacting transcription factor using DNA pull-down assays and mass spectrometry. The present study aimed to elucidate the role of FOKK1 in regulating miR-32 expression in CRC. The expressions of *FOKK1*, miR-32, transmembrane protein 245 gene (*TMEM245*) and *PTEN* were compared between CRC and normal colonic tissues. Levels of miR-32, *TMEM245*, *PTEN* and the proliferation and apoptosis of CRC cells were studied using FOKK1-overexpression or knockdown, or by simultaneously interfering with FOKK1 and miR-32 expression. Direct FOKK1 binding to the miR-32 promoter was verified using chromatin immunoprecipitation (ChIP) and dual-luciferase reporter assays. The results showed elevated *FOKK1*, miR-32 and *TMEM245* expression, and significantly decreased *PTEN* expression in CRC, compared with normal colonic tissues. Correlations between the expressions of *TMEM245* and miR-32, *FOKK1* and miR-32, and *FOKK1* and *TMEM245* were positive and significant. FOKK1-knockdown led to decreased miR-32 and *TMEM245* expression and increased *PTEN* expression, whereas FOKK1-overexpression had the opposite effect. Overexpressed FOKK1 promoted the malignancy of CRC cells *in vitro* by stimulating proliferation and reducing apoptosis; whereas FOKK1-depletion suppressed such malignancy and a miR-32 inhibitor partially

reversed the effects of FOKK1. The results of ChIP and dual-luciferase reporter assays indicated that FOKK1 directly binds to the promoter of *TMEM245/miR-32*. Thus, the FOKK1-miR-32-PTEN signaling axis may play a crucial role in the pathogenesis and development of CRC.

Introduction

Colorectal cancer (CRC) is the fourth most commonly diagnosed malignant disease worldwide (1). Despite surgery, chemotherapy, targeted therapy and other options, it is estimated that colorectal cancer ranks as the fourth and fifth most common cause of cancer-associated death among women and men, respectively, in China (1). The CRC mortality rate in China increased annually between 1991 and 2011 (2). By 2012, tobacco smoking, alcohol consumption, obesity, low levels of physical activity, low fruit and vegetable intake and high intake of red and processed meat were responsible for ~46% of CRC cases and associated deaths in China (3). The development of CRC is a multistep process that includes the progressive destruction of epithelial cell proliferation, apoptosis, differentiation and survival mechanisms (4).

MicroRNAs (miRNAs) are single-stranded RNAs of ~20 nucleotides in length that can degrade and inhibit the translation of target mRNA (5). Some miRNAs function as CRC inducers or regulators and can be considered as biomarkers and therapeutic targets (5). miRNAs are regulated at multiple levels, including transcription, Drosha and Dicer processing, loading onto Argonaute proteins and miRNA turnover (6). Each level is mediated by multiple factors, including transcription factors, RNA-binding proteins, protein-modifying enzymes, RNA-modifying enzymes, exoribonucleases and endoribonucleases (6). Intronic miR-32 is located within intron 14 of the transmembrane protein 245 (*TMEM245*) gene.

We previously found that miR-32 is upregulated in CRC tissues and that high miR-32 levels are closely associated with lymph-node and distant metastases (7). Overall, survival rates are low among patients with CRC that express abundant miR-32 (7). miR-32-overexpression in CRC cells results in enhanced proliferation, migration, invasion and reduced apoptosis through the repression of phosphatase and tensin homolog (PTEN) expression (8). The mechanisms of miR-32 upregulation in CRC were explored in more detail and luciferase reporter assays revealed that miR-32 binds to

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the 3'-untranslated region of PTEN (8). The promoter region (~2 kb) of *TMEM245/miR-32* was cut into fragments of different lengths, cloned into the 5' end of a luciferase reporter vector and then transfected into CRC cells. The results of the dual-luciferase reporter assays suggested that the core promoter region is located within -320 to -1 bp of the 5'-flanking region. Protein binding to the core promoter was analyzed using DNA pull-down assays and mass spectrometry. Transcription factor (TF) analyses identified forkhead box K1 (FOXK1) as a potentially interactive TF (9).

Forkhead box K1 is involved in tumorigenesis and cancer development. It promotes ovarian cancer cell proliferation and metastasis (10), esophageal cancer cell proliferation and migration, inhibits apoptosis and is associated with poor differentiation and prognosis (11). Wu *et al.* (12) found that FOXK1 induces the epithelial-mesenchymal transition and facilitates CRC cell invasion *in vitro* and *in vivo*, and that enhanced FOXK1 expression indicates a poor prognosis in patients with CRC.

The present study aimed to provide novel insights into the molecular mechanisms underlying CRC by defining FOXK1 expression in CRC tissues and investigating the relationship between FOXK1 and miR-32 expression. In addition, the study aimed to clarify the role of FOXK1 in miR-32 regulation and CRC cell proliferation and apoptosis.

Materials and methods

Patients and specimens. In total, 35 primary CRC and 31 non-cancerous colonic tissue samples were obtained from 66 patients that had been treated by surgical resection or colonoscopy at the Affiliated Hospital of Guangdong Medical University (Guangdong, China) between July 2017 and September 2019. All the patients were Han Chinese. The mean age of CRC patients was 59±13 years old (range, 28-84 years; 25 males, 10 females). Patients with newly diagnosed CRC and >18 years old were included. The patients declined participation, as well as had received either radiotherapy or chemotherapy before surgery or colonoscopy were excluded. The study protocol was approved by The Medical Ethics Committee at the Affiliated Hospital of Guangdong Medical University and written informed consent was obtained from all participants. The diagnoses of the samples were verified by pathologists who were independent from the present study. Data on clinicopathological characteristics, including sex, age, tumor diameter, differentiation, lymphatic and distant metastasis and staging were collected. Median *FOXK1* expression (0.00492) served as the cut-off for separating the 35 patients with CRC into groups with high (n=18) and low (n=17) *FOXK1* expression. After collection tissues were immediately frozen in liquid nitrogen and stored at -80°C until use.

Cell transfection. HCT-116 and HT-29 CRC cells were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. The HT-29 cell line was authenticated by STR profiling. The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C under a 5% CO₂ atmosphere. No antibiotics were used for cell culture. To generate the FOXK1 expression plasmid, the human FOXK1 coding sequence was amplified using PCR, as

later described. The PCR product was cloned and inserted into a pcDNA3.1 vector (Promega Corp.). The inserted fragment was confirmed by sequencing. The FOXK1-overexpressing plasmid, pcDNA3.1-FOXK1, was synthesized at Guangzhou RiboBio Co., Ltd. The pcDNA3.1 empty vector was used as the negative control (NC) of pcDNA3.1-FOXK1. For plasmid/siRNA/mimic/inhibitor transfection, cells were seeded at a density of 3x10⁵ cells per well in 6-well plates, or 1x10⁵ cells per well in 12-well plates, or 5x10⁴ cells per well in 24-well plates, or 6x10³ cells per well in 96-well plates. Twenty-four hours later, pcDNA3.1-FOXK1 (2.0 µg/ml), pcDNA3.1 (2.0 µg/ml), FOXK1 small interfering (si)RNA (siFOXK1) (150 nM), siRNA-NC (150 nM), miR-32 mimic (200 nM), mimic-NC (200 nM), miR-32 inhibitor (200 nM) and inhibitor-NC (200 nM) (all RiboBio Co., Ltd.) were transfected as described by the manufacturer into HCT-116 and HT-29 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in Opti-MEM™ I medium (Gibco; Thermo Fisher Scientific, Inc.). Cells were harvested 48 h after transfection for RNA and protein detection, dual-luciferase reporter assay and apoptosis assay, and 24, 48, 72 and 96 h for CCK-8 assays, respectively. The siRNA-NC (cat. no. siN0000001-1-5), mimic-NC (cat. no. miR1N0000001-1-5), inhibitor-NC (cat. no. miR2N0000001-1-5) were non-targeting and designed and synthesized by RiboBio Co., Ltd. The following siRNA sequence was used: siFOXK1, 5'-CCAGTTCACGTCGCTCTAT-3'. The sequences of miR-32 mimic and inhibitor were as follows: miR-32 mimic, sense: 5'-UAUUGCACAUUACUAAGUUGCA-3', and antisense: 5'-UGCAACUUAGUAAUGUGCAAUA-3'; mimic-NC, sense: 5'-UUUGUACUACACAAAAGUACUG-3', and antisense: 5'-CAGUACUUUUGUGUAGUACAAA-3'; miR-32, inhibitor: 5'-UGCAACUUAGUAAUGUGCAAUA-3' and inhibitor-NC: 5'-CAGUACUUUUGUGUAGUACAAA-3'.

