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Tumor and Stem Cell Biology

Cancer Research

FOXQ1 Is Overexpressed in Colorectal Cancer and Enhances Tumorigenicity and Tumor Growth

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Abstract

Forkhead box Q1 (FOXQ1) is a member of the forkhead transcription factor family, and it has recently been proposed to participate in gastric acid secretion and mucin gene expression in mice. However, the role of FOXQ1 in humans and especially in cancer cells remains unknown. We found that *FOXQ1* mRNA is overexpressed in clinical specimens of colorectal cancer (CRC; 28-fold/colonic mucosa). A microarray analysis revealed that the knockdown of *FOXQ1* using small interfering RNA resulted in a decrease in $p21^{CIP1/WAF1}$ expression, and a reporter assay and a chromatin immunoprecipitation assay showed that *p21* was one of the target genes of FOXQ1. Stable FOXQ1-overexpressing cells (H1299/FOXQ1) exhibited elevated levels of p21 expression and inhibition of apoptosis induced by doxorubicin or camptothecin. Although cellular proliferation was decreased in H1299/FOXQ1 cells *in vitro*, H1299/FOXQ1 cells significantly increased tumorigenicity [enhanced green fluorescent protein (EGFP): 2/15, FOXQ1: 7/15] and enhanced tumor growth (437 ± 301 versus 1735 ± 769 mm^3, $P < 0.001$) *in vivo*. Meanwhile, stable p21 knockdown of H1299/FOXQ1 cells increased tumor growth, suggesting that FOXQ1 promotes tumor growth independent of p21. Microarray analysis of H1299/EGFP and H1299/FOXQ1 revealed that FOXQ1 overexpression upregulated several genes that have positive roles for tumor growth, including *VEGFA, WNT3A, RSPO2*, and *BCL11A*. CD31 and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining of the tumor specimens showed that FOXQ1 overexpression mediated the angiogenic and antiapoptotic effect *in vivo*. In conclusion, FOXQ1 is overexpressed in CRC and enhances tumorigenicity and tumor growth presumably through its angiogenic and antiapoptotic effects. Our findings show that FOXQ1 is a new member of the cancer-related FOX family. *Cancer Res; 70(5); 2053–63. ©2010 AACR.*

Introduction

The forkhead box (*Fox*) gene family is a large and diverse group of transcription factors that share certain characteristics of a conserved, ~100 amino acid DNA-binding motif known as the forkhead or winged helix domain; over 100 proteins with forkhead domains have been identified, comprising at least 17 subclasses to date (1). The Fox gene family plays various important roles, not only in biological processes including development, metabolism, immunology, and senescence but also in cancer development (2, 3).

Forkhead box Q1 (*FOXQ1*, also known as HFH1) is a member of the FOX gene family and contains the core DNA binding domain, whereas the flanking wings of FOXQ1 contribute to its sequence specificity (4). As a transcription factor, FOXQ1 is known to repress the promoter activity of smooth muscle–specific genes, such as telokin and SM22α, in A10 vascular muscle cells (5), and FOXQ1 expression is regulated by Hoxa1 in embryonic stem cells (6). The biological function of *Foxq1* has been clearly identified in hair follicle differentiation in satin (sa) homozygous mice (7); interestingly, satin mice also exhibit suppressed natural killer cell function and T-cell function, suggesting a relation with immunology. Satin mice have provided evidence that Hoxc13 regulates foxq1 expression and that "cross-talk" occurs between Homeobox and Fox (8). Foxq1 mRNA is widely expressed in murine tissues, with particularly high expression levels in the stomach and bladder (5). Recently, two important findings have been reported regarding its involvement in stomach surface cells. Foxq1-deficient mice exhibit a lack of gastric acid secretion in response to various secretagogue stimuli (9). On the other hand, Foxq1 regulates gastric MUC5AC synthesis, providing clues as to the lineage-specific cell differentiation in gastric surface epithelia (10). Despite accumulating evidence supporting the biological function of the murine foxq1 gene in hair follicle

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morphogenesis and gastric epithelial cells, no data regarding the cellular and biological functions of human *FOXQ1*, especially in cancer cells, are available.

$p21^{CIP1/WAF1}$ (hereafter called p21) is a member of the cip/kip family of cyclin kinase inhibitors, and initial reports have shown that p21 functions as a G_1 cyclin kinase inhibitor (11, 12) and a downstream molecule of p53 (13). p21 possesses a variety of cellular functions, including the negative modulation of cell cycle progression (14), cellular differentiation (15), and the regulation of p53-dependent antiapoptosis (reviewed in ref. 16). The expression of p21 is regulated by both p53-dependent and p53-independent mechanisms at the transcriptional level. Other regulatory mechanisms of p21 expression involve proteasome-mediated degradation, mRNA stability, alterations in the epigenetic silencing of the p21 promoter, and secondary decreases resulting from viral activity targeting p53, such as the activities of human papilloma virus and hepatitis C virus (17). However, its expression is considered to be regulated mainly at the transcriptional level (18). Accumulating data indicate that many molecules from diverse signaling pathways can activate or repress the p21 promoter, including p53, transforming growth factor-β (TGF-β), c-jun, Myc, Sp1/Sp3, signal transducers and activators of transcriptions, CAAT/enhancer binding protein-α (C/EBP-α), C/EBP-β, basic helix-loop-helix proteins, and myogenic differentiation 1 (reviewed in ref. 19). Thus, p21 is integrally involved in both cell cycle and apoptosis; therefore, identifying its regulatory molecules is of great importance.

We performed a microarray analysis of clinical samples of paired colorectal cancer (CRC) specimens and normal colonic mucosa specimens to identify genes that were overexpressed in CRC. Our results revealed that *FOXQ1* gene expression was ~28-fold higher in CRC than in normal colonic mucosa, and we hypothesized that FOXQ1 may play a role in CRC. In the present study, we investigated the biological function of FOXQ1.

Materials and Methods

Antibodies. The following antibodies were used: anti-p21, anti-p53, anti-cdk2, anti-cdk4, anti-cyclin D, anti-phosphorylated Rb, anti-poly(ADP-ribose) polymerase (PARP), anti-cleaved PARP, anti-caspase-3, anti-cleaved caspase-3, secondary antibodies, and Myc-tag mouse antibody (Cell Signaling), as well as anti-β-actin (Santa Cruz Biotechnology). A mouse anti-CD31 monoclonal antibody was purchased from BD Biosciences.

Cell lines and cultures. The DLD-1, MKN74, H1299, SBC3, and U251 cell lines were cultured in RPMI 1640 (Sigma). The WiDr, CoLo320DM, and human embryonic kidney cell line 293 (HEK293) cell lines were cultured in DMEM (Sigma), and the LoVo cell line was cultured in Ham/F12 medium [Life Technologies Bethesda Research Laboratories (BRL)]. All media were supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies BRL), and the cell lines were maintained in a 5% CO_2-humidified atmosphere at 37°C.

