**Abstract.** Forkhead box K1 (FOXK1) is a member of the FOX transcription factor family, which plays an important role in oncogenesis. However, the exact function and mechanism of FOXK1 in human colorectal cancers (CRCs) remain unclear. In the present study, we first screened for potential FOXK1 target genes by ectopically expressing FOXK1 in SW480 cells and examined the subsequent changes in the expression levels of major oncogenes using RT-PCR. We also evaluated the effects of FOXK1 regulation on growth and apoptosis. In addition, we investigated the biological impact of FOXK1 knockdown on CRC cells in vitro and in vivo. We found that FOXK1 overexpression increased the expression of multiple oncogenes in vitro. FOXK1 promoted serum-dependent and anchorage-dependent and -independent cell growth. Knockdown of FOXK1 induced G0/G1 cell cycle arrest in CRC cells. Moreover, FOXK1 suppression induced apoptosis and increased cell susceptibility to 5-fluorouracil (5-FU)-induced apoptosis. Furthermore, a xenograft model was established to explore FOXK1 shRNA-mediated tumorigenesis in vivo. A strong antitumorigenic effect of FOXK1-shRNA was enhanced when combined with 5-FU treatment. These findings implicate FOXK1 as a cell cycle and growth modulator that inhibits apoptosis in colon cancer cells. FOXK1-shRNA may serve as a novel and potent therapeutic agent, alone or with 5-FU, against colon cancer.

**Introduction.**

As one of the major causes of cancer-related mortality worldwide, colorectal cancer (CRC) is surgically curable at early stages, but advanced disease at the metastatic stage is associated with high mortality rates (1,2). CRCs progress from local adenomas in the intestinal epithelium to invasive carcinomas that typically metastasize to the liver (3). Approximately half of all patients with local CRCs may develop metastases. Current therapies show a 5-year survival of less than 5% for metastatic disease (4).

The forkhead box (Fox) gene family encodes a large and diverse group of transcription factors that share certain characteristics in a conserved, ~100 amino acid DNA-binding motif known as the forkhead or winged helix domain; over 150 proteins with forkhead domains have been identified, comprising at least 17 subclasses, to date (5,6). Through the transcriptional control of gene expression, many FOX protein members reportedly play important roles in embryonic development and organogenesis and in the regulation of various physiological processes, such as the cell cycle (7), progression (8), cell survival (9), cellular metabolism (10), life span (11) and immune responses (12). Consequently, dysregulation of the function, subcellular localization and expression of FOX transcription factors leads to the development and progression of diseases, particularly cancer (13,14). Several other FOX family proteins have been shown to have either a tumor-promoting [FOXA1 (15), FOXQ1 (16), FOXM1 (17) and FOXC2 (18)] or suppressive [FOXD3 (19), FOXP1 (20), FOXI1 (21) and FOXP2 (22)] role in the cellular signaling that is associated with proliferation and/or suppression.

The human forkhead box K1 (FOKK1) gene encodes predicted proteins that are most homologous to the mouse myocyte nuclear factor MNF/Forkhead box K1 (Foxk1) protein. FOXK1 is predominantly expressed in many malignant tissues and in the brain, colon and lymph nodes (23). This gene has been implicated in normal and neoplastic developmental processes. Wang et al demonstrated that FOXK1 and FOXK2 positively regulate Wnt/β-catenin signaling by translocating...
Dishevelled (DVL) into the nucleus. Moreover, FOXK1 and FOXK2 protein levels are elevated in human CRCs (24). However, the underlying mechanism of FOXK1 induction in multiple cellular events, such as growth, apoptosis and chemotherapy resistance, remains unclear.

In the present study, we demonstrated a novel and effective way to knock down FOXK1, thereby significantly inducing apoptosis, inhibiting tumorigenesis and tumor growth and strongly enhancing the antitumor activity of 5-fluorouracil (5-FU) in vitro and in vivo. Our results showed that lenti-shRNA-FOXK1 is a promising candidate for the gene therapy of colon cancer.

Materials and methods

Reagents and cell culture. 5-FU was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse antibodies against FOXK1 (G-4), cyclin D1 (A-12), CDK4 (H-22), CDK6 (C-21), caspase 3 p11 (C-6), caspase 8 p18 (E-8), caspase 9 p35 (A-9), PARP-1 (H-300), GAPDH (G-9), horseradish peroxidase-conjugated anti-goat IgG, and anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse antibodies against PPAR-1 (H-300), GAPDH (G-9), horseradish peroxidase-conjugated anti-goat IgG, and anti-mouse IgG were purchased from Sigma-aldrich (St. Louis, MO, USA). Mouse antibodies against 5-FU was purchased from Trevigen, Inc. (Gaithersburg, MD, USA) according to the manufacturer's instructions. The antibodies were then visualized using an enhanced chemiluminescence detection system.