Reverse-transcription quantitative PCR (RT-qPCR). Levels of *FOXK1*, *TMEM245* and miR-32 expression in tissues and transfected cells were measured using RT-qPCR. Total RNA was extracted from tissues and transfected cells using RNAiso Plus (Takara Bio, Inc.) that was then reverse transcribed using PrimeScript™ RT Master mix (Perfect Real Time; Takara Bio, Inc.) for *FOXK1* and *TMEM245* and Mir-X™ miRNA First-Strand Synthesis kits (Clontech Laboratories, Inc.) for miR-32 as described by the manufacturers. The endogenous controls were β-actin mRNA for *FOXK1* and *TMEM245*, and U6 small-nuclear RNA for miR-32. Quantitative PCR was performed using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus; Takara Bio, Inc.) and a LightCycler® 480 II system (Roche Diagnostics) under a cycling program comprising of 30 sec at 95°C for 1 cycle, then 5 sec at 95°C and 20 sec at 60°C for a total of 40 cycles. Table I lists the qPCR primers. The mRQ 3'-primer was included in the Mir-X™ miRNA First-Strand Synthesis kit, but sequence information of this primer was unavailable. Relative levels of miR-32 and *FOXK1* and *TMEM245* mRNA were determined using the 2^{-ΔΔCq} method (13).

Western blotting. Cells transfected with pcDNA3.1-FOXK1/siFOXK1/pcDNA3.1-FOXK1 + miR-32 inhibitor/siFOXK1+miR-32 mimic were incubated at 37°C for

Table I. Sequence information for the primers used in the quantitative PCR assay.

Gene	Forward primer, 5'-3'	Reverse primer, 5'-3'
<i>miR-32</i>	CGCGCTATTGCACATTACTAAGTTGC	mRQ 3' primer
<i>U6</i>	CTCGCTTCGGCAGCACCA	mRQ 3' primer
<i>FOXK1</i>	TTCCAGGAGCCGCACTTCTA	GGAAGGTACACTGCTTGGGC
<i>TMEM245</i>	GACATTCTGGACTGGCAGGA	AGTGGTGAACAGCAGGCTCA
<i>PTEN</i>	AAAGGGACGAACTGGTGTAAATG	TGGTCCTTACTTCCCCATAGAA
<i>β-actin</i>	GCGGCAACACCATGTACCCT	AGGGGCCGGACTCGTCATACT

miR, microRNA; FOXK1, forkhead box K1; TMEM245, transmembrane protein 245; PTEN, phosphatase and tensin homolog.

48 h and then were digested and total protein was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology), and quantified using BCA protein assay kits (Genstar Technologies Co., Inc.). In total, 30 μg protein was loaded per lane, electrophoresed and separated using 12% SDS-PAGE and was then transferred to polyvinylidene difluoride membranes (MilliporeSigma; Merck KGaA). Non-specific protein binding was blocked by incubating the membranes with skimmed milk at room temperature for 1 h, then the membranes were incubated overnight at 4°C with anti-PTEN (1:1,000; cat. no. 9559S; Cell Signaling Technology, Inc.), anti-FOXK1 (1:1,000; cat. no. ab18196; Abcam) and anti-GAPDH (1:1,000; cat. no. AG019; Beyotime Institute of Biotechnology) antibodies. The membranes were then incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1:1,000; cat. no. A0208; Beyotime Institute of Biotechnology) or horseradish peroxidase-labeled goat anti-mouse IgG (1:1,000; cat. no. A0216; Beyotime Institute of Biotechnology) secondary antibody for 1 h at room temperature, washed three times with TBST, then signals were detected using MilliporeSigma™ Immobilon™ Western Chemiluminescent HRP Substrate (MilliporeSigma; Merck KGaA). Images were acquired using an Azure c600 system (Azure Biosystems, Inc.). Protein bands were quantified using Quantity One software (version 4.6.2; Bio-Rad Laboratories, Inc.). All experiments were repeated at least three times.

Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR). JASPAR (<http://jaspar.genereg.net/>) and HOCOMOCO (<https://hocomoco11.autosome.ru/>) bioinformatics analysis (performed by Guangzhou RiboBio Co., Ltd.) was used to identify the binding sites of FOXK1 to the *TMEM245/miR-32* promoter. Two putative FOXK1-binding sites were located within -320 to -1 bp of the *TMEM245/miR-32* promoter region at -90 to -77 bp (site one) and -166 to -153 (site two) bp. The ChIP assay was carried out with the Pierce Agarose ChIP Kit (cat. no. 26156; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Cells were cross-linked with 1% paraformaldehyde and genomic DNA was digested to an average size of 100-1,000 bp. Total sheared chromatin was incubated with FOXK1 antibody (5 μg; cat. no. ab18196; Abcam) or normal rabbit IgG (2 μl; cat. no. 2729S; Cell Signaling Technology, Inc.) at 4°C overnight. The NC was non-specific IgG. Immunoprecipitated DNA was analyzed using qPCR on a LightCycler® 480 II system. The

FOXK1-binding sites one and two in the *TMEM245/miR-32* promoter region were amplified using the respective primers: Site one, 5'-TTCCCCATTTCCCCTTC-3' and 5'-CGAGAT TGTGGGAGTTGTAG-3'; and site two, 5'-CCTCCAGGA AGATATAGACCC-3' and 5'-TCCCGAAGGGGAAATG-3'. The primers were synthesized by Sangon Biotech Co., Ltd. Target enrichment was expressed as % input according to the formula: Fold enrichment = $2^{-\Delta\Delta Cq(\text{ChIP/IgG})}$ (14).

Dual-luciferase reporter assays. HCT-116 cells were cultured in 24-well plates (5x10⁴ cells per well), and were transfected with pGL3-basic (Promega Corporation) luciferase reporter constructs harboring wild-type (WT) or mutant (MUT) *TMEM245/miR-32* promoter target sequences to evaluate the binding potential of FOXK1 to this promoter. The DNA fragments containing binding sites one and two were amplified and subcloned into vector pGL3-basic to create the luciferase pGL3-promoter-WT and the mutated pGL3 reporters: MUT1, MUT2 And MUT1+2, which were mutated at binding sites one, two or both, respectively (Fig. 1). All WT and MUT plasmids were synthesized by RiboBio Co, Ltd. The pRL-TK (Promega Corporation) plasmid was used as an internal control to standardize by Renilla luciferase activity. Plasmid pGL3-promoter-WT, MUT1, MUT2 or MUT1+2 and pcDNA 3.1-FOXK1 or pcDNA 3.1 vector and pRL-TK were co-transfected into HCT-116 cells in 24-well plates (5x10⁴ cells per well) at 37°C for 48 h. Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect cells with the plasmids following the manufacturer's instructions. Luciferase activities were measured using the Dual-Luciferase Reporter Assay system (Promega Corp.). Firefly luciferase activity was normalized to that of Renilla luciferase.

Cell Counting Kit-8 (CCK-8) assay. Cell proliferation was assessed using CCK-8 assays (Dojindo Molecular Technologies, Inc.). HCT-116 or HT-29 cells (6x10³ cells per well) were seeded into 96-well plates for 24 h, transfected, then incubated for 24, 48, 72 and 96 h. The cells were incubated with CCK-8 reagent for 1 h at 37°C, then absorbance at 450 nm was determined by spectrophotometry.

Apoptosis assays. Apoptosis was assayed using Annexin V-FITC Apoptosis Detection kit (Dojindo Molecular Technologies, Inc.). At 48 h after transfection, CRC cells were digested, resuspended in Annexin V Binding Solution,

