Pre-Clinical Research Report

Journal of INTERNATIONAL MEDICAL RESEARCH

Journal of International Medical Research
48(12) 1–14

DOI: 10.1177/0300060520971453
journals.sagepub.com/home/imr
SAGE

Knockdown of Forkhead box A1 suppresses the tumorigenesis and progression of human colon cancer cells through regulating the phosphatase and tensin homolog/Akt pathway

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Abstract

Background: This study aimed to evaluate the role and the underlying mechanisms of Forkhead box A1 (encoded by *FOXA1*) in colon cancer.

Methods: We analyzed *FOXA1* mRNA and protein expression in colon cancer tissues and cell lines. We also silenced *FOXA1* expression in HCT116 and SW480 cells to evaluate the effects on cell proliferation, cell cycle, migration, and invasion by using MTT, colony formation, flow cytometry, and the Transwell assay, respectively.

Results: FOXA1 immunostaining was higher in colon cancer tissues than adjacent healthy tissues. *FOXA1* mRNA and protein expression was significantly increased in human colon cancer cells compared with a normal colonic cell line. *FOXA1* expression was also significantly higher in colorectal cancer tissues from TCGA data sets and was associated with worse prognosis in the R2 database. *FOXA1* expression was negatively correlated with the extent of its methylation, and

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its knockdown reduced proliferation, migration, and invasion, and induced G2/M phase arrest in HCT116 and SW480 cells by suppressing the phosphatase and tensin homolog/Akt signaling pathway and inhibiting epithelial–mesenchymal transition.
Conclusion: *FOXA1* may act as an oncogene in colon cancer tumorigenesis and development.

Keywords
Colon cancer, Forkhead box A1, proliferation, migration, invasion, phosphatase and tensin homolog/Akt signaling pathway

Date received: 12 March 2020; accepted: 14 October 2020

Introduction

Colon cancer is one of the most common types of malignancy worldwide.[1] Currently, more than 1 million new cases are reported each year, and an estimated 700,000 deaths are caused by colon cancer annually.[2] Tumor invasion and metastasis are major treatment challenges and the main cause of colon cancer mortality. The occurrence and development of colon cancer is a complex but poorly understood process involving multiple factors, such as the activation of oncogenes and the inactivation of tumor suppressor genes.[3] A better understanding of the pathogenesis of colon cancer development, invasion, and metastases is important in its early diagnosis and treatment.[4]

Forkhead box A1 (encoded by the *FOXA1* gene), also known as hepatic nuclear factor 3α, is a member of the human forkhead box transcription factor family, which is critical for both early embryonic development and late or end-stage epithelial differentiation.[5] Accumulating evidence has demonstrated that FOXA1 participates in the development and progression of cancer because it regulates a series of biological processes, including cell proliferation, apoptosis, differentiation, migration, and invasion.[6] Recent studies have detected amplification of *FOXA1* in various types of cancers, such as prostate,[7] ovarian,[8] and endometrial[9] cancers, suggesting that FOXA1 could be a novel biomarker for cancer diagnosis and prognosis.

FOXA1 can act as an oncogenic or tumor suppressor gene depending on the type of tumor.[10] In pancreatic cancer, the expression of FOXA1 in cancerous tissues was significantly lower than in healthy epithelium and precancerous lesions, and loss of FOXA1/2 was observed during the epithelial–mesenchymal transition (EMT).[11] Together, these findings indicate a potentially dynamic perturbation of FOXA1 in cancer progression. However, only two studies have reported the role of FOXA1 in human colorectal cancer (CRC).[12,13] Therefore, we aimed to elucidate the potential biological function and the underlying mechanism of FOXA1 in human colon cancer.

We observed increased expression of *FOXA1* in human colon cancer tissues and cell lines. Knockdown of *FOXA1* suppressed the proliferation, colony formation, invasion, and migration of colon cancer cells by inactivating the phosphatase and tensin homolog (PTEN)/Akt signaling pathway. Our findings suggest that *FOXA1* functions as an oncogene in the tumorigenesis and development of colon cancer.