Patients and samples. Paired CRC and noncancerous colonic mucosa samples were evaluated using a microarray analysis in the first consecutive 10 patients. These samples and another 36 CRC samples were analyzed using real-time reverse transcription–PCR (RT-PCR). The RNA extraction method and the quality check protocol have been previously described (20). This study was approved by the institutional review board of the National Cancer Center Hospital, and written informed consent was obtained from all the patients.

Plasmid construction, viral production, and stable transfectants. The cDNA fragment encoding human full-length FOXQ1 was isolated using PCR and Prime STAR HS DNA polymerase (TaKaRa) with 5′-GGG AAT TCG CGG CCA TGA AGT TGG AGG TCT TCG TC-3′ and 5′-CCC TCG AGC GCT ACT CAG GCT AGG AGC GTC TCC AC-3′ sense and antisense primers, respectively. The methods used in this section have been previously described (21). Short hairpin RNA (shRNA) targeting p21 was constructed using oligonucleotides encoding small interfering RNA (siRNA) directed against p21 and a nonspecific target as follows: 5′-CTA AGA GTG CTG GGC ATT TTT-3′ for p21 shRNA and 5′-TGT TCG CAG TAC GGT AAT GTT-3′ for control shRNA. They were cloned into an RNAi-Ready pSIREN-RetroQZsGreen vector (Clontech) according to manufacturer's protocol. The stable transfectants expressing enhanced green fluorescent protein (EGFP) or FOXQ1 or FOXQ1 with shRNA targeting p21 for each cell line were designated as HEK293/EGFP, HEK293/FOXQ1, CoLo320/EGFP, CoLo320/FOXQ1, H1299/EGFP, H1299/FOXQ1, H1299/FOXQ1/sh-control, and H1299/FOXQ1/sh-p21. The FOXQ1 human cDNA was tagged at the NH_2 terminus with the myc epitope using the pCMV-Myc vector (Clontech) for chromatin immunoprecipitation (ChIP) assay.

SiRNA transfection. Two different sequences of siRNA targeting human FOXQ1 and negative control siRNA were purchased from QIAGEN. The sequences of FOXQ1 and control siRNA were as follows: FOXQ1#1 sense, 5′-CCA UCA AAC GUG CCU UAA A-3′ and antisense, 5′-UUU AAG GCA CGU UUG AUG G-3′; FOXQ1#4 sense, 5′-CGC GGA CUU UGC ACU UUG A-3′ and antisense, 5′-UCA AAG UGC AAA GUC CGC G-3′; control siRNA (scramble) sense, 5′-UUC UCC GAA CGU GUC ACG U-3′ and antisense, 5′-ACG UGA CAC GUU CGG AGA A-3′; control siRNA (GFP) sense, 5′-GCA AGC UGA CCC UGA AGU UCA U-3′ and antisense, 5′-GAA CUU CAG GGU CAG CUU GCC G-3′. The methods of transfection have been previously described (22).

Real-time RT-PCR and Western blot analysis. The methods used in this section have been previously described (21). The primers used for real-time RT-PCR were purchased from Takara as follows: FOXQ1 forward, 5′-CGC GGA CTT TGC ACT TTG AA-3′ and reverse, 5′-AGC TTT AAG GCA CGT TTG ATG GAG-3′; p21 forward, 5′-TCC AGC GAC CTT CCT CAT CCA C-3′ and reverse, 5′-TCC ATA GCC TCT ACT GCC ACC ATC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPD) forward, 5′-GCA CCG TCA AGG CTG AGA AC-3′ and reverse, 5′-ATG GTG GTG AAG ACG CCA GT-3′. The experiment was performed in triplicate.

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Luciferase reporter assay. The human p21 promoter reporter vector was constructed according to a previously described method (13). The p21 promoter fragment was cut between the *Kpn*I and *Xho*I restriction sites and was transferred into the luciferase reporter vector pGL4.14 (Promega). All sequences were verified using DNA sequencing. The empty and p21 promoter-containing reporter vectors were designated as pGL4.14-mock and pGL4.14-p21, respectively. All the samples were examined in triplicate.

ChIP. ChIP was carried out using the ChIP-IT Express Enzymatic kit (Active Motif) according to manufacturer's protocol. HEK293 cells were transfected with empty vector (Myc) or Myc-tagged FOXQ1 vector. The putative region of the p21 promoter (–2264 to –1971) was amplified with the following primers: 5′-TTG AGC TCT GGC ATA GAA GA-3′ (forward) and 5′-TAC CCA GAC ACA CTC TAA GG-3′ (reverse). As a negative control, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) second intron promoter was amplified with the following primers: 5′-AAT GAA TGG GCA GCC GTT AG-3′ (forward) and 5′-AGC TAG CCT CGC TCC ACCTGA C-3′ (reverse).

Xenograft studies. Two separate xenograft studies were performed independently. Nude mice (*BALB/c nu/nu*; 6-week-old females; CLEA Japan, Inc.) were used for the *in vivo* studies and were cared for in accordance with the recommendations for the Handling of Laboratory Animals for Biomedical Research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animals Experiments, Kinki University. The ethical procedures followed and met the requirements of the United Kingdom Coordinating Committee on Cancer Research guidelines (23). To assess tumorigenicity, suspensions of 1×10^6 H1299/EGFP or H1299/FOXQ1 cells (in 0.1 mL PBS) were s.c. injected into the left or right flanks of nude mice (n = 15), respectively. To evaluate tumor growth, a suspension of 6×10^6 H1299/EGFP, H1299/FOXQ1, H1299/FOXQ1/sh-control, and H1299/FOXQ1/sh-p21 cells (in 0.1 mL PBS) were s.c. inoculated (n = 10) into nude mice. The tumor volume was calculated as length × width^2 × 0.5. The tumor formation was assessed every 2 to 3 d. At the end of the experiment, the mice were sacrificed and the xenografts were resected, fixed in 10% buffered formalin for 6 to 10 h, and processed for histologic analysis.

Immunohistochemical and immunofluorescence staining. The methods used in this section have been previously described (24, 25).