Flow cytometric analysis. For cell cycle analysis, SW480 cells were harvested 48 h after transfection and lysed in lysis buffer [10 mM Tris-HCl (pH 7.4), 1% SDS, 10% glycerol, 5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 5 µg/ml leupeptin and 21 µg/ml aprotinin]. A total of 30 µg of each protein lysate was separated by SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. The primary antibodies were diluted according to the manufacturer's recommendations. The antibodies were then visualized using an enhanced chemiluminescence detection system.

Morphological detection of apoptosis. Apoptosis was detected using the Annexin V-FITC kit (Trevigen, Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. Briefly, cells with various treatments were collected and stained with Annexin V-FITC and PI in the dark for 15 min before being subjected to flow cytometry and analyzed using FlowJo software.

Cell growth assay. Cells were seeded at 5.0x10⁴/well into 96-well plates. Cell proliferation was determined by measuring the absorption of the cell proliferation reagent WST-1 (Roche Molecular Biochemicals, Basel, Switzerland) according to the manufacturer's protocol. The ratio of the absorbance of treated cells at 450 nm relative to that of the untransfected cells was calculated from the resultant DNA histograms using FlowJo software.

Transient siRNA transfection. Ablation of FOXK1 was performed by transfection with small interfering RNA (siRNA) duplex oligos, which were synthesized by GenePharma (Shanghai, China). Control siRNA (scrambled RNA) and FOXK1-specific siRNA [sense, 889-GAGACAGCCCAAGGAUGA (dTdT)-908 and antisense, 908-UCAUUCCUGGGGCUGUCUC(dTdT)-889] were transfected using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, western blot analysis was performed.

Western blot analysis. For western blot analysis, cells were harvested 48 h after transfection and lysed in lysis buffer [10 mM Tris-HCl (pH 7.4), 1% SDS, 10% glycerol, 5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 5 µg/ml leupeptin and 21 µg/ml aprotinin]. A total of 30 µg of each protein lysate was separated by SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. The primary antibodies were diluted according to the manufacturer’s recommendations. The antibodies were then visualized using an enhanced chemiluminescence detection system.

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Constructs and establishment of stable transfectants. Complementary DNA (cDNA) corresponding to full-length human testis cDNA with primers specific to FOXK1. The PCR products were subcloned into the mammalian expression vector pcDNA3.1 (Invitrogen).

To establish stable cell lines, cells transfected with the empty pcDNA3.1 vector or with pcDNA3.1-FOXK1 were passaged at 1:15 (vol/vol) and were cultured in RPMI-1640 medium supplemented with geneticin (G418; Calbiochem, Darmstadt, Germany) at 1,000 µg/ml for 4 weeks. Stably transfected clones were selected by immunoblotting for FOXK1 expression.
Construction of lentiviral vectors with FOXK1 short hairpin RNA. To further investigate the effect of siRNA-induced downregulation of FOXK1 expression on the in vivo tumor growth of CRC, a FOXK1-RNAi lentiviral vector (GV208-FOXK1-shRNA) was constructed by Shanghai GeneChem Co., Ltd. (Shanghai, China). Double-stranded oligonucleotides encoding human FOXK1-vshRNA (NM_00103765; CCGGGACACGGCCAAAGGATGTAC AAGAGTCATCCTTGGGCTGTCTCTTCTTGG) were annealed and inserted into the short hairpin RNA (shRNA) expression vector GV208-GFP (Shanghai GeneChem Co., Ltd.). A GFP lentiviral vector (GV208-GFP) was used as a negative control. Clone identity was verified by sequencing.

Recombinant lentiviral vector was produced by co-transfecting HEK293FT cells with the lentiviral expression vector and packing the plasmid mix using Lipofectamine™ 2000 according to the manufacturer's instructions. Infectious lentiviral particles were harvested at 48 h post-transfection and were then filtered through 0.45 μm cellulose acetate filters. The virus was concentrated, and the titer was determined by serial dilution of 293T cells.

For lentivirus transduction, the SW480 cells were subcultured at 1x10^5 cells/well into 6-well culture plates. Cells were transduced with the FOXK1-siRNA-expressing (FOXK1-siRNA) or scramble (scr)-siRNA-expressing lentivirus at a multiplicity of infection (MOI) of 50. Cells were then harvested 72 h after infection, and the transduction efficiency was evaluated by counting the percentage of GFP-positive cells.