Materials and methods

Reagents

Roswell Park Memorial Institute (RPMI)-1640, Dulbecco's Modified Eagle Medium (DMEM), and 10% fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl-tetrazolium bromide (MTT) and propidium iodide (PI) were purchased from Sigma Chemical (St. Louis, MO, USA). Crystal violet, radioimmunoprecipitation assay (RIPA) lysis buffer, and the bicinchoninic acid (BCA) protein assay kit were from Beyotime Biotechnology (Shanghai, China). 3, 3-diaminobenzidine (DAB) was obtained from Dako (Santa Clara, CA, USA). The Transwell chamber was purchased from Millipore (Bedford, MA, USA). Primary antibodies against FOXA1, p-Akt, Akt, PTEN, vimentin, N-cadherin, E-cadherin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Abcam (Cambridge, UK). TRIzol reagent, PrimeScript™ RT reagent Kit with gDNA Eraser and SYBR Premix Ex Taq were purchased from Takara Bio Inc. (Dalian, China). Lipofectamine® 3000 was obtained from Invitrogen (Carlsbad, CA, USA).

Tissue samples

Tissue samples were obtained from 20 patients with primary colon carcinoma who had undergone surgery at Fujian Medical University Union Hospital. All patients were diagnosed by pathological examinations, and did not receive radiotherapy or chemotherapy before surgery. The Research Ethics Committee of Fujian Medical University Union Hospital approved this study and written informed consents were obtained from all patients. Colon cancerous and corresponding healthy tissue samples (at least 5 cm from the carcinoma tissue) were immediately snap-frozen in liquid nitrogen after surgery and stored at −80°C for Western blotting.

Cell culture

Human colon cancer cell lines HCT116, HT-29, SW480, LOVO, and normal human CCD-18co colon cells were purchased from the Shanghai Institute of Cell Resource Center Life Science (Shanghai, China). 293T cells were purchased from the Shanghai Institute of Cell Resource Center Life Science (Shanghai, China). Cells were cultured in RPMI-1640 or DMEM supplemented with 10% FBS, 100 U/mL penicillin–streptomycin in a humidified incubator at 37°C with 5% CO_2 and maintained in a logarithmic growth phase for all experiments.

Bioinformatics analysis of FOXA1

To detect the differential expression of *FOXA1* between tumor and healthy tissues, we carried out Gene Expression Profiling Interactive Analysis (GEPIA)[14] of The Cancer Genome Atlas (TCGA) dataset (http://gepia.cancer-pku.cn/). Additionally, we investigated the prognostic value of FOXA1 for CRC patients using the R2: Genomics Analysis and Visualization Platform[15] (http://r2.amc.nl), and determining the best gene cut-off value. *FOXA1* methylation data between CRC and healthy tissues from TCGA were obtained from MethHC,[16] which is a database for human pan-cancer gene expression, methylation, and microRNA expression (http://biit.cs.ut.ee/methsurv). Additionally, the Gene Expression Omnibus (GEO) GSE24747 dataset, including $CD133^+$ and $CD133^-$ Caco-2 cells, was downloaded and used to verify differential expression between colon cancers and colon cancer stem cells.

Construction and transfection of the pLKO.1-FOXA1 short hairpin (shRNA) lentivirus vector

To stably knock down *FOXA1* expression in HCT116 and SW480 cells, shRNA interference was performed using the pLKO.1-FoxA1 shRNA (sh-FoxA1) recombinant silencing vector constructed by Genepharma (Shanghai, China). The pLKO.1-scramble shRNA (sh-NC) recombinant vector with no homology to *FOXA1* mRNA or other genes was used as a control. The vectors were transduced into 293T cells using a ViraDuctin™ Lentivirus Transduction Kit (Cell Biolabs, San Diego, CA, USA). Briefly, the virapower lentiviral packaging mix was uniformly mixed with recombinant vector and co-transfected into 293FT cells using Lipofectamine® 3000. Cells were then incubated for 48 hours in a 5% CO_2 incubator, then viral supernatant was collected and added to HCT116 and SW480 cells (multiplicity of infection = 10), which were seeded into six-well culture plates and grown to 70%–90% confluence. Positive clones were selected by 2 μg/mL penicillin. Finally, *FOXA1* down-regulation was measured by quantitative real-time (qRT)-PCR.