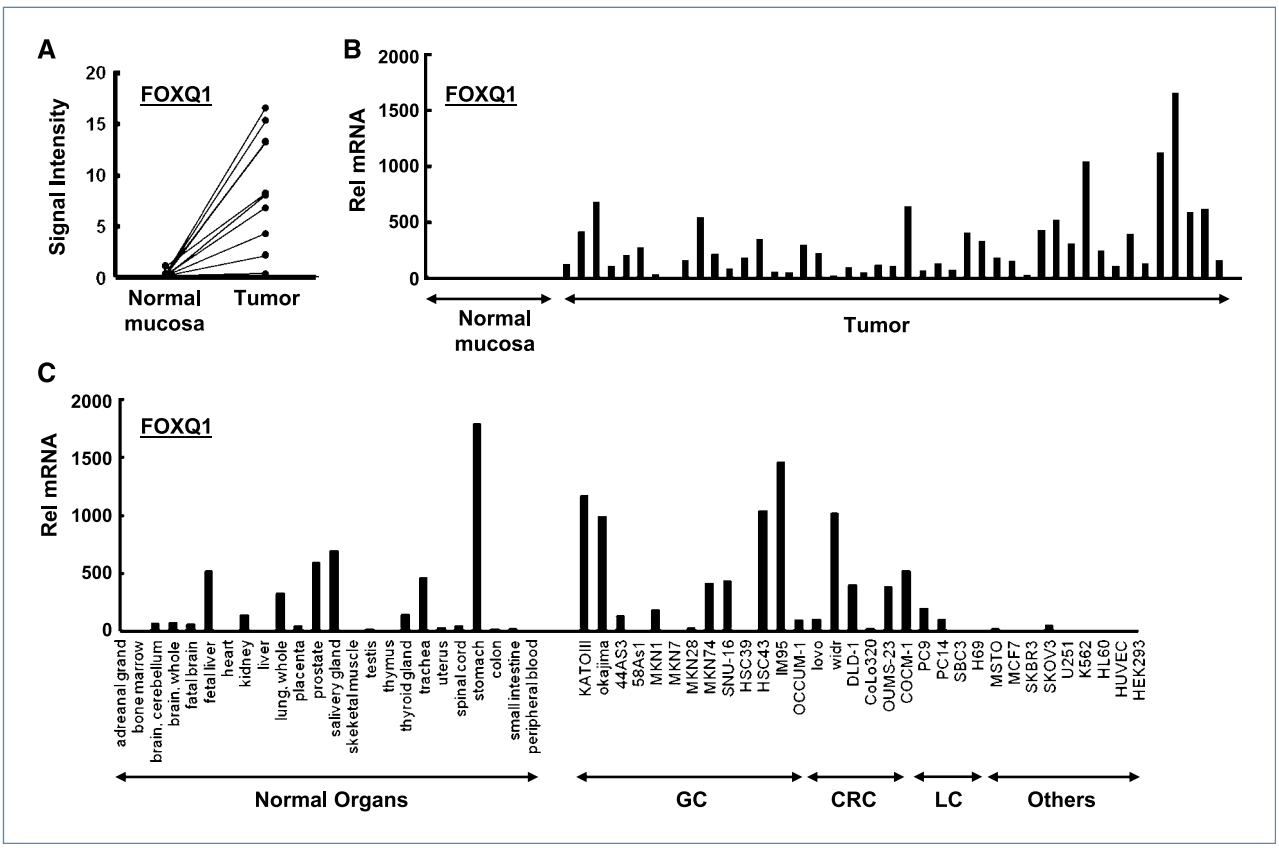

Figure 1. *FOXQ1* expression in CRC. A, mRNA expression of *FOXQ1* obtained from a microarray analysis of 10 CRC and paired normal mucosa specimens. The values indicate the normalized signal intensity. B, the mRNA expression levels of *FOXQ1* were determined using real-time RT-PCR for 10 paired and an additional 36 CRC samples. C, the mRNA expression levels of *FOXQ1* were determined using a real-time RT-PCR analysis of human normal tissue (left) and 30 human cancer cell lines, HEK293, and human umbilical vascular endothelial cell (HUVEC) cell lines (right). GC, gastric cancer; LC, lung cancer; Rel mRNA, normalized mRNA expression levels (*FOXQ1/GAPD* × 10^4).

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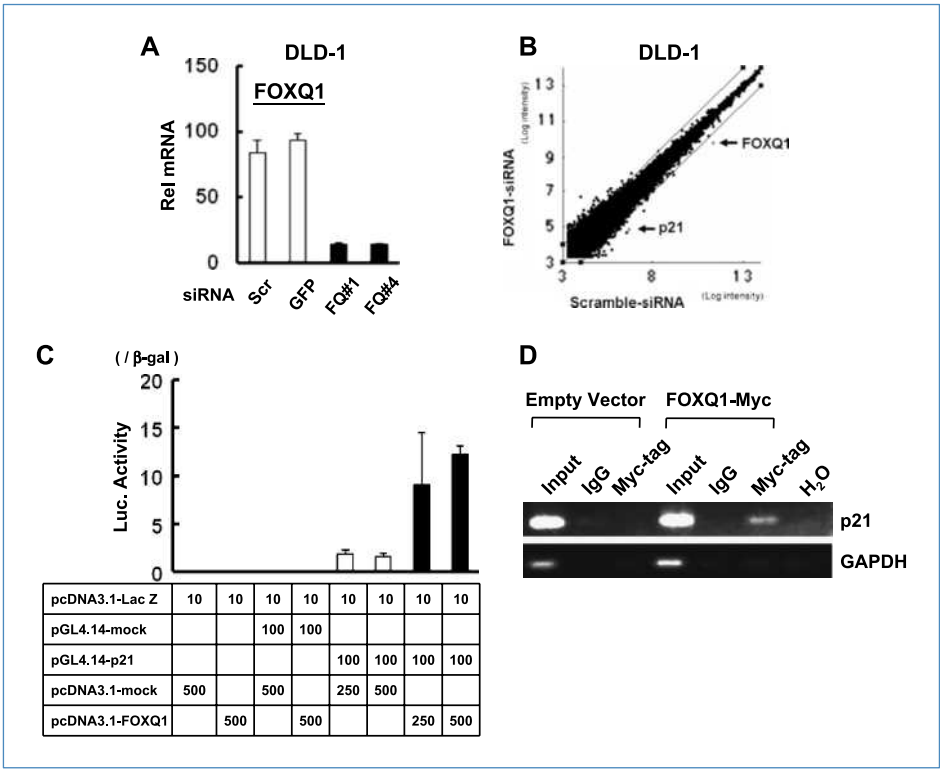

pcDNA3.1-Lac Z	10	10	10	10	10	10	10	10
pGL4.14-mock			100	100				
pGL4.14-p21					100	100	100	100
pcDNA3.1-mock	500		500		250	500		
pcDNA3.1-FOXQ1		500		500			250	500

Figure 2. FOXQ directly regulates *p21* transcription. A, FOXQ1-targeting siRNA (FQ#1 and FQ#4) suppressed FOXQ1 expression in DLD-1 cells. The mRNA expression levels of *FOXQ1* were determined using real-time RT-PCR. B, microarray analysis of DLD-1 cells transfected with control-siRNA or FOXQ1-siRNA. The longitudinal axis indicates the mRNA expression of FOXQ1-siRNA transfected cells and the horizontal axis indicates that of control-siRNA. Arrow, FOXQ1 or p21 expression. Each point indicates the normalized and log base 2 transformed microarray data. C, induction of p21 promoter activity by FOXQ1. Luciferase vectors with either an empty or p21 promoter (pGL4.14-mock or pGL4.14-p21) were transiently cotransfected with a mock or FOXQ1 expression plasmid (pcDNA3.1-mock or pcDNA3.1-FOXQ1) expressing β-galactosidase as an internal control. The results were normalized to β-galactosidase activity and are representative of at least three independent experiments. D, ChIP of FOXQ1 on the promoter of *p21*. HEK293 cells were transfected with empty vector (Myc) or Myc-tagged FOXQ1 vector. Agarose gel shows PCR amplification (35 cycles) of the *p21* promoter using inputs (1% of chromatin used for ChIP) or ChIPs as templates. Primers to the *GAPDH* promoter were used as the negative control.