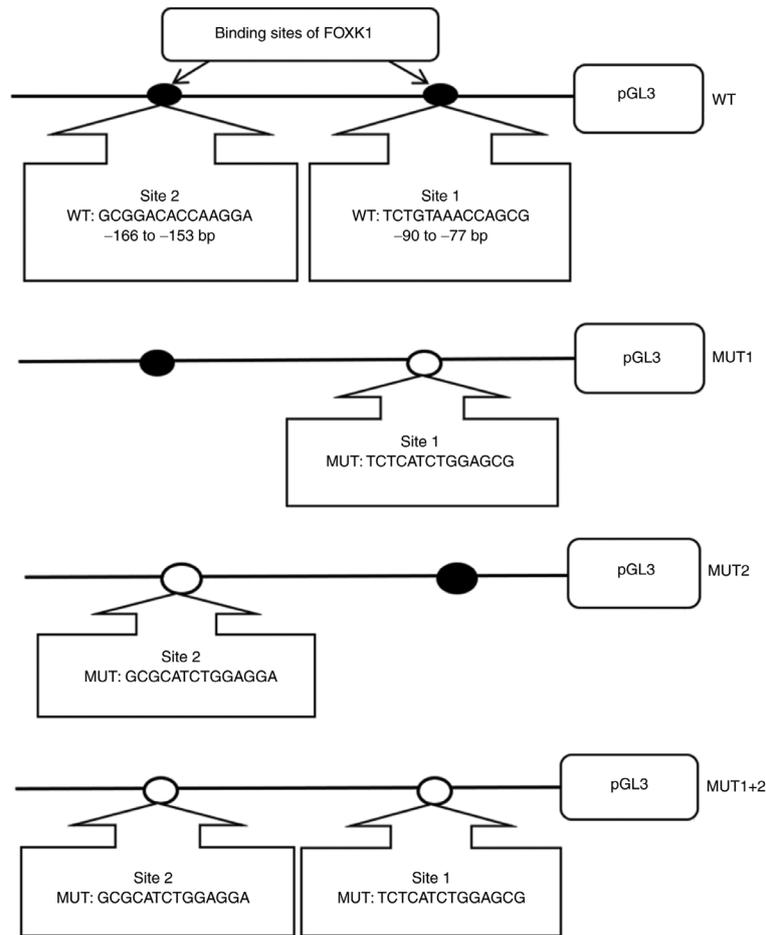


Figure 1. Schematic diagram of wild-type transmembrane protein 245/microRNA-32 promoter along with the three mutants of the predicted binding sites in the promoter. WT, wild-type; MUT, mutant; FOXK1, forkhead box K1.

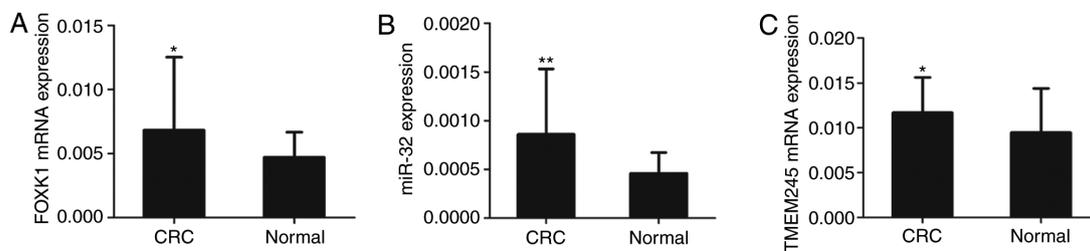


Figure 2. Levels of *FOXK1* mRNA, *TMEM245* mRNA and miR-32 are upregulated in CRC. Expression of (A) *FOXK1* mRNA, (B) miR-32 and (C) *TMEM245* mRNA analyzed using reverse-transcription quantitative PCR using specific primers in CRC and normal colonic tissues. * $P < 0.05$ and ** $P < 0.01$ vs normal. FOXK1, forkhead box K1; TMEM245, transmembrane protein 245; miR, microRNA; CRC, colorectal cancer.

mixed with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide as described by the manufacturer instructions, and then analyzed by flow cytometry using a FACScan (BD Biosciences). The ratios (%) of Annexin V-FITC-positive cells identified by flow cytometry using FACSDiva software (version 6.1.3; BD Biosciences) represented the apoptotic population.

Statistical analysis. All data were statistically analyzed using SPSS v19.0 software (IBM Corp.). Data are presented as means \pm standard deviation. Two groups were compared using unpaired Student's t-tests. Associations between clinical and pathological features with *FOXK1* expression was analyzed

with Fisher's exact test. Correlations between *TMEM245* and miR-32, *FOXK1* and miR-32 and *FOXK1* and *TMEM245* expression were determined using Pearson's correlation coefficients. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of *FOXK1*, *TMEM245* and miR-32 in CRC tissues. Levels of miR-32, *FOXK1* and *TMEM245* mRNA expression in CRC and normal colonic tissues were determined using RT-qPCR. The results showed that the expressions of *FOXK1* (Fig. 2A), miR-32 (Fig. 2B) and *TMEM245* (Fig. 2C) were

Table II. *FOXK1* expression and clinicopathological features of the patients with colorectal cancer.

Clinicopathological features	<i>FOXK1</i> expression		P-value
	High, n=18	Low, n=17	
Age, years			0.315
<60	8	11	
≥60	10	6	
Sex			0.471
Male	14	11	
Female	4	6	
Diameter, cm			0.738
<5	9	7	
≥5	9	10	
Differentiation			0.691
High	5	3	
Middle/low	13	14	
Lymphatic metastasis			0.027 ^a
Positive	16	9	
Negative	2	8	
Distal metastasis			>0.999
Positive	2	2	
Negative	16	15	
Stage			0.027 ^a
I-II	2	8	
III-IV	16	9	

^aP<0.05 was considered to indicate a statistically significant difference. FOXK1, forkhead box K1.

upregulated in CRC compared with normal tissues (all P<0.05 or P<0.01).

The relationship between increased *FOXK1* expression and the clinicopathological features of patients with CRC was explored. Median *FOXK1* expression served as the cut-off for separating the 35 patients with CRC into groups with high (n=18) and low (n=17) *FOXK1* expression. *FOXK1* expression was significantly associated with lymphatic metastasis and tumor stage (P=0.027 for both; Table II). These results implied a potential role for FOXK1 in CRC progression.

Correlations between FOXK1, miR-32 and TMEM245 expression in CRC tissues. The expression of *TMEM245* mRNA and miR-32 was significantly and positively correlated (r=0.453; P<0.05; Fig. 3A). Increased *FOXK1* mRNA expression was positively correlated with upregulated miR-32 and *TMEM245* mRNA (r=0.336 and 0.469, respectively; P<0.05; Fig. 3B and C). These results suggest that miR-32 and host gene *TMEM245* may be regulated by FOXK1.

Expression of miR-32, TMEM245 and PTEN is modulated by FOXK1 in CRC cells. *FOXK1* was overexpressed or knocked down in CRC cells that were respectively transfected with either pcDNA3.1-FOXK1 or siFOXK1, to determine whether FOXK1 regulated miR-32, *TMEM245* and *PTEN*

expression using RT-qPCR and western blotting. The level of FOXK1 was significantly increased in HCT-116 and HT-29 cells transfected with pcDNA3.1-FOXK1 compared with control cells transfected with the empty vector pcDNA3.1 (P<0.01; Fig. 4A and B). The level of FOXK1 was significantly decreased after FOXK1-knockdown compared with control cells transfected with siRNA-NC (all P<0.05 or P<0.01; Fig. 4C and D). Fig. 4E-H shows that *FOXK1*-overexpression significantly increased miR-32 and *TMEM245* mRNA expression and suppressed PTEN protein levels compared with the control empty vector (all P<0.05 or P<0.01). In contrast, *FOXK1*-knockdown resulted in significantly decreased miR-32 and *TMEM245* mRNA and increased PTEN protein expression compared with siRNA-NC transfected cells (all P<0.05 or P<0.01; Fig. 4I-L) without significant changes in *PTEN* mRNA expression. Taken together, these results suggested that FOXK1 could enhance miR-32 and *TMEM245* expression, and suppress PTEN protein expression.

FOXK1 promotes CRC cell proliferation. The potential function of FOXK1 in CRC cell proliferation was investigated using CCK-8 assays. Overexpressed FOXK1 significantly promoted the proliferation of HCT-116 cells at 24, 48, 72 and 96 h and of HT-29 cells at 72 and 96 h after transfection (all P<0.05 or P<0.01; Fig. 5A). In contrast, *FOXK1* inhibition restricted proliferation of HCT-116 cells at 48, 72 and 96 h and of HT-29 cells at 24, 48 and 96 h after transfection (all P<0.05 or P<0.01; Fig. 5B).

FOXK1 suppresses apoptosis in CRC cells. Apoptosis was assessed using flow cytometry. Apoptosis in CRC cells was significantly decreased and increased by FOXK1-overexpression and knockdown, respectively, compared with controls (all P<0.05 or P<0.01; Fig. 6). Collectively, these results suggested that FOXK1 acts as an oncogene in CRC cells.

FOXK1 directly binds to the TMEM245/miR-32 promoter. The findings of the bioinformatics analysis using JASPAR and HOCOMOCO (RiboBio Co., Ltd.) predicted that FOXK1 could bind to the core promoter of *TMEM245/miR-32*. Putative FOXK1-binding sites one (-90 to -77 bp) and two (-166 to -153 bp) were identified within the upstream region of the *TMEM245/miR-32* gene by bioinformatics analysis. ChIP-qPCR was used to determine whether endogenous FOXK1 could directly bind to these sites in CRC cells. The results revealed that FOXK1 was strongly bound to both sites of the *TMEM245/miR-32* promoter in HCT-116 cells compared with the IgG control. Fig. 7A shows substantial enrichment of endogenous FOXK1 at both sites in the *TMEM245/miR-32* promoter, indicating that this promoter was a direct target of FOXK1.