Immunohistochemistry

Colon cancer and corresponding healthy tissue samples were separated from the surgical specimen, embedded in paraffin, cut into 4-μm-thick sections by a RM2235 microtome (Leica, Hamburg, Germany), and mounted on slides. Slides were deparaffinized, rehydrated, and boiled in a citrate antigen retrieval solution at 95°C for 15 minutes. Subsequently, they were cooled to room temperature and incubated in 5% bovine serum albumin-blocking solution for 30 minutes. Then they were incubated with an anti-FOXA1 antibody (1:1000 dilution) at 4°C overnight, washed twice with phosphate-buffered saline and Tween 20 (PBST), and incubated with an HRP-conjugated anti-rabbit secondary antibody (1:500 dilution) for 2.5 hours at room temperature. After washing again with PBST, the slides were stained with DAB and hematoxylin (Baso, Zuhai, China) and then visualized under an Olympus IX81 Microscope (Olympus, Tokyo, Japan). The staining intensity was scored as follows: 0 (no staining), 1 (light yellow staining), 2 (brown staining), and 3 (deep brown staining). The percentage of positive cells was scored as follows: 0 (5% positive cells), 1 (5%–25% positive cells), 2 (25%–50% positive cells), 3 (50%–75% positive cells), and 4 (>75% positive cells). The final score was determined by multiplying these two scores: 0, for negative (–); 1 to 4, for weakly positive (+); 5 to 8, for positive (++); and 9 to 12, for strongly positive (+++).

Cell proliferation assay

Cell proliferation was evaluated by an MTT assay. HCT116 and SW480 cells were seeded in 96-well plates (4×10^3 cells/well), cultured overnight, and then transfected with sh-FoxA1 or sh-NC. After 48 hours, 10 μL MTT reagent (5 mg/ml) was added per well and the cells were cultured for 3 hours at 37°C. The medium was removed, 150 μL dimethyl sulfoxide was added, and the absorbance was measured at a wavelength of 570 nm on a microplate reader (MK3, Thermo Fisher Scientific, Dreieich, Germany).

Colony formation assay

After transfecting with sh-FoxA1 or sh-NC, HCT116 and SW480 cells were seeded into six-well plates at a density of 3×10^2 cells/well and cultured for 15 days. They were then stained with crystal violet for 10 minutes, and colony images were obtained

by a digital camera (Canon, Tokyo, Japan). Colonies of $\geq$50 cells were manually counted and used to calculate the colony formation rate.

Cell migration and invasion assay

Cell migration and invasion were evaluated by the Transwell assay. For the cell migration assay, 5×10^4 cells in serum-free DMEM were seeded in the upper chamber (BD Biosciences, San Jose, CA, USA), while the lower chamber was filled with 600 μL media containing 10% FBS. After incubation for 48 hours at 37°C, cells in the lower membrane were fixed in 4% formaldehyde for 10 minutes, stained with crystal violet solution for 15 minutes at room temperature, and then counted under a light microscope. The cell invasion assay was similarly performed except that the upper chamber was prepared with Matrigel (BD Biosciences). At least five randomly selected fields were counted and photographed in each well. All experiments were performed in triplicate.

Cell cycle analysis

The cell cycle was analyzed by flow cytometry. HCT116 and SW480 cells (4×10^5 cells) were transfected with sh-FoxA1 or sh-NC. After 48 hours of transfection, cells were collected and fixed in 75% ice-cold ethanol overnight. The fixed cells were harvested by centrifugation at $750 \times g$, and then re-suspended in 500 μL PBS containing 1 mg/mL RNase and 50 μg/mL PI at 37°C in the dark for 30 minutes. Finally, the samples were analyzed by FACScan flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

Western blotting

Protein from cells and tissues was extracted by ice-cold RIPA lysis buffer and the concentration measured by the BCA Protein Assay Kit. Equal amounts of protein (50 μg) were electrophoresed using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then, they were transferred to polyvinylidene fluoride membranes and blocked in Tris-buffered saline, 0.1% Tween 20 containing 5% BSA at room temperature for 1 hour. The membranes were washed twice with PBST and incubated with primary antibodies (dilution 1:1000) at 4°C overnight, followed by incubation with HRP-conjugated secondary antibodies (dilution 1:1000) at 37°C for 2 hours. Protein bands were detected using enhanced chemiluminescence reagents. All experiments were performed at least three times. Densitometry analysis was performed using ImageJ2 software (NIH, Bethesda, MD, USA), and data were presented as means $\pm$standard deviation (SD).