Microarray analysis. The microarray procedure and analysis were performed according to the Affymetrix protocols and BRB Array Tools software, Ver. 3.3.0,[4] developed by Dr. Richard Simon and Dr. Amy Peng, as reported previously (21, 26).

Statistical analysis. The statistical analyses were performed using Microsoft Excel (Microsoft) to calculate the SD and to test for statistically significant differences between the samples using a Student's *t* test. A *P* value of <0.05 was considered statistically significant.

Results

FOXQ1 mRNA was overexpressed in CRCs. A microarray analysis for 10 paired CRC samples identified 30 genes as being significantly upregulated by >10-fold in CRC ($P < 0.001$; Supplementary Table S1). *FOXQ1*, an uncharacterized transcription factor, was upregulated by 28-fold in the CRC specimens (Fig. 1A), exhibiting the fourth highest level of upregulation [after interleukin-8, matrix metalloproteinase-1 (MMP), and MMP-3]. Real-time RT-PCR for the 10 paired samples and an additional 36 CRC samples showed that *FOXQ1* mRNA was markedly overexpressed in the CRC samples but was only expressed at a very low level in noncancerous colonic mucosa ($P < 0.001$; Fig. 1B). The average levels of *FOXQ1* expression were 299 ± 326 and 4.0 ± 5.0 ($\times 10^4$/GAPD), respectively.

FOXQ1 expression in normal tissues and cancer cell lines. To investigate the expression of *FOXQ1*, we analyzed the mRNA expression levels of *FOXQ1* in panels of human normal tissues and cancer cell lines using real-time RT-PCR. High levels of *FOXQ1* expression were observed in the stomach, salivary gland, prostate, trachea, and fetal liver among the 24 normal tissues that were examined (Fig. 1C, left). Relatively weak expression levels were detected in brain-derived tissues, kidney, lung, placenta, and thyroid gland. These results were consistent with those of a previous report (27).

[4] http://linus.nci.nih.gov/BRB-ArrayTools.html

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In the cancer cell line panel, the mRNA expression levels of *FOXQ1* were higher in gastric cancer, CRC, and lung cancer cell lines than in the other cancer cell lines, indicating that the expression of *FOXQ1* varies among specific cancers (Fig. 1C, right). Interestingly, the overexpression of *FOXQ1* in CRC arose from normal colonic mucosa with very low expression levels during carcinogenesis.

p21 is a target gene of FOXQ1. To examine the function of FOXQ1 as a transcription factor and to explore its target genes, we performed a microarray analysis using a CRC cell line, DLD-1, transfected with FOXQ1-targeting siRNA or control siRNA. Two sequences of FOXQ1-siRNA, FQ#1 and FQ#4, were used to exclude the off-target effect of siRNA. Real-time RT-PCR showed that both sequences of FOXQ1-siRNA suppressed *FOXQ1* mRNA expression by ~80% in DLD-1 cells (Fig. 2A); thus, FQ#4 was used as the FOXQ1-siRNA in the following experiments. A microarray analysis showed that 19 genes were downregulated by FOXQ1-siRNA (Fig. 2B; Supplementary Table S2); *p21* was the fifth most-downregulated gene. Because p21 is a key regulator of cell cycle and apoptosis, we focused on p21 as a target molecule of FOXQ1.

To confirm the microarray data, p21 downregulation by FOXQ1-siRNA was examined using real-time RT-PCR and a