The relative luciferase activity of pGL3-promoter-WT, -MUT1 and MUT-2 was significantly increased when co-transfected with pcDNA3.1-FOXK1 compared with pcDNA3.1 vector (P<0.05; Fig. 7B). However, there was no significant difference in relative luciferase activity between pcDNA3.1-FOXK1 and pcDNA3.1 vector when cotransfected with pGL3-promoter-MUT1+2 (P>0.05; Fig. 7B). These results indicated that FOXK1 enhanced the transcriptional activities of the *TMEM245/miR-32* promoter and the involvement of

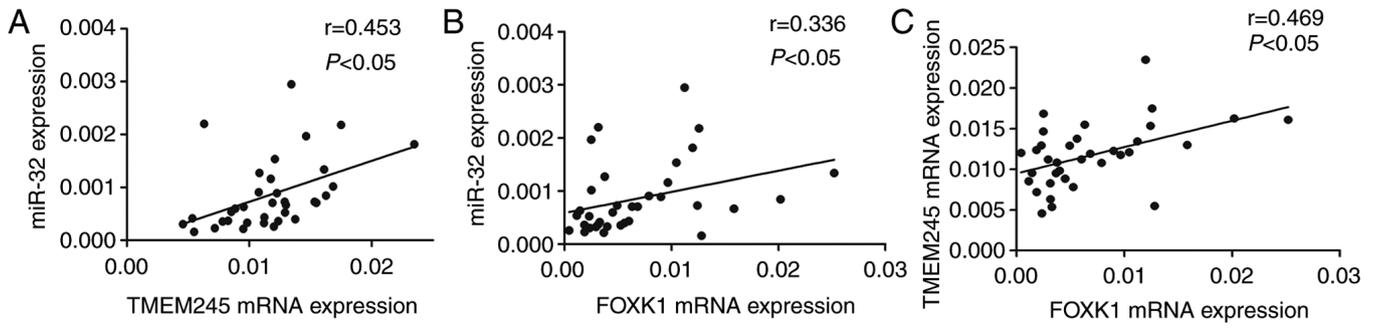


Figure 3. Correlations between miR-32, *FOXK1* and *TMEM245* mRNA levels in colorectal cancer tissues. Levels of (A) *TMEM245* and (B) *FOXK1* mRNA positively correlate with those of miR-32. (C) Levels of *FOXK1* mRNA positively correlate with those of *TMEM245* mRNA. FOXK1, forkhead box K1; TMEM245, transmembrane protein 245; miR, microRNA.

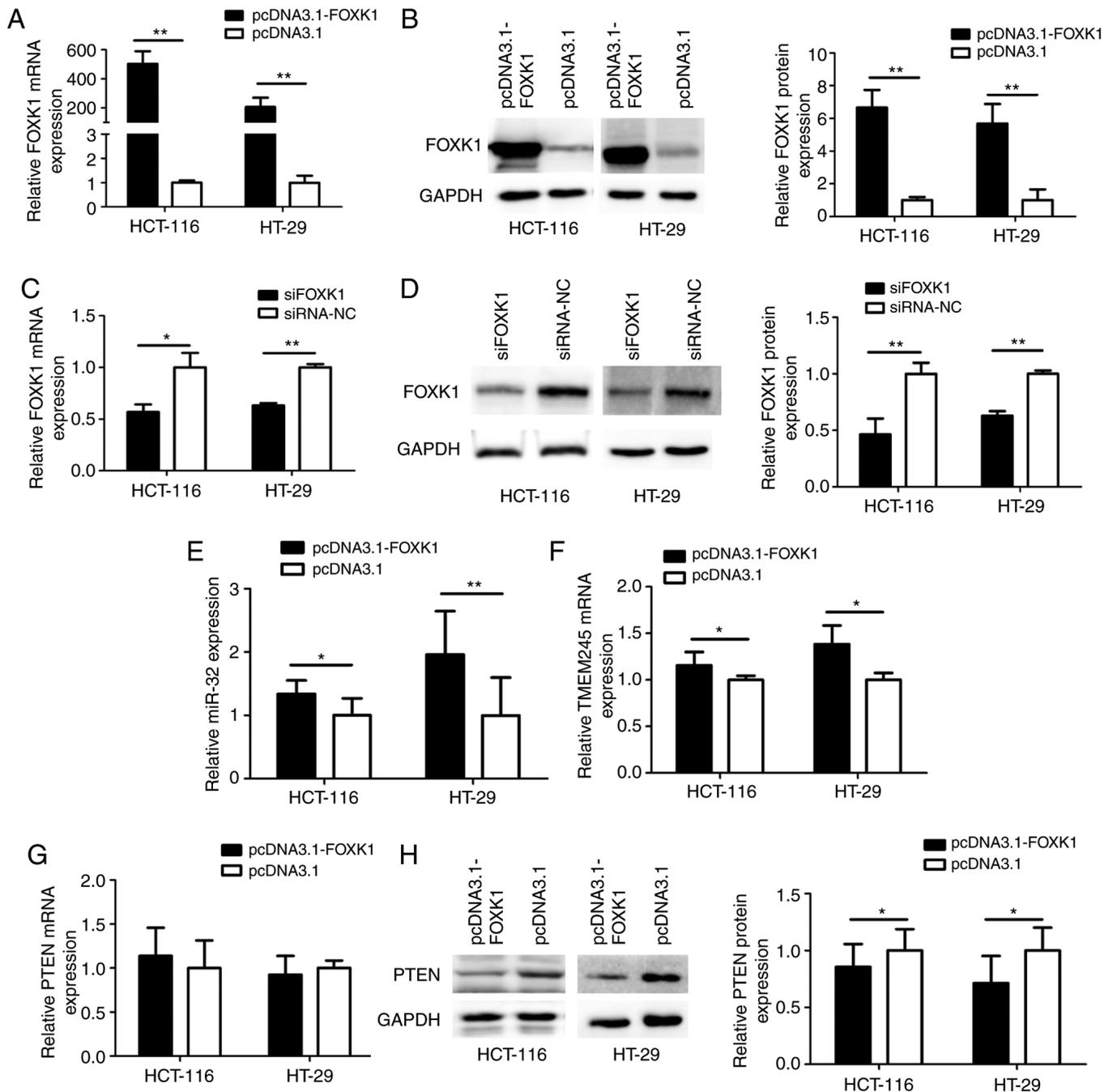


Figure 4. Continued.

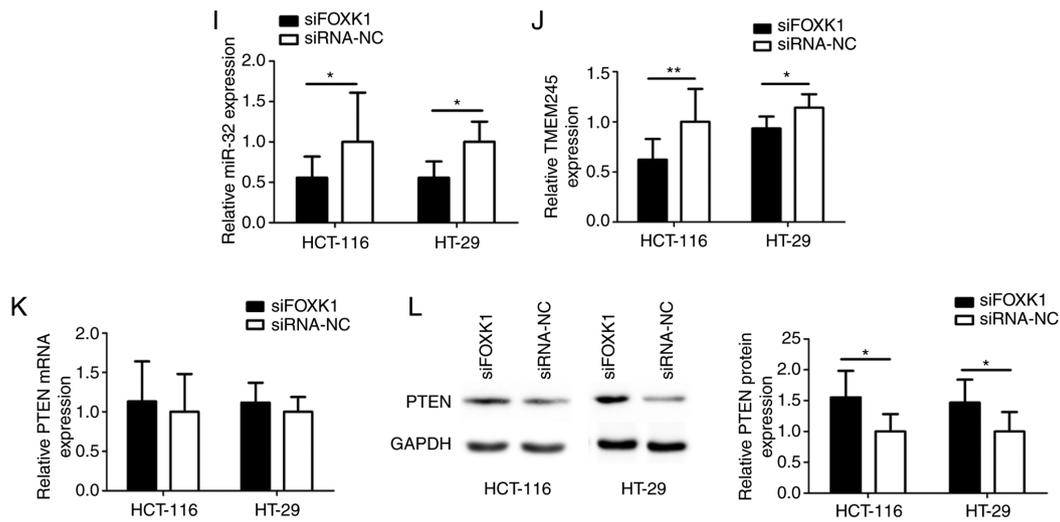


Figure 4. Expressions of miR-32, *TMEM245* and PTEN are modulated by *FOXK1* in CRC cells. mRNA and protein expression was detected using reverse-transcription quantitative PCR and western blotting, respectively. (A) Expression of *FOXK1* mRNA and (B) *FOXK1* protein in CRC cells transfected with pcDNA3.1. (C) Expression of *FOXK1* mRNA and (D) *FOXK1* protein in CRC cells transfected with siFOXK1. (E-H) Expression of miR-32, *TMEM245* and PTEN in CRC cells transfected with pcDNA3.1-*FOXK1*. (I-L) Expression of miR-32, *TMEM245* and PTEN in CRC cells with *FOXK1*-knockdown. * $P < 0.05$ and ** $P < 0.01$ vs corresponding control groups. FOXK1, forkhead box K1; *TMEM245*, transmembrane protein 245; miR, microRNA; CRC, colorectal cancer; NC, negative control; si-, short interfering.