RT-qPCR

Total RNA from colon cancer cell lines was extracted with TRIzol reagent and reverse-transcribed into cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser. RT-qPCR amplification was performed using UltraSYBR Mixture and all PCR reactions were performed at least in triplicate. The thermal cycling conditions were as follows: 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 1 minute, with a final extension at 72°C for 5 minutes. Primer sequences were as follows: FOXA1, forward: 5′-AGATCTACCAGTGGATCATGGA-3′ and reverse: 5′-TCATTGAAGGACAGCGAGTG-3′; PTEN, forward: 5′-GCAGTATAGAGCGTGCAGATAA-3′ and reverse: 5′-GTATCGGTTGGCTTTGTCTTTATT-3′; Akt, forward: 5′-CTACAACCAGGACCATGAGAAG-3′ and reverse: 5′-TCTTGAGCAGCCCTGAAAG-3′; N-cadherin, forward: 5′-GGATGAAACGCCG GGATAAA-3′ and reverse: 5′-TCTTCTTCTCCTCCACCTTCTT-3′; vimentin, forward: 5′-GCTCGTCACCT

TCGTGAATA-3′ and reverse: 5″-AGTTT GGAAGAGGCAGAGAAA-3′; E-cadherin, forward: 5′-CTTCTGCTGATCCT GTCTGATG-3′ and reverse: 5′-TGCTG TGAAGGGAGATGTATTG-3′; GADPH forward: 5′-GGTGTGAACCATGAGAA GTATGA-3′ and reverse: 5′-GAGTCCT TCCACGATACCAAAG-3′; The $2^{-\Delta\Delta Ct}$ method was used for data analysis. GAPDH expression levels were used for comparison with the relative mRNA expression levels of each sample.

Statistical analysis

All statistical analyses were performed using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). Data are presented as means ± SD and compared through a one-way analysis of variance analysis. Immunohistochemistry score data were analyzed by the Wilcoxon rank-sum test. All experiments were performed in triplicate. A p-value <0.05 was considered statistically significant.

Results

FOXA1 expression was up-regulated in human colon cancer tissues and cell lines

To examine the clinical relevance of FOXA1 in human colon carcinoma, we first determined its expression in 20 pairs of colon cancerous and adjacent healthy tissues. As shown in Figure 1a and b, FOXA1 expression was significantly up-regulated in human colon cancer tissues as shown by western blotting ($p < 0.001$). Similarly, immunohistochemistry revealed increased staining of FOXA1 in carcinoma than paracarcinoma tissue (Figure 1c). To evaluate the expression of *FOXA1* in human colon cancer, various human colon cancer cell lines were subjected to RT-qPCR analyses. Compared with the normal colonic epithelial cell line CCD-18Co, *FOXA1* mRNA expression was significantly higher in HCT116, HT-29, SW480, and LOVO cells, as shown in Figure 1d ($p < 0.001$). FOXA1 protein expression in these four tumor cell lines was also confirmed by western blot analysis to be higher than in CCD-18Co cell lines (data not shown, $p < 0.01$).

We next examined the expression of *FOXA1* in different types of tumors using GEPIA of TCGA data sets. As shown in Figure 1e, *FOXA1* expression was higher in cancerous tissues than in adjacent healthy tissues in both colon and rectal cancer. Taken together, these results suggest that *FOXA1* expression is a clinically relevant marker for tumorigenesis and progression in colon cancer.

Validation of the prognostic value of FOXA1 in R2

To further evaluate the prognostic value of *FOXA1* expression in colon cancer, we used the R2: Genomics Analysis and Visualization Platform to generate Kaplan–Meier overall survival curves using data sets "Tumor Colon (Core Exon)-Sveen-333", "Mixed Colon Adenocarcinoma-TCGA-174", "Tumor Colon (Core Transcript)-Sveen-333", and "Tumor Colon MSI status(Core Exon)-Sveen-95". As shown in Figure 2a, b, d, and e, high *FOXA1* expression was associated with significantly worse event-free survival ($p = 0.018$, Bonf $p = 1.000$; $p < 0.01$, Bonf $p = 0.024$; $p < 0.01$, Bonf $p = 1.000$; and $p = 0.042$, Bonf $p = 1.000$). Similarly, high expression of *FOXA1* was correlated with significantly poorer overall survival ($p = 0.043$, Bonf $p = 1.000$; Figure 2c).