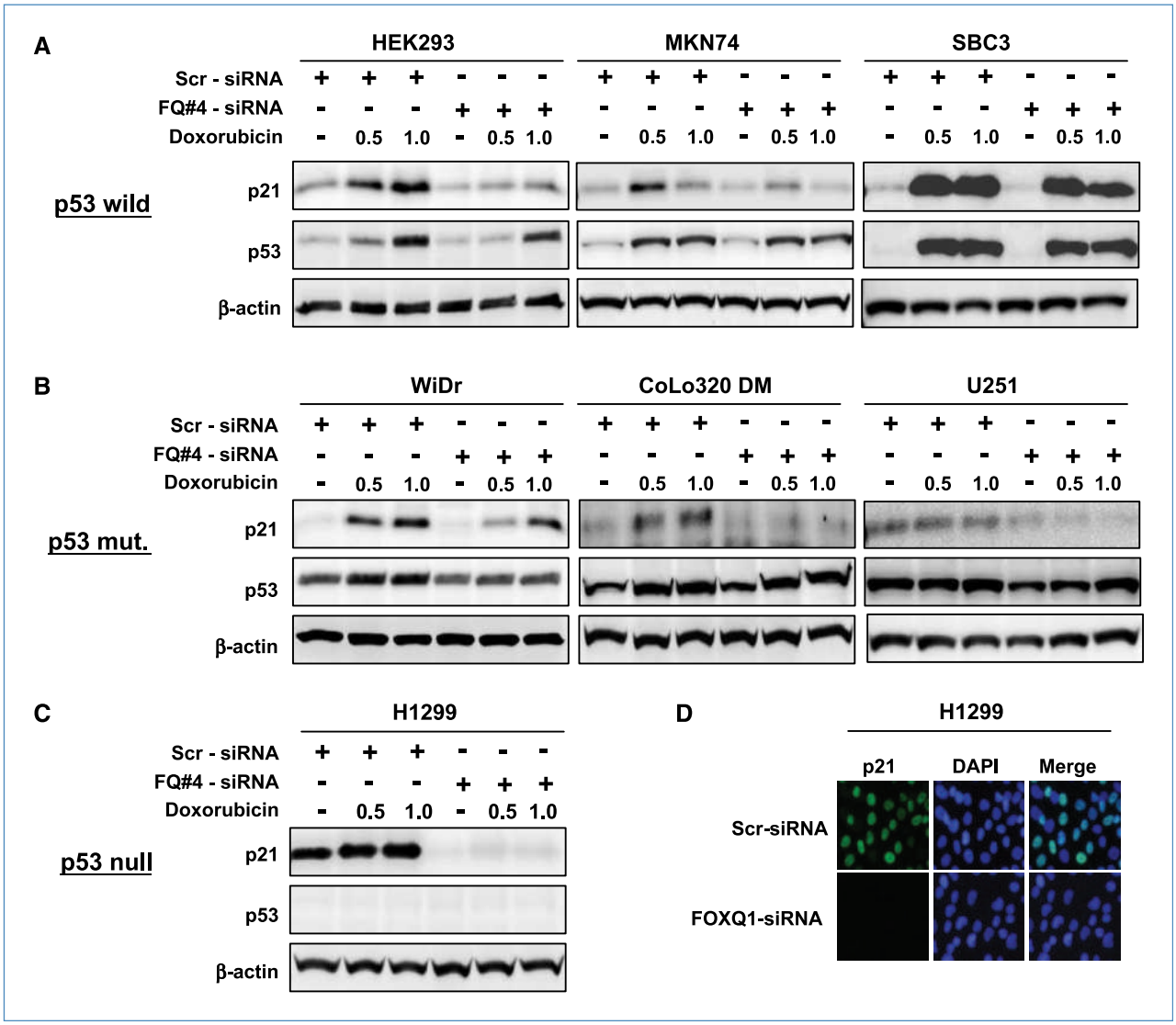

Figure 3. p21 induction by FOXQ1 and p53 status in cancer cells. The seven cell lines were transfected with control-siRNA or FOXQ1-siRNA for 24 h, and the cells were exposed to doxorubicin at a final concentration of 0.5 or 1 μmol/L for a further 24 h to enhance p21 induction. Western blot analyses for p21 and p53 were performed in three p53–wild type cell lines (A), three p53-mutant cell lines (B), and one p53-null cell line (C). The experiment was performed in duplicate. D, immunofluorescence p21 staining and 4′,6-diamidino-2-phenylindole (DAPI) staining for H1299 cells transfected with control-siRNA (top) or FOXQ1-siRNA (bottom) for 48 h. Scr, scramble-siRNA (control); FQ#4, FOXQ1-targeting siRNA. β-Actin was used as an internal control.

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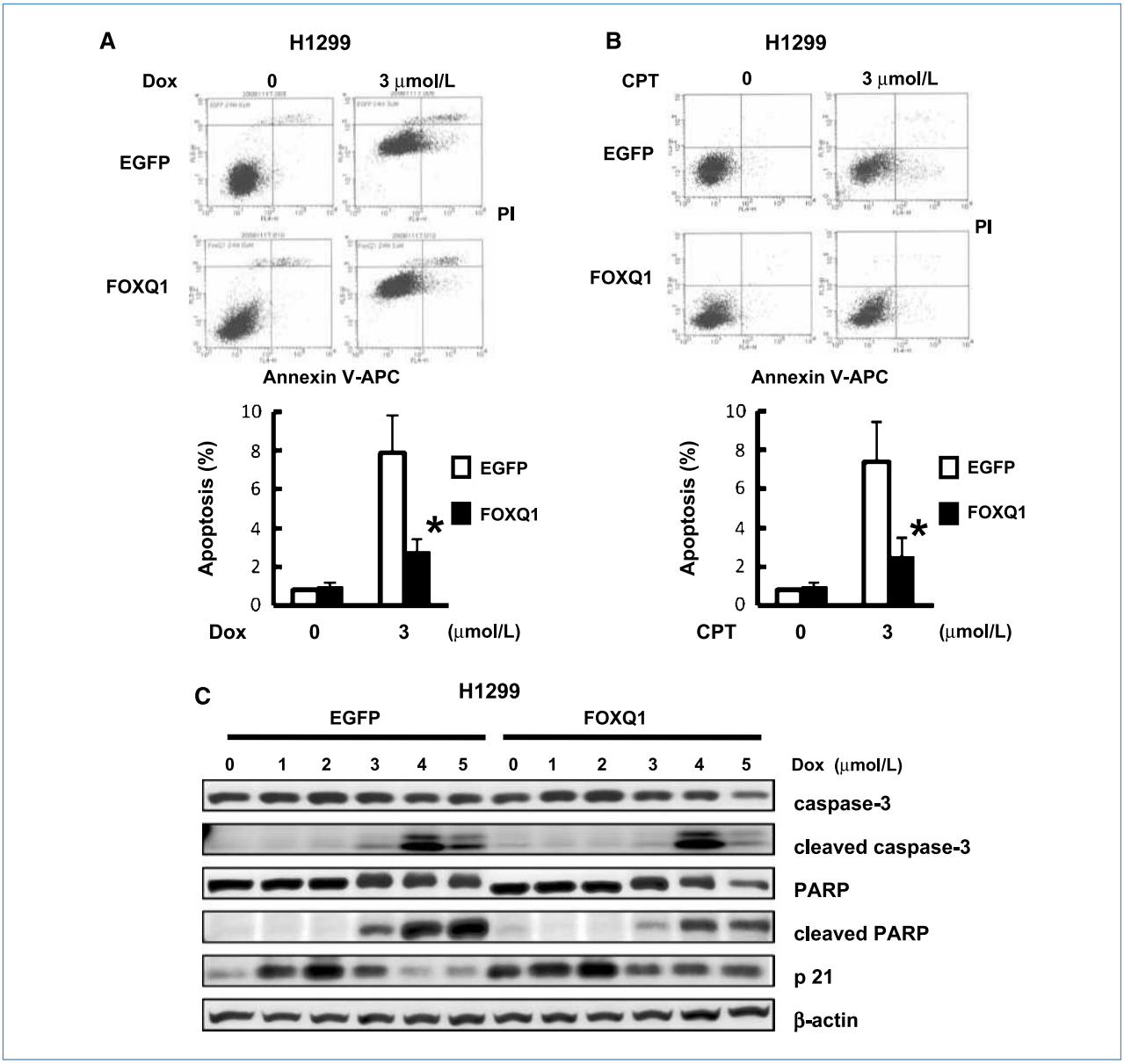

Figure 4. Overexpression of FOXQ1 promotes an antiapoptotic effect. Stable H1299 cell lines expressing EGFP or FOXQ1 (H1299/EGFP, H1299/FOXQ1) were exposed to doxorubicin (A) or camptothecin (B) at a final concentration of 3 μmol/L. Apoptotic cells were detected by Annexin V and propidium iodide (PI) using flow cytometry. C, Western blot analysis for apoptosis-related molecules. EGFP- or FOXQ1-expressing cells were exposed to doxorubicin at the indicated doses (0–5 μmol/L) for 24 h. β-Actin was used as an internal control. Dox, doxorubicin; CPT, camptothecin; EGFP, H1299/EGFP; FOXQ1, H1299/FOXQ1. *, $P < 0.05$.

Western blot analysis in DLD-1 cells. The results indicated that both sequences of FOXQ1-siRNA (FQ#1 and FQ#4) downregulated p21 expression at both the mRNA and protein levels. In addition, we confirmed the downregulation of p21 by FOXQ1-siRNA in other cell lines (WiDr and HEK293), obtaining similar results (Supplementary Fig. S1).