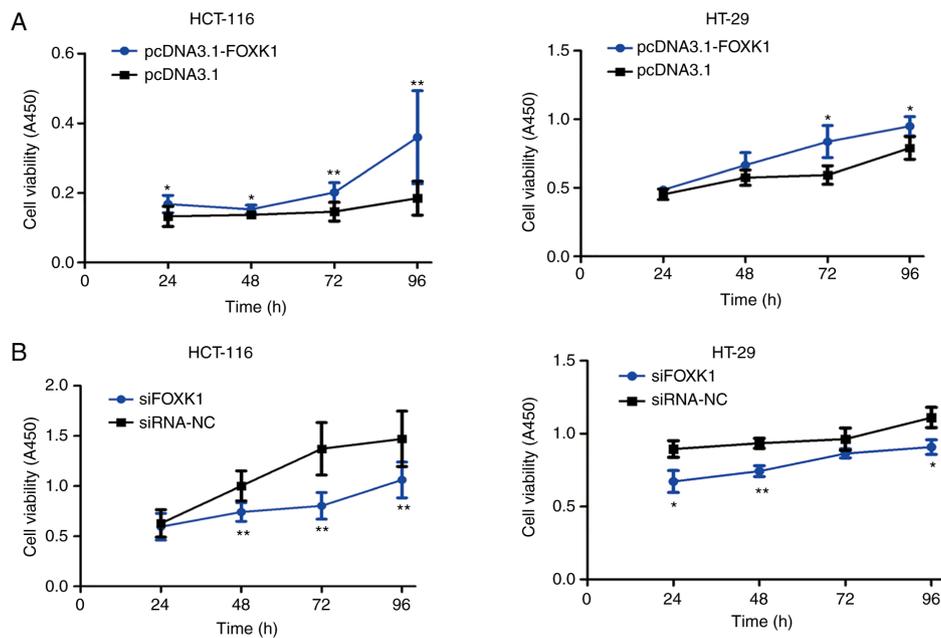


Figure 5. *FOXK1* promotes CRC cell proliferation. Effects of (A) pcDNA3.1-*FOXK1* and (B) siFOXK1 on CRC cell proliferation was determined using Cell Counting Kit-8 assays. * $P < 0.05$ and ** $P < 0.01$ vs corresponding control groups. CRC, colorectal cancer; FOXK1, forkhead box K1; si-, short interfering; NC, negative control.

the two binding sites. Collectively, these data indicated that *FOXK1* directly binds to the *TMEM245*/miR-32 promoter.

Downregulation of miR-32 reverses the effects of FOXK1 on PTEN expression and cell proliferation and apoptosis. miR-32 was explored as a functional target of *FOXK1* by co-transfecting HCT-116 and HT-29 cells with pcDNA3.1-*FOXK1* and miR-32 inhibitor, or siFOXK1 and miR-32 mimic. Transfection with the miR-32 mimic and inhibitor significantly increased and decreased miR-32 expression, respectively (all $P < 0.05$ or $P < 0.01$; Fig. 8A

and B). Co-transfection with miR-32 inhibitor partially reversed the decrease in PTEN expression induced by *FOXK1*-overexpression ($P < 0.05$; Fig. 8D), whereas the miR-32 mimic reversed the PTEN upregulation caused by *FOXK1*-knockdown ($P < 0.05$ or $P < 0.01$; Fig. 8F). Levels of *PTEN* mRNA did not significantly change (Fig. 8C and E). Proliferation ability was decreased, whereas apoptosis was increased after co-transfection with the *FOXK1*-overexpression plasmid and miR-32 inhibitor compared with inhibitor-NC (Fig. 8G and I). Proliferation ability was increased, whereas apoptosis was decreased after co-transfection with siFOXK1

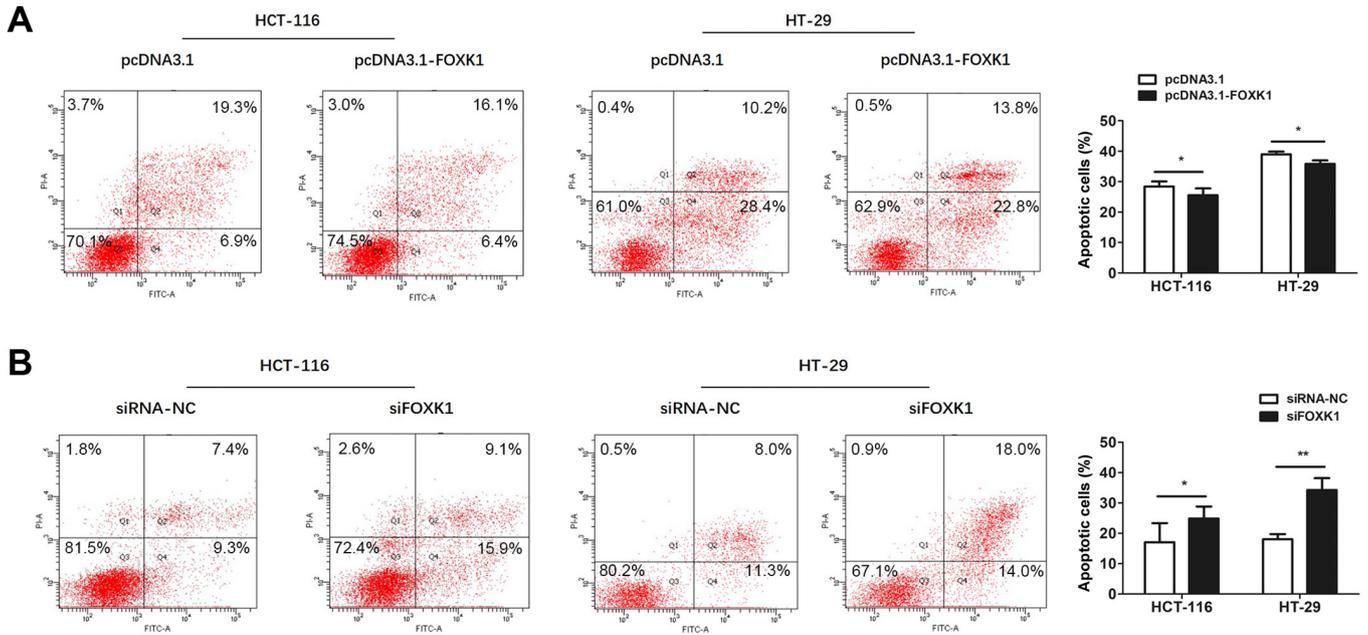


Figure 6. Apoptosis of CRC is suppressed by FOXK1. Representative images show effects of (A) pcDNA3.1-FOXK1 or (B) siFOXK1 on CRC apoptosis, which was determined using flow cytometry. Q2 + Q4 quadrants show ratios (%) of apoptotic cells in total cell population. * $P < 0.05$ and ** $P < 0.01$ vs corresponding control groups. FOXK1, forkhead box K1; si-, short interfering; CRC, colorectal cancer; NC, negative control.