In vivo tumor growth assay. The present study was conducted in strict accordance with the recommendations in the Guide for the Institutional Animal Care and Use Committee (IACUC), and the protocol was approved by the Committee on the Ethics of Animal Experiments of Nanfang Hospital. All (IacUc), and the protocol was approved by the committee with NS. Twelve hours after transduction, 5x10^6 viable cells of 50 with lenti-scr-shRNA or lenti-FOXK1-shRNA infected for the Institutional animal care and Use committee.

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Results

Ectopic overexpression of FOXK1 increases the expression of multiple oncopgenes in vitro. We developed in vitro models to examine the mechanistic role of FOXK1 in CRC biology, and we established stable transfectants with FOXK1 sense and vector (pcDNA3.1) plasmids. The overexpression of FOXK1 was confirmed by western blot analysis (Fig. 1A).

Next, we screened for potential FOXK1 target genes by ectopically expressing FOXK1 in SW480 cells and examining the subsequent changes in the expression levels of 10 major oncopgenes that are reportedly involved in proliferation, transformation or apoptosis inhibition (27). The mRNA expression levels of ZEB1, ID1, Spl, cyclin D1, β-catenin, Myc, TWIST, TERT, survivin and FOXK1 were upregulated in the stable transfectants of FOXK1 (Fig. 1B).

Our results suggest that FOXK1 activates the expression of multiple oncopgenes in CRC.

FOXK1 promotes cell growth by stimulating cell proliferation. To analyze the effect of FOXK1 on cell growth, we assessed the proliferation of the stable transfectants cultured in complete cell culture medium at various time points. The cell growth percentages relative to those of transfectants carrying an empty vector were calculated. We showed that the growth rates of the SW480-FOXK1 transfectants were 103.1±1.5, 169.6±3.1 and 214.3±4.0% after culture for 24, 48 and 72 h, respectively (Fig. 1C). Significant differences between the FOXK1- and empty vector-transfected cells were found at 48 and 72 h (p<0.05).

Docking-dependent growth in one pivotal characteristic of malignant transformation. To determine whether forced expression of FOXK1 may alter the oncogenicity of CRC cells, we examined the anchorage-independent growth of the stable transfectants with the empty vector or with FOXK1 in soft agar after 14 days. SW480 and empty vector-transfected cells formed colonies in soft agar as expected, while forced expression of FOXK1 markedly enhanced colony formation in soft agar (Fig. 1D). FOXK1 expression increased the clonogenicity of the SW480 cells by 155% (Fig. 1E). Conversely, we also tested the effect of RNAi-mediated FOXK1 knockdown in the
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SW480 cells. FOXK1-siRNA knockdown caused a significant loss of colony-forming capacity. The clonogenicity of the suppressed SW480 cells was reduced by 71.4% (Fig. 1E).

Therefore, the overexpression of FOXK1 markedly promoted the proliferation of CRC cells.

Knockdown of FOXK1 induces G0/G1 cell cycle arrest. To assess in more detail the effect of constitutive FOXK1 expression on the growth characteristics of CRC cells in vitro, we evaluated the cell cycle distribution of the cells by flow cytometry. FOXK1 knockdown increased the proportion of cells in the G0/G1 phase and concomitantly decreased the proportion of cells in the G2/M phase of the cell cycle (Fig. 2A).

Furthermore, cell cycle-related protein expression was assessed by western blotting. Consistent with the accumulation of cells in G0/G1 phase, cyclin D1, CDK4 and CDK6 were significantly decreased in the FOXK1-siRNA-treated SW480 and SW1116 cells (Fig. 2B). The cyclin-dependent kinases (CDKs) CDK4 and CDK6, along with cyclin D1 proteins, are involved in the progression of cells through G1 phase and their entry into S phase. Collectively, these findings indicate that reduced expression of FOXK1 slowed cell cycle progression through the G1 phase.

Knockdown of FOXK1 induces CRC cell apoptosis. To investigate the mechanism by which FOXK1-siRNA induces growth suppression, we performed an apoptosis assay. Apoptosis was detected by FACS analysis after double staining with PI and Annexin V-FITC. As shown in Fig. 3A, FOXK1-siRNA induced more apoptosis than scr-siRNA in the SW480 cells, indicating that FOXK1-siRNA inhibited cell growth by inducing apoptosis. Apoptotic induction was further confirmed by Hoechst 33258 staining at the single-cell level (Fig. 3B and C). Moreover, FOXK1-siRNA activated caspases 3, 8 and 9, as evidenced by the increased protein level of cleaved caspases compared with that in the scr-siRNA-treated cells (Fig. 3D). Taken together, these results support the idea that knockdown of FOXK1 promotes the apoptosis of CRC cells.