FOXQ1 directly increases the transcription activity of p21. We performed a luciferase reporter assay to determine whether FOXQ1 regulates p21 expression at the transcriptional level. A 2.4-kb section of the p21 promoter region was subcloned into a luciferase vector according to a previously described method (13, 28). The p21 promoter activity was increased by >8-fold when cotransfected with a FOXQ1 expression vector, compared with an empty vector (Fig. 2C). To determine whether FOXQ1 directly binds to p21 promoter, we transfected Myc or Myc-tagged FOXQ1 vectors into HEK293 cells and then conducted ChIP experiments. A segment of the *p21* promoter containing putative FOXQ1 binding site (−2264 to −1971) is precipitated with specific antibody, only if, FOXQ1 was induced (Fig. 2D).

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The result indicates that FOXQ1 binds to the *p21* promoter and upregulates p21 transcriptional activity.

p53-independent p21 induction by FOXQ1 in cancer cells. Because p53 is the most important regulatory molecule of p21, we examined the downregulation of p21 by FOXQ1-siRNA in several cell lines with p53–wild type, p53-mutant, or p53-null statuses. These cell lines were transfected with control-siRNA or FOXQ1-siRNA, and p21 induction was enhanced by doxorubicin (29–31). The experiments were performed using three p53–wild type cell lines, three p53-mutation cell lines, and one p53-null cell line (Fig. 3A–C). Without doxorubicin exposure, all seven cell lines showed that p21 expression was downregulated by FOXQ1-siRNA. Notably, with doxorubicin exposure, considerable p21 downregulation by FOXQ1-siRNA was observed in the p53-mutation and p53-null cell lines, compared with in the p53–wild type cell lines. In the p53-null H1299 cell line, FOXQ1-siRNA completely suppressed p21 expression. These results suggest that p21 induction by FOXQ1 is p53 independent. An immunofluorescence study of p21 in H1299 cells also showed that p21 was completely downregulated by FOXQ1-siRNA (Fig. 3D).

Overexpression of FOXQ1 increases p21 expression and exhibits an antiapoptotic effect in cancer cells. Next, we established a stable *FOXQ1*-overexpressing cell line to confirm the induction of p21 expression by FOXQ1 and to detect any changes in the cellular phenotype of the cancer cells. FOXQ1 overexpression induced p21 expression (both mRNA and protein) in HEK293 and CoLo320 cells (Supplementary Fig. S1). Notably, p21 protein expression was markedly induced by >10-fold in the H1299/FOXQ1 cells (Supplementary Fig. S1). These results indicated that FOXQ1 robustly induces p21 expression, consistent with the findings of the siRNA study.

p21 induces an antiapoptotic effect and exerts a protective role against apoptosis induced by DNA damage. To

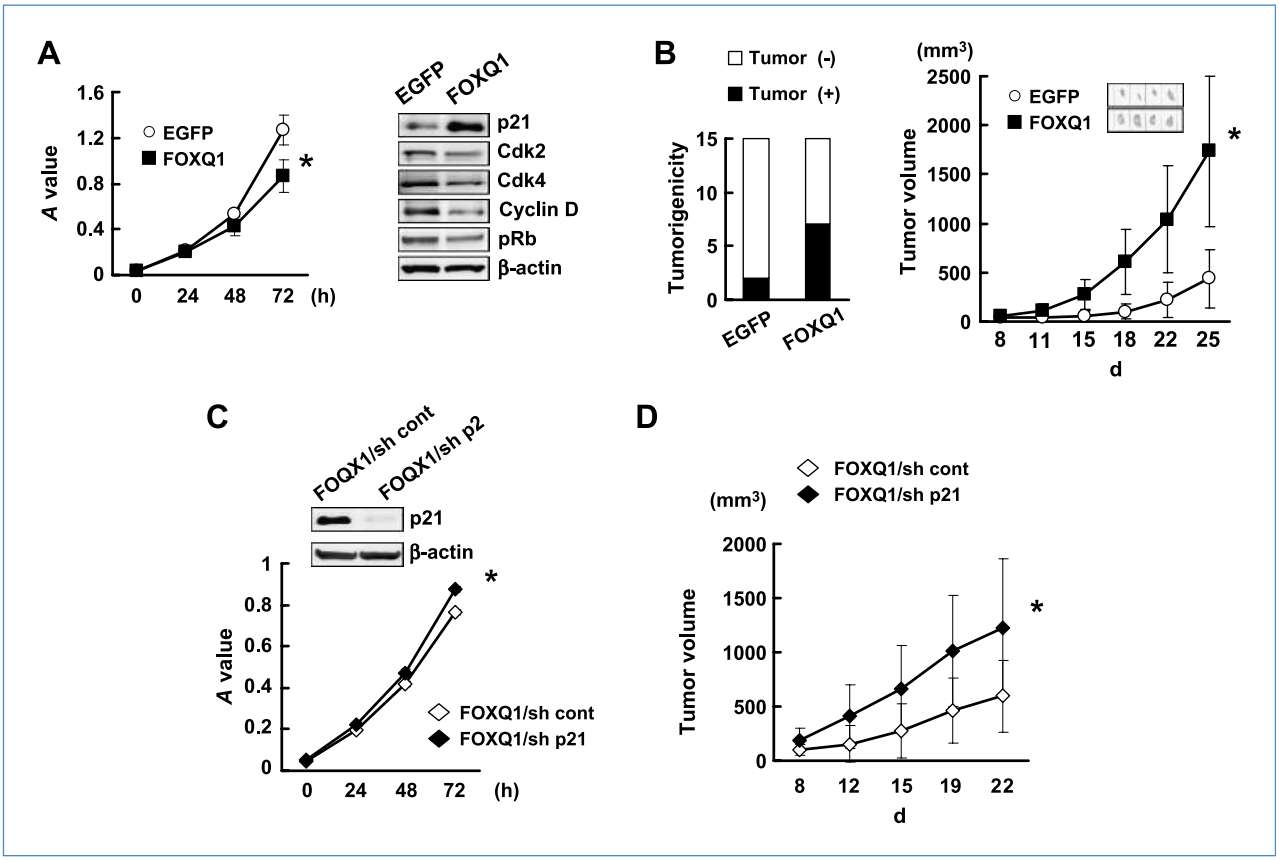

Figure 5. Overexpression of FOXQ1 enhances tumorigenicity and tumor growth *in vivo*. A, cellular growth and immunoblotting analysis of H1299 cell lines stably expressing EGFP or FOXQ1 (H1299/EGFP, H1299/FOXQ1). A total of 2×10^3 cells of each cell line were seeded in 96-well plates and evaluated after 0, 24, 48, and 72 h using MTT assay. Error bars, SD. Protein levels of H1299/EGFP and H1299/FOXQ1 cells were examined by Western blotting using specific antibody to p21, Cdk2, Cdk4, cyclin D, and phosphorylated Rb (pRb) protein. β-Actin was used as an internal control. EGFP, stable EGFP-overexpressing cells; FOXQ1, stable FOXQ1-overexpressing cells. B, H1299/EGFP and H1299/FOXQ1 cells were evaluated for their tumorigenicity *in vivo*. Mice ($n = 15$) were s.c. inoculated with a total of 1×10^6 cells. The numerical data indicate the number of mice. A total of 6×10^6 H1299/EGFP or H1299/FOXQ1 cells were s.c. inoculated into the right flank of each mouse to evaluate the tumor growth *in vivo* ($n = 12$). Representative H&E staining of tumor specimens was also shown. C, stable p21 knockdown or control cells obtained from H1299/FOXQ1 cells (H1299/FOXQ1/sh-control and H1299/FOXQ1/sh-p21) were evaluated for cellular growth and immunoblotting analysis. D, a total of 6×10^6 H1299/FOXQ1/sh-control or H1299/FOXQ1/sh-p21 cells were s.c. inoculated into the right flank of each mouse to evaluate the tumor growth ($n = 10$). *, $P < 0.05$.