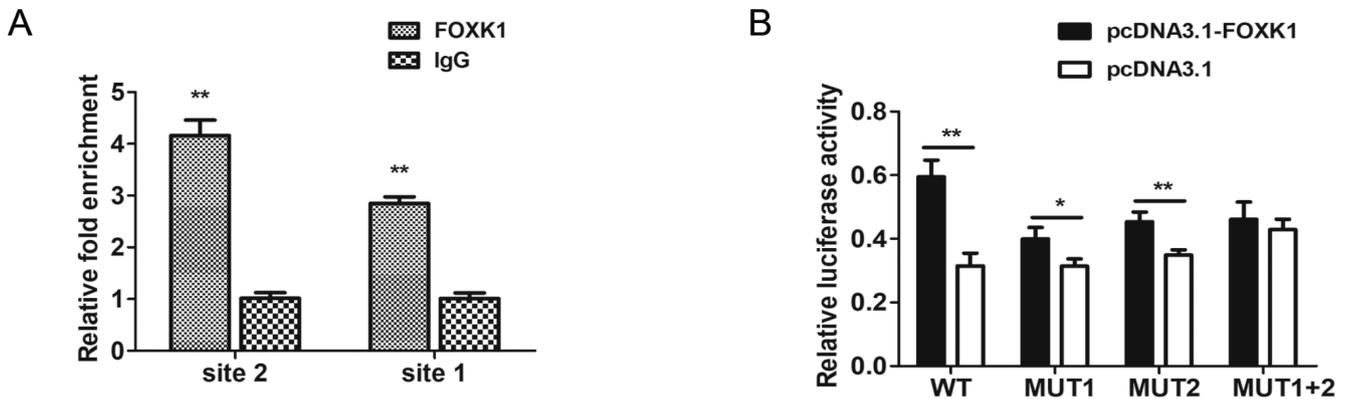


Figure 7. FOXK1 directly binds to *TMEM245/miR-32* promoter. (A) Binding of FOXK1 to the *TMEM245/miR-32* promoter in HCT-116 cells was determined with chromatin immunoprecipitation quantitative PCR assays using IgG or FOXK1-specific antibodies. (B) Relative luciferase activities in HCT-116 cells co-transfected with pcDNA3.1-FOXK1 or pcDNA3.1 and pGL3-promoter-WT, pGL3-promoter-MUT1, -MUT2 or -MUT1+2. * $P < 0.05$ and ** $P < 0.01$ vs corresponding control groups or WT. FOXK1, forkhead box K1; *TMEM245*, transmembrane protein 245; miR, microRNA; WT, wild-type; MUT, mutant.

and miR-32 mimic compared with mimic-NC (Fig. 8H and J). These results indicate that the promotive effects of FOXK1 on CRC cells are largely mediated by miR-32 expression and can be reversed by downregulating miR-32.

Discussion

Numerous regulatory mechanisms are involved in the differential processing of miRNA. Mechanisms that regulate miRNA expression include transcriptional regulation, such as changes in host gene expression and hypermethylation of host or miRNA gene promoters, and post-transcriptional regulation, including changes in miRNA processing and stability (15). The transcription of miRNA can be regulated by using TFs that bind to specific promoters (16). For example,

the TF p65/NF κ B can bind to the miR-224 promoter and acts as a direct transcriptional regulator of miR-224 expression, and such binding increases in cells incubated with lipopolysaccharides, tumor necrosis factor- α or lymphotoxin- α (17). The myogenic regulatory factor, MyoD, regulates miR-206 expression by directly binding to its promoter (18). It can also directly bind to the miR-182 promoter and upregulate miR-182 expression (19). The basic leucine zipper TF, C/EBP β , binds to the *let-7f* miRNA promoter and positively modulates *let-7f* expression (14). It was determined that FOXK1 was a potentially interactive TF binding to *TMEM245/miR-32* promoter in our previous study (9). Therefore, the present study aimed to clarify the role of FOXK1 in miR-32 regulation.

miRNAs are classified as intronic or intergenic according to their genomic location. Some intronic miRNAs can be

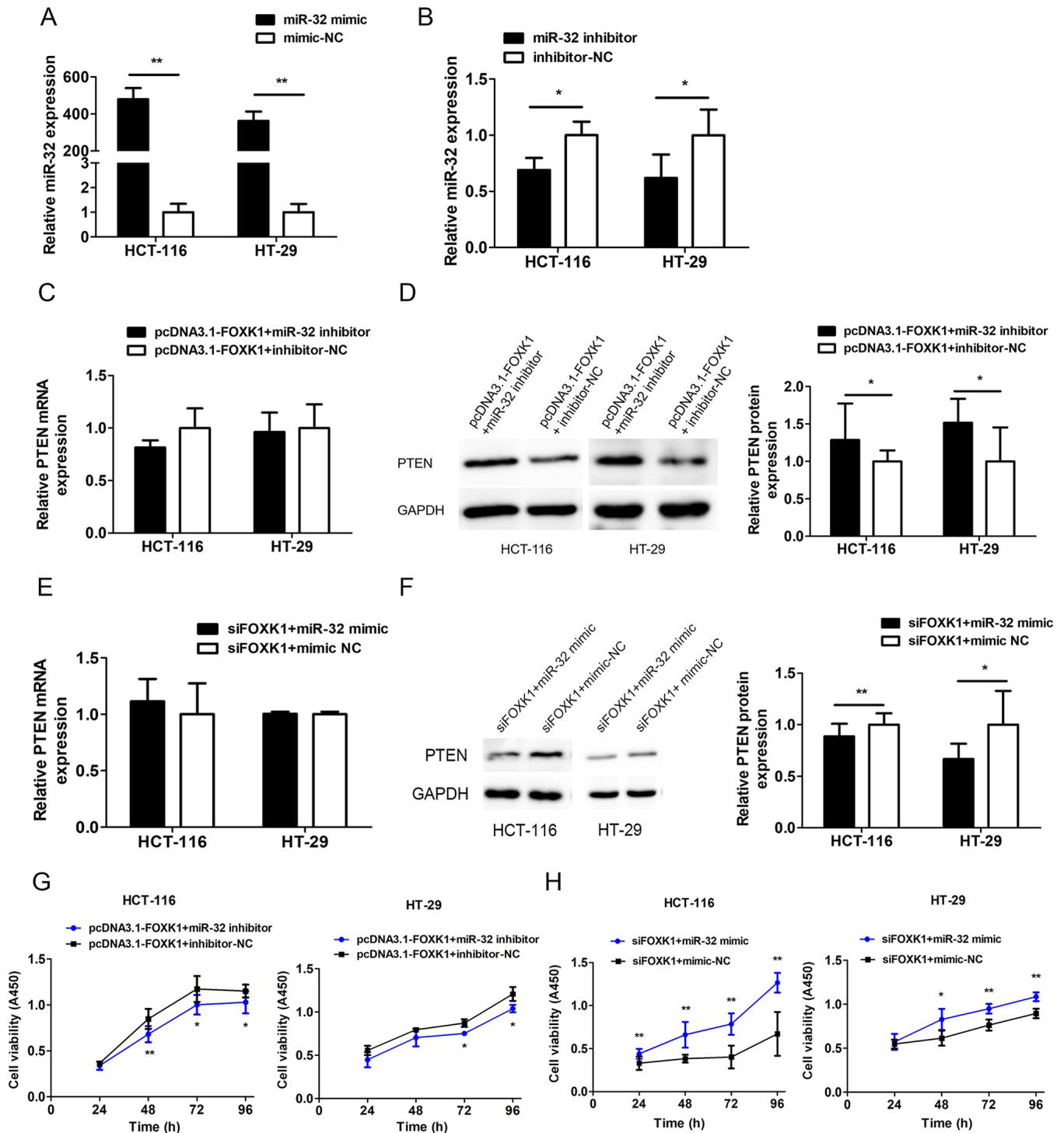


Figure 8. Continued.

transcribed with their host genes, whereas others are not co-expressed. The co-transcription of intronic miRNA with host genes might be regulated by the host gene promoter (20). Baskerville and Bartel (21) showed that intronic miRNAs are closely associated with their host genes. The upstream regions of pre-miRNA are considered the promoters for intergenic and intronic miRNA that are independently transcribed (20). Lerner *et al* (22) showed that the putative tumor suppressor gene, deleted in lymphocytic leukemia 2 (*DLEU2*), is the host gene of miR-15a/miR-16-1 and that Myc binding to

two alternative *DLEU2* promoters reduces levels of *DLEU2* transcription and mature miR-15a/ -16-1. The host gene of miR-196b-5p, homeobox protein A10 (*HOXA10*), is over-expressed in human gastric cancer tissues, and is positively correlated with miR-196b-5p expression levels. The expression of *HOXA10* and miR-196b-5p in gastric cancer cells increases when the *HOXA10* promoter is demethylated (23).