Silencing of FOXK1 suppresses the growth of xenograft tumors in nude mice. To explore the effects of FOXK1 knockdown in vivo, xenograft tumors were generated by injecting SW480 cells that were stably infected with either scr-shRNA or FOXK1-shRNA. The cells were subcutaneously injected into the flanks of nude mice (Fig. 4A). As shown in Fig. 4B, compared with the scr-shRNA group, the tumors derived from FOXK1-shRNA-infected cells grew much more slowly throughout the experiment, suggesting that knockdown of FOXK1 markedly impaired the tumorigenic growth of the SW480 cells.

We next examined the protein expression of cell proliferation (Ki-67) and angiogenesis markers (CD105) in xenograft tumors. Representative immunohistochemical images of the

Figure 1. FOXK1 regulates multiple oncogenes and promotes the proliferation and anchorage-independent growth of CRC cells. (A) FOXK1 expression in stable transfectants of SW480 cells harboring the empty vector or expressing FOXK1, as detected by western blotting. GAPDH was used as the internal control. (B) The mRNA expression levels of ZEB1, ID1, Sp1, cyclin D1, β-catenin, Myc, TWIST, TERT, survivin and FOXK1 were upregulated in stable transfectants of FOXK1. (C) Significant differences between FOXK1- and empty vector-transfected cells were found at 48 and 72 h (p<0.05). (D) SW480 and empty vector-transfected cells formed colonies in soft agar as expected, while forced expression of FOXK1 markedly enhanced colony formation in soft agar. (E) FOXK1 expression increased the clonogenicity of SW480 cells by 155%.
Figure 2. FOXK1 modulates cell cycle progression. (A) The effects of FOXK1-siRNA and scr-siRNA on FOXK1 expression and on the cell cycle distribution of SW480 cells were determined by flow cytometry after staining with PI. (B) Whole-cell lysates of parental SW480 cells were prepared, and protein expression was detected by western blotting. GAPDH was used as the internal control.

Figure 3. Knockdown of FOXK1 induces CRC cell apoptosis. (A) Cells transfected with scr-siRNA or FOXK1-siRNA were double-stained with annexin V-FITC and PI, and then subjected to flow cytometric analysis. (B) SW480 cells were treated with scr-siRNA or FOXK1-siRNA, the nuclei were stained with Hoechst 33258, and the cells were visualized under a fluorescence microscope (arrows denote cells exhibiting nuclear fragmentation and condensed chromatin). (C) Histogram of the percentages of apoptotic cells after staining with Hoechst 33258. The values are expressed as the mean ± SEM of 3 separate experiments; *p<0.01. (D) SW480 cells were transfected with scr-siRNA or FOXK1-siRNA, and cell lysates were prepared after 48 h. The expression levels of pro- and cleaved caspase 3, 8 and 9, and PARP-1 were detected by western blotting with GAPDH as the internal control.
tumors are shown in Fig. 4C. The knockdown of FOXK1 in the FOXK1 group showed significantly inhibited proliferation rates and lower tumor vessel density relative to the scr-shRNA group. These findings suggest that FOXK1-shRNA may have an inhibitory effect on tumorigenesis in vivo.

**Knockdown by FOXK1-shRNA increases cell susceptibility to apoptotic stimuli in vitro and in vivo.** To evaluate the role of FOXK1-siRNA-mediated knockdown in the susceptibility of cells to chemotherapy-induced apoptosis, SW480 cells were treated with or without 5-FU (50 µg/ml, using NS as the vehicle) for 48 h in vitro. Double staining with Annexin V and PI was then performed, followed by flow cytometric analysis to determine the apoptosis rate. As shown in Fig. 5A, the apoptotic indices of the cells transfected with FOXK1-siRNA + 5-FU and scr-siRNA + 5-FU were significantly increased relative to the scr-siRNA controls.

Similarly, by Hoechst 33258 staining, the proportion of condensed nuclei-positive cells was higher in the SW480 cells transfected with FOXK1-siRNA + 5-FU (p<0.001 compared with FOXK1-siRNA + 5-FU) and scr-siRNA + 5-FU (p<0.01 compared with scr-siRNA + 5-FU) than in SW480 cells transfected with the scr-siRNA (Fig. 5B).