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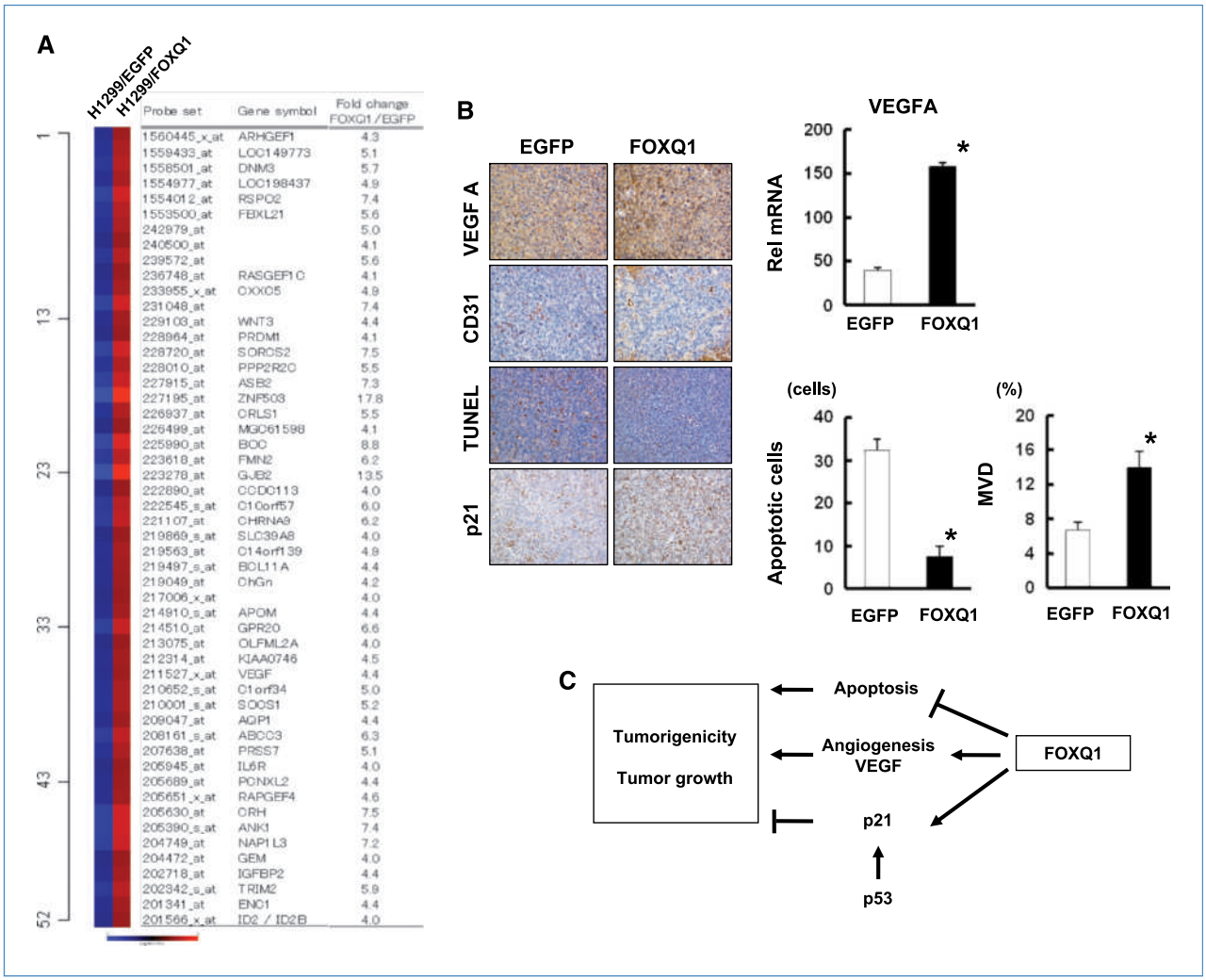

Figure 6. FOXQ1 promotes angiogenic and antiapoptotic effects *in vivo*. A, microarray analysis for H1299/EGFP or H1299/FOXQ1 cells. The upregulated genes over 4-fold by FOXQ1 were shown in the list. B, the mRNA expression levels of *VEGFA* were determined using a real-time RT-PCR analysis. Rel mRNA, normalized mRNA expression levels (*VEGFA*/*GAPD* × 10^4). VEGF, CD31, TUNEL, and p21 staining of tumor specimens inoculated with H1299/EGFP or H1299/FOXQ1 cells. Microvessel density (MVD) was determined by CD31-positive endothelial cells in tumor specimens using computer-assisted image analysis (Image J software package). C, diagram of a proposed mechanism of FOXQ1 for tumorigenicity and tumor growth. *, $P < 0.05$.

elucidate the role of apoptosis induced by FOXQ1 in cancer cells, we examined the apoptotic effect in H1299/EGFP and H1299/FOXQ1 cells using anticancer drugs. The overexpression of FOXQ1 inhibited the apoptosis induced by doxorubicin (H1299/EGFP: 7.9 ± 1.9%, H1299/FOXQ1: 2.7 ± 0.7%; Fig. 4A). Similarly, camptothecin-induced apoptosis was also inhibited in FOXQ1-overexpressing cells (H1299/EGFP: 7.4 ± 2.1%, H1299/FOXQ1: 2.5 ± 1.0%; Fig. 4B). Western blotting revealed that FOXQ1 overexpression decreased the levels of cleaved caspase-3 and cleaved PARP induced by doxorubicin (Fig. 4C). These results are consistent with those obtained using flow cytometry.