Intronic miR-32 is encoded by *TMEM245*. Levels of *TMEM245* and miR-32 transcripts positively correlate in prostate tumors (24). The present study found that *TMEM245*

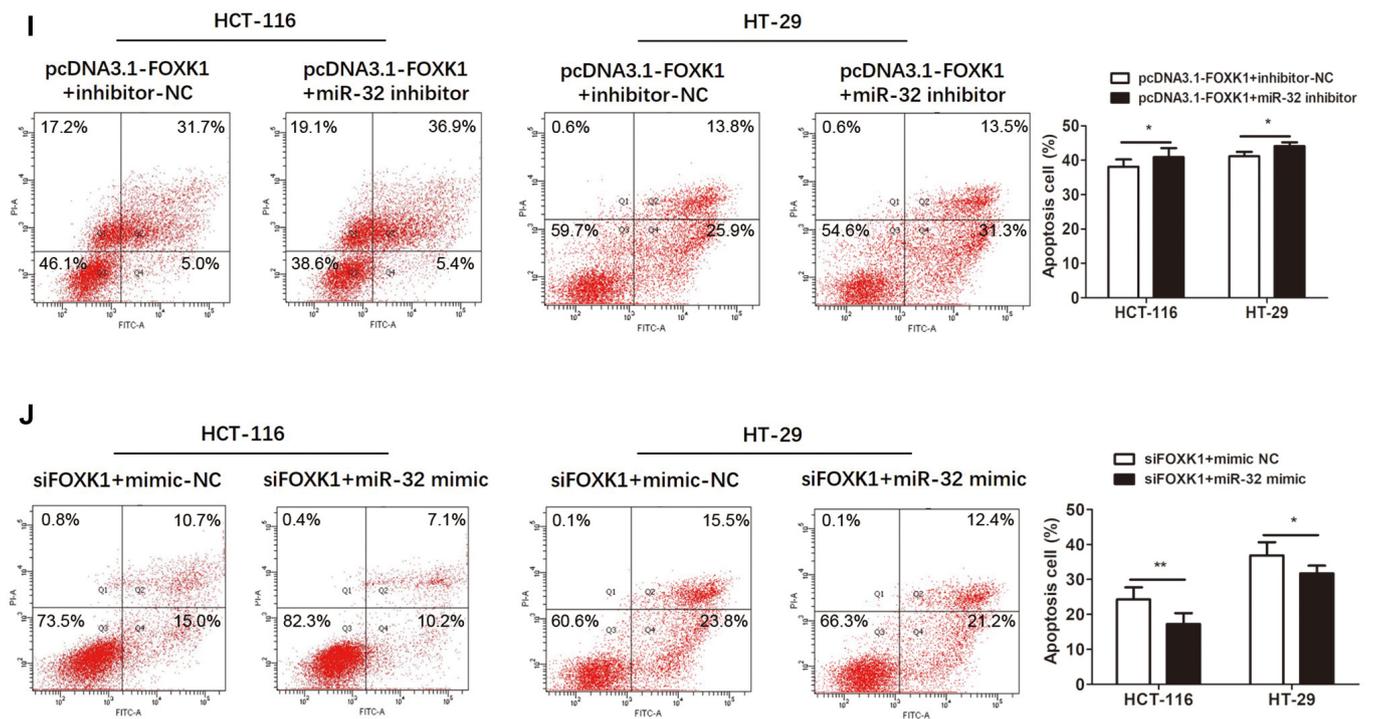


Figure 8. Knockdown of miR-32 reverses FOXK1 effects. (A and B) Expression of miR-32 in CRC cells co-transfected with a miR-32 mimic or inhibitor. (C and D) Expression of PTEN in CRC cells co-transfected with pcDNA3.1-FOXK1 and miR-32 inhibitor. (E and F) Expression of PTEN in CRC cells co-transfected with either pcDNA3.1-FOXK1 and miR-32 inhibitor or siFOXK1 and miR-32 mimic using Cell Counting Kit-8 assays and flow cytometry, respectively. * $P < 0.05$ and ** $P < 0.01$ vs corresponding control groups. miR, microRNA; FOXK1, forkhead box K1; PTEN, phosphatase and tensin homolog; si-, short interfering; CRC, colorectal cancer; NC, negative control.

mRNA expression was positively correlated with miR-32 levels in CRC tissues. The evolutionarily conserved FOX proteins comprise of a TF superfamily that is characterized by a 'forkhead' or 'winged-helix' DNA-binding domain, including FOXK1 and FOXK2 (25). The vital TF FOXK1 protein regulates numerous biological activities, including cell cycle progression in myogenic progenitor cells (26), aerobic glycolysis (27), insulin-like growth factor-1 receptor-mediated signaling involved in cell proliferation and metabolism (28) and diseases such as cancer (25). This protein also plays a crucial role in various cancer types by acting as an oncogene. Li *et al* (29) found that FOXK1 expression is significantly increased in human hepatocellular carcinoma tissues and cell lines, and that FOXK1-knockdown significantly suppresses hepatocellular carcinoma cell proliferation, migration and invasion, in part by inactivating Wnt/ β -catenin signaling. Moreover, FOXK1 expression is increased in various malignancies, including CRC, gastric cancer, glioma and prostate cancer (30-33). Huang *et al* (34) reported that FOXK1 promotes the epithelial-mesenchymal transition, tumor invasion and metastasis by transactivating cysteine-rich angiogenic inducer 61 expression in CRC cells.

The present study found that upregulated FOXK1 expression in CRC tissues was positively correlated with miR-32 or TMEM245 expression. Furthermore, FOXK1 promoted CRC cell proliferation and reduced apoptosis, thus acting as an oncogene. However, the proliferation inhibition of siFOXK1 on HT-29 cells at 72 h was not significant, which may be due to the large differences within the groups. Bioinformatics analysis predicted

two binding sites for FOXK1 in the *TMEM245/miR-32* core promoter region. Gain- and loss-of-function studies revealed that FOXK1 upregulated the expression of miR-32 and TMEM245 and downregulated PTEN protein levels. However, there was no significant change in PTEN mRNA expression. This may be because the main mechanism of miR-32-induced PTEN suppression is post-transcriptional, which is consistent with the results in our previous research (8). The binding of FOXK1 to the *TMEM245/miR-32* promoter was identified using ChIP and dual-luciferase reporter assays. Thus, the present findings revealed that FOXK1 binds to the *TMEM245/miR-32* promoter and induces miR-32 expression, leading to increased CRC cell proliferation. In order to determine the mechanism through which FOXK1 regulates miR-32 functions in cell proliferation and apoptosis, PTEN expression was assessed and CCK-8 and Annexin V-FITC apoptosis assays were conducted after simultaneously interfering with FOXK1 and miR-32 expression. Knockdown of miR-32 in cells overexpressing FOXK1 resulted in decreased cell proliferation, increased apoptosis and the upregulation of PTEN protein. Therefore, the promotive effects of FOXK1 on CRC cell proliferation were mediated, at least in part, by upregulated miR-32.

However, there were several limitations in the present study. As a TF, FOXK1 regulates several other genes, including p21, CCDC43 and Snail (10,35,36). Moreover, miRNA can be regulated by multiple TFs as well as non-coding RNAs, such as long non-coding and circular RNA (37,38). Thus, FOXK1 or miR-32 might affect the onset and development of CRC via pathways other than the FOXK1-miR-32-PTEN

axis. Interactions between FOXK1 or miR-32 with other genes remain to be clarified. Animal experiments should be conducted to further evaluate the mechanisms of FOXK1 in miR-32 regulation.

The findings of the present study suggested that FOXK1 expression is increased in CRC tissues and positively correlates with miR-32 levels. FOXK1 was shown to directly bind the *TMEM245/miR-32* promoter to activate miR-32 expression and downregulate PTEN. Furthermore, miR-32-knockdown mitigated FOXK1-promoted CRC cell proliferation and FOXK1-inhibited apoptosis *in vitro*. Thus, the FOXK1-miR-32-PTEN signaling axis might play a crucial role in the pathogenesis and development of CRC.