Moreover, the antitumor effect of FOXK1-shRNA and/or 5-FU in vivo was demonstrated by a xenograft model in nude mice (Fig. 5C). We subcutaneously injected SW480 cells that were stably infected with either scr-shRNA or FOXK1-shRNA into the right flanks of nude mice, respectively. When tumor nodules became visible (~3-5 mm in diameter), 5-FU was intraperitoneally injected as a co-treatment. The tumor sizes were then monitored weekly. As shown in Fig. 5C, the tumor volumes of the scr-shRNA-treated mice were markedly greater than those of the FOXK1-shRNA + 5-FU- and scr-shRNA + 5-FU-treated mice 4 weeks after injection of the SW480 cells.

Tumors injected with the combination of FOXK1-shRNA and/or 5-FU exhibited the greatest percentage of apoptotic cells. In contrast, tumors injected with scr-shRNA exhibited relatively few TUNEL-positive cells (Fig. 5D). These findings suggest that FOXK1-shRNA enhances the susceptibility of cancer cells to apoptotic triggers that are induced by 5-FU.

**Discussion**

In the present study, we found that the overexpression of FOXK1 increased the expression of multiple oncogenes. We also demonstrated that the antitumor efficacy of shRNA-mediated FOXK1 inhibition in cells and nude mouse xenograft models was due to the induction of cell cycle arrest and apoptosis. These results indicate that FOXK1-shRNA may
represent a novel and potent therapeutic agent, on its own or in conjunction with 5-FU, for the treatment of colon cancer.

Members of the Fox transcription factor family have been identified in several vertebrate cell lineages. Fox proteins have similar binding specificity for a core DNA sequence, (T/C)(A/C)AA(C/T)A (28,29), and they display conserved amino acid sequences in the putative recognition helix. There is evidence that FOX proteins have a central function in established cancers. For example, FOXA1, the most extensively studied member of the family, is upregulated in nearly all cancers, including breast (30), bladder (31), prostate (32) and pancreatic cancers (33). This protein contributes to many of the typical characteristics of cancer, including increased proliferation, resistance to cell death, and increased invasion and metastasis. Wang et al. showed that FOXK1 protein levels are elevated in human CRCs (24). However, the use of FOXK1 as a target in CRC biological therapy has yet to be established.

The present study showed that ectopic FOXK1 expression significantly induced the RNA levels of multiple oncogenes that are involved in neoplastic transformation, apoptosis, the cell cycle and proliferation, including ZEB1, ID1, Sp1, cyclin D1, β-catenin, Myc, TWIST, c-Jun and survivin. Among these targets, Myc and cyclin D1 are involved in cell cycle regulation. Survivin and β-catenin are involved in neoplastic transformation. The transcription factors TWIST, AP-1, Sp1, ZEB1 and ID1, as well as epithelial-to-mesenchymal transition inducers, underlie the phenotypic conversion from epithelial cells to mesenchymal cells. Thus, FOXK1 acts as an oncogene in CRC.

The oncogenic function of FOXK1 in colon cancer was further investigated using in vitro and in vivo assays.
siRNA-mediated FOXK1 knockdown had a marked growth suppression effect under both anchorage-dependent and -independent culture conditions, and it also reduced tumor size in nude mice. Flow cytometry revealed that FOXK1 knockdown caused significant G1/G0 arrest, and a concomitant reduction of CDK4/CDK6 expression was apparent. Furthermore, we found that FOXK1 knockdown increased apoptosis, a finding further confirmed by staining with Hoechst 33258. Notably, apoptotic induction by FOXK1-siRNA was mediated through the intrinsic and extrinsic caspase-dependent pathways. Taken together, these results indicate that FOXK1 is involved in cell growth and apoptosis in CRC.

The drug 5-FU is an important component of standard chemotherapy protocols for various solid tumors, including CRCs (34,35). In the present study, we further investigated the in vitro and in vivo effects of combination treatment with FOXK1-shRNA and 5-FU on colon cancer growth. According to flow cytometric analysis and Hoechst 33258 staining, knockdown of FOXK1 resulted in a greater proportion of 5-FU-induced apoptotic cells. We also found that the volumes of xenograft tumors were significantly reduced when treated with a combination of FOXK1-shRNA and 5-FU. Furthermore, TUNEL staining demonstrated that FOXK1-shRNA could further enhance 5-FU-induced apoptosis. To the best of our knowledge, no studies have yet shown the therapeutic value of RNAi-mediated FOXK1 knockdown in conjunction with 5-FU in CRC.

The present study demonstrates that FOXK1 acts as an oncogene in CRC. FOXK1-shRNA inhibits cell growth and promotes apoptosis in CRC cells in vitro. Furthermore, FOXK1-shRNA hinders tumor progression and results in outright regression when combined with 5-FU in vivo. Thus, our current data provide direct evidence that a combination of RNAi-mediated FOXK1 knockdown and 5-FU may be a potent strategy for colon cancer therapy.

References