***Overexpression of FOXQ1 decreases cellular proliferation but enhances tumorigenicity and tumor growth* in vivo.** Stable H1299/FOXQ1 cells showed decreased cellular proliferation compared with control cells *in vitro* (Fig. 5A). Expressions of Cdk4, cyclin D1, and Cdk2 were decreased by FOXQ1 expression in H1299/FOXQ1 cells and resulted in a decrease of phosphorylated Rb expression (Fig. 5A). To examine the biological functions of FOXQ1 overexpression *in vivo*, we evaluated tumorigenicity and tumor growth using H1299/EGFP or H1299/FOXQ1 cells. H1299/FOXQ1 cells exhibited a significantly elevated level of tumorigenesis *in vivo* (GFP 2/15, FOXQ1 7/15, $P < 0.05$; Fig. 5B). In addition, the tumor volume was markedly larger in H1299/FOXQ1 cells than in H1299/EGFP cells (EGFP: 437 ± 301, FOXQ1: 1735 ± 769 mm^3, $P < 0.001$; Fig. 5B) on day 25.

***p21 does not contribute to FOXQ1-mediated tumor growth* in vivo.** Because emerging evidence has indicated that p21 may have dual functions with regard to tumor

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progression and the suppression of cancer cells (32, 33), the shRNA targeting p21 or shRNA control viral vectors were further introduced into the H1299/FOXQ1 cells to elucidate the involvement of p21 in increased FOXQ1-mediated tumorigenicity and tumor growth *in vivo*. Stable H1299/FOXQ1/sh-p21 cells were slightly increased in cellular proliferation *in vitro* (Fig. 5C). In addition, tumor growth of H1299/FOXQ1/sh-p21 cells was increased compared with control cells *in vivo* (Fig. 5D). The results clearly indicate that p21 has negative roles for cellular proliferation and tumor growth in FOXQ1-overexpressing cells, suggesting that p21 does not contribute to FOXQ1-mediated tumor growth in FOXQ1-overexpressing cells *in vivo*.

***Overexpression of FOXQ1 promotes angiogenesis and antiapoptosis* in vivo.** To gain an insight into the mechanism by which FOXQ1 enhances tumor growth *in vivo*, we performed the microarray analysis on H1299/EGFP and H1299/FOXQ1 cells. Fifty-two genes were upregulated over 4-fold by overexpression of FOXQ1 including several genes that have positive roles for tumor growth, such as *VEGFA, WNT3A, RSPO2*, and *BCL11A* (Fig. 6A). Overexpression of FOXQ1 upregulated the *VEGFA* expression for 4.4-fold, suggesting the possibility of enhanced angiogenesis. Real-time RT-PCR for these cells and vascular endothelial growth factor (VEGF) staining of tumor specimens confirmed the result (Fig. 6B). Furthermore, CD31 staining of the tumor specimens showed that FOXQ1 overexpression significantly increased the angiogenesis *in vivo*.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and p21 immunostaining of the tumor specimens showed that p21 expression was increased and apoptosis was inhibited in H1299/FOXQ1 cells (Fig. 6B). These results strongly suggest that FOXQ1 promotes tumorigenicity and tumor growth with its angiogenic and antiapoptotic properties *in vivo* (Fig. 6C).

Discussion

FOX transcription factors are an evolutionarily conserved superfamily that control a wide spectrum of biological processes. Several Fox gene family members are involved in the etiology of cancer. Only the FOXO family has been regarded as *bona fide* tumor suppressors that promote apoptosis and cell cycle arrest at G_1 (34, 35). The loss of FOXO function observed in alveolar rhabdomyosarcoma through chromosomal translocation was first identified in relation to cancer. Many target genes of FOXO have been reported to date, including p21, cyclin D, Bim, TRAIL, and ER-α (36). On the other hand, the overexpression of FOXM is observed in head and neck cancer, breast cancer, and cervical cancer, and it enhances proliferation and tumor growth *in vitro* (37), suggesting that *FOXM* may be an oncogene. Although the available evidence is not conclusive, FOXP, FOXC, and FOXA have been linked to tumorigenesis and progression of certain cancers (36). Thus, the FOX family is thought to act as either an oncogene or a tumor suppressor. In the present study, we showed that the overexpression of FOXQ1 played a tumor-promoting role in CRC.

The p21 promoter region contains several definitive DNA regulatory elements, such as the p53-binding domain, E-box, Smad binding element, and TGF-β response elements. In the case of the other FOX family member FOXO, a recent report showed that the p21 promoter contains a consensus forkhead binding element (GGATCC) immediately upstream of the first Smad binding element and that the FOXO and Smad complexes activate p21 expression, whereas the FOXG1 protein binds to FOXO and blocks p21 induction (38). On the other hand, the consensus binding sequence (5′-NA(A/T)TGTTTA(G/T)(A/T)T-3′) has been defined for human FOXQ1 (4). The p21 promoter region contains several putative FOXQ1 binding sites according to its consensus binding sequence. Indeed, we have shown that FOXQ1 binds to a segment of the p21 promoter, indicating that FOXQ1 directly transactivates the p21 gene expression.

The initial descriptions of p21 were thought to indicate a tumor suppressor–like role, and p21 was almost solely regarded as a modulator with the principal function of inhibiting a cyclin-dependent kinase activity and, hence, cell cycle progression, because it was originally identified as a mediator of p53-induced growth arrest. However, emerging evidence has indicated that p21 may have dual functions with regard to tumor progression and the suppression of cancer cells, with examples of other genes with dual functions including TGF-β, Notch, Runx3, E2F, and p21 (32). Besides its growth inhibitory role, p21 is known to have a positive effect on cell proliferation (39–41). A more recent study on leukemic stem cells showed a p21-dependent cellular response that leads to reversible cell cycle arrest and DNA repair; such data clearly illustrate the oncogenic potential of p21 (33). We have shown that p21 has negative roles for tumor growth using FOXQ1-overexpressing cells with knockdown of p21 (Fig. 5D).

Recently, accumulating evidence has shown that FOX transcriptional factors are involved in VEGF regulation and angiogenesis. For example, forkhead has exhibited a positive role in mediating induction of VEGF (42–44). In the present study, we identified *VEGFA* as a candidate target gene of FOXQ1 by microarray analysis and showed that FOXQ1 increased angiogenesis *in vivo*. Interestingly, although overexpression of FOXQ1 decreases cellular proliferation *in vitro*, it enhances tumorigenicity and tumor growth *in vivo*. We consider that this discrepancy can be explained by these angiogenic and antiapoptotic effects of FOXQ1 contribute to enhanced tumor growth *in vivo*, although p21 negatively functions.

We showed that the overexpression of FOXQ1 inhibited doxorubicin-induced and camptothecin-induced apoptosis in p53-inactivated cancer cells. Therefore, we speculated that FOXQ1 might be a new determinant factor of resistance to drug-induced apoptosis and might represent a poor prognostic factor for CRC patients.

In conclusion, FOXQ1 is markedly overexpressed in CRC and enhances tumorigenicity and tumor growth *in vivo*. We have elucidated a biological function of FOXQ1, which directly upregulates p21 transcription and promotes angiogenesis and antiapoptosis. Our findings support FOXQ1

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as a new member of the cancer-related FOX family in cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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