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Availability of data and materials

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

Author's contributions

WW designed the study. WW, YC, SY, HY and JY carried out the experiments. SY collected the samples. WW, YC and JQ analyzed the data. WW and JQ drafted the manuscript. JQ revised the manuscript. WW, YC, SY, HY, JY and JQ confirm the authenticity of all raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Medical Ethics Committees at the Affiliated Hospital of Guangdong Medical University (Guangdong, China) approved this study and written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Geng F, Wang Z, Yin H, Yu J and Cao B: Molecular targeted drugs and treatment of colorectal cancer: Recent progress and future perspectives. *Cancer Biother Radiopharm* 32: 149-160, 2017.
- Fang JY, Dong HL, Sang XJ, Xie B, Wu KS, Du PL, Xu ZX, Jia XY and Lin K: Colorectal cancer mortality characteristics and predictions in China, 1991-2011. *Asian Pac J Cancer Prev* 16: 7991-7995, 2015.
- Gu MJ, Huang QC, Bao CZ, Li YJ, Li XQ, Ye D, Ye ZH, Chen K and Wang JB: Attributable causes of colorectal cancer in China. *BMC Cancer* 18: 38, 2018.
- Sun L, Xue H, Jiang C, Zhou H, Gu L, Liu Y, Xu C and Xu Q: LncRNA DQ786243 contributes to proliferation and metastasis of colorectal cancer both *in vitro* and *in vivo*. *Biosci Rep* 36: e00328, 2016.
- Strubberg AM and Madison BB: MicroRNAs in the etiology of colorectal cancer: Pathways and clinical implications. *Dis Model Mech* 10: 197-214, 2017.
- Ha M and Kim VN: Regulation of microRNA biogenesis. *Nat Rev Mol Cell Biol* 15: 509-524, 2014.
- Wu W, Yang P, Feng X, Wang H, Qiu Y, Tian T, He Y, Yu C, Yang J, Ye S and Zhou Y: The relationship between and clinical significance of microRNA-32 and phosphatase and tensin homologue expression in colorectal cancer. *Genes Chromosomes Cancer* 52: 1130-1140, 2013.
- Wu W, Yang J, Feng X, Wang H, Ye S, Yang P, Tan W, Wei G and Zhou Y: MicroRNA-32 (miR-32) regulates phosphatase and tensin homologue (PTEN) expression and promotes growth, migration, and invasion in colorectal carcinoma cells. *Mol Cancer* 12: 30, 2013.
- Wu W, Tan W, Ye S, Zhou Y and Quan J: Analysis of the promoter region of the human miR-32 gene in colorectal cancer. *Oncol Lett* 17: 3743-3750, 2019.
- Li L, Gong M, Zhao Y, Zhao X and Li Q: FOXK1 facilitates cell proliferation through regulating the expression of p21, and promotes metastasis in ovarian cancer. *Oncotarget* 8: 70441-70451, 2017.
- Chen D, Wang K, Li X, Jiang M, Ni L, Xu B, Chu Y, Wang W, Wang H, Kang H, *et al*: FOXK1 plays an oncogenic role in the development of esophageal cancer. *Biochem Biophys Res Commun* 494: 88-94, 2017.
- Wu Y, Peng Y, Wu M, Zhang W, Zhang M, Xie R, Zhang P, Bai Y, Zhao J, Li A, *et al*: Oncogene FOXK1 enhances invasion of colorectal carcinoma by inducing epithelial-mesenchymal transition. *Oncotarget* 7: 51150-51162, 2016.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
- Ayyar K and Reddy KVR: Transcription factor CCAAT/enhancer-binding protein- β upregulates microRNA, let-7f-1 in human endocervical cells. *Am J Reprod Immunol* 78. Epub 2017 Sep 16 2017.
- Gulyaeva LF and Kushlinskiy NE: Regulatory mechanisms of microRNA expression. *J Transl Med* 14: 143, 2016.
- Treiber T, Treiber N, Plessmann U, Harlander S, Daiß JL, Eichner N, Lehmann G, Schall K, Urlaub H and Meister G: A compendium of RNA-binding proteins that regulate microRNA biogenesis. *Mol Cell* 66: 270-284.e13, 2017.
- Scisciani C, Vossio S, Guerrieri F, Schinzari V, De Iaco R, D'Onorio de Meo P, Cervello M, Montalto G, Pollicino T, Raimondo G, *et al*: Transcriptional regulation of miR-224 upregulated in human HCCs by NF κ B inflammatory pathways. *J Hepatol* 56: 855-861, 2012.
- Koutalios D, Koutsoulidou A, Mastroiannopoulos NP, Furling D and Phylactou LA: MyoD transcription factor induces myogenesis by inhibiting Twist-1 through miR-206. *J Cell Sci* 128: 3631-3645, 2015.
- Dodd RD, Sachdeva M, Mito JK, Eward WC, Brigman BE, Ma Y, Dodd L, Kim Y, Lev D and Kirsch DG: Myogenic transcription factors regulate pro-metastatic miR-182. *Oncogene* 35: 1868-1875, 2016.
- Zhang HM, Kuang S, Xiong X, Gao T, Liu C and Guo AY: Transcription factor and microRNA co-regulatory loops: Important regulatory motifs in biological processes and diseases. *Brief Bioinform* 16: 45-58, 2015.
- Baskerville S and Bartel DP: Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. *RNA* 11: 241-247, 2005.
- Lerner M, Harada M, Lovén J, Castro J, Davis Z, Oscier D, Henriksson M, Sangfelt O, Grandér D and Corcoran MM: DLEU2, frequently deleted in malignancy, functions as a critical host gene of the cell cycle inhibitory microRNAs miR-15a and miR-16-1. *Exp Cell Res* 315: 2941-2952, 2009.
- Shao L, Chen Z, Peng D, Soutto M, Zhu S, Bates A, Zhang S and El-Rifai W: Methylation of the HOXA10 promoter directs miR-196b-5p-dependent cell proliferation and invasion of gastric cancer cells. *Mol Cancer Res* 16: 696-706, 2018.

24. Ambs S, Prueitt RL, Yi M, Hudson RS, Howe TM, Petrocca F, Wallace TA, Liu CG, Volinia S, Calin GA, *et al*: Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer. *Cancer Res* 68: 6162-6170, 2008.
25. Liu Y, Ding W, Ge H, Ponnusamy M, Wang Q, Hao X, Wu W, Zhang Y, Yu W, Ao X and Wang J: FOXK transcription factors: Regulation and critical role in cancer. *Cancer Lett* 458: 1-12, 2019.
26. Shi X and Garry DJ: Sin3 interacts with Foxk1 and regulates myogenic progenitors. *Mol Cell Biochem* 366: 251-258, 2012.
27. Sukonina V, Ma H, Zhang W, Bartesaghi S, Subhash S, Heglind M, Foyn H, Betz MJ, Nilsson D, Lidell ME, *et al*: FOXK1 and FOXK2 regulate aerobic glycolysis. *Nature* 566: 279-283, 2019.
28. Sakaguchi M, Cai W, Wang CH, Cederquist CT, Damasio M, Homan EP, Batista T, Ramirez AK, Gupta MK, Steger M, *et al*: FoxK1 and FoxK2 in insulin regulation of cellular and mitochondrial metabolism. *Nat Commun* 10: 1582, 2019.
29. Li P, Yu Z, He L, Zhou D, Xie S, Hou H and Geng X: Knockdown of FOXK1 inhibited the proliferation, migration and invasion in hepatocellular carcinoma cells. *Biomed Pharmacother* 92: 270-276, 2017.
30. Wu M, Wang J, Tang W, Zhan X, Li Y, Peng Y, Huang X, Bai Y, Zhao J, Li A, *et al*: FOXK1 interaction with FHL2 promotes proliferation, invasion and metastasis in colorectal cancer. *Oncogenesis* 5: e271, 2016.
31. Zhang H, Wu X, Xiao Y, Wu L, Peng Y, Tang W, Liu G, Sun Y, Wang J, Zhu H, *et al*: Coexpression of FOXK1 and vimentin promotes EMT, migration, and invasion in gastric cancer cells. *J Mol Med (Berl)* 97: 163-176, 2019.
32. Ji ZG, Jiang HT and Zhang PS: FOXK1 promotes cell growth through activating wnt/ β -catenin pathway and emerges as a novel target of miR-137 in glioma. *Am J Transl Res* 10: 1784-1792, 2018.
33. Chen F, Xiong W, Dou K and Ran Q: Knockdown of FOXK1 suppresses proliferation, migration, and invasion in prostate cancer cells. *Oncol Res* 25: 1261-1267, 2017.
34. Huang X, Xiang L, Li Y, Zhao Y, Zhu H, Xiao Y, Liu M, Wu X, Wang Z, Jiang P, *et al*: Snail/FOXK1/Cyr61 signaling axis regulates the epithelial-mesenchymal transition and metastasis in colorectal cancer. *Cell Physiol Biochem* 47: 590-603, 2018.
35. Wang J, Liu G, Liu M, Xiang L, Xiao Y, Zhu H, Wu X, Peng Y, Zhang W, Jiang P, *et al*: The FOXK1-CCDC43 axis promotes the invasion and metastasis of colorectal cancer cells. *Cell Physiol Biochem* 51: 2547-2563, 2018.
36. Xu H, Huang S, Zhu X, Zhang W and Zhang X: FOXK1 promotes glioblastoma proliferation and metastasis through activation of Snail transcription. *Exp Ther Med* 15: 3108-3116, 2018.
37. Zhang Z, Li M and Zhang Z: LncRNA MALAT1 modulates oxaliplatin resistance of gastric cancer via sponging miR-22-3p. *Onco Targets Ther* 13: 1343-1354, 2020.
38. Zhang L and Ding F: Hsa_circ_0008945 promoted breast cancer progression by targeting miR-338-3p. *Onco Targets Ther* 12: 6577-6589, 2019.



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