Knockdown of FOXA1 suppressed cell proliferation, induced cell cycle arrest, and inhibited human colon cancer cell migration and invasion

To investigate the effects of FOXA1 on human colon cancer cells, SW480 and

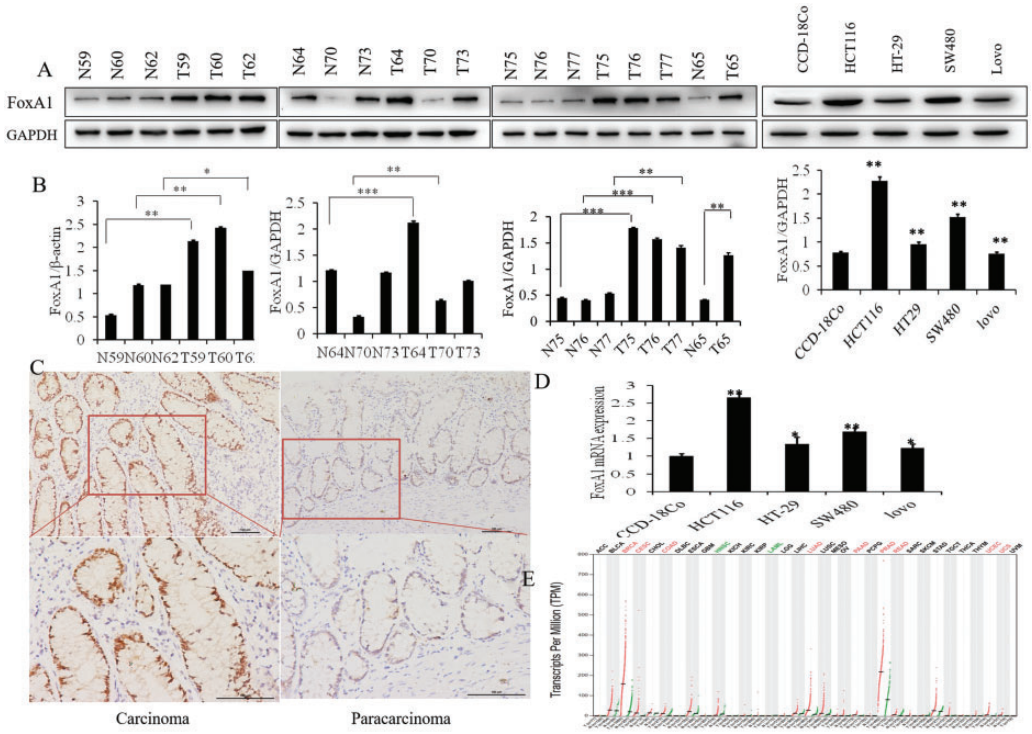

Figure 1. Expression of FOXA1 in colon cancer patients. (a) Western blotting for FOXA1 expression in human colon cancer and paired healthy colon tissues, and various human colon cancer cells. (b) Histograms showing relative band intensity ratios generated from western blotting experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.01$ *vs.* paired healthy colon tissues. (c) Immunohistochemical staining of FOXA1 expression in human colon cancer and paired healthy colon tissue. Scale bar = 100 and 200 μm. (d) RT-qPCR for *FOXA1* mRNA expression in various human colon cancer cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* normal human CCD-18co colon cells. (e) *FOXA1* expression in different tumor types as evaluated by GEPIA. Red line shows tumor tissue expression; green line shows adjacent healthy tissue expression. At the top of the panel, red font represents significantly higher expression in tumor tissue ($p<0.05$); black font represents no difference in expression; green font represents significantly lower expression in tumor tissue ($p<0.05$).
FOXA1, Forkhead box A1; RT-qPCR, quantitative real-time PCR.

HCT116 with higher endogenous expression of FOXA1 were transfected with sh-FoxA1 or sh-NC vectors. *FOXA1* knockdown significantly decreased the proliferation of SW480 and HCT116 cells as shown by the MTT assay, with rates decreased by 50% in both cell lines ($p < 0.01$; Figure 3a). Moreover, *FOXA1* knockdown significantly decreased the clone formation ability of SW480 and HCT116 cells ($p < 0.01$; Figure 3b). To further explore the anti-proliferativ effects of *FOXA1* knockdown, the cell cycle distribution was examined and quantified by flow cytometry. After *FOXA1* knockdown in HCT116 and SW480 cells, the proportion of G2/M phase cells exhibited a remarkable increase together with a significant decrease in the number of S phase cells as shown in Figure 3c ($p < 0.05$).

We next investigated the regulatory effects of FOXA1 on the migration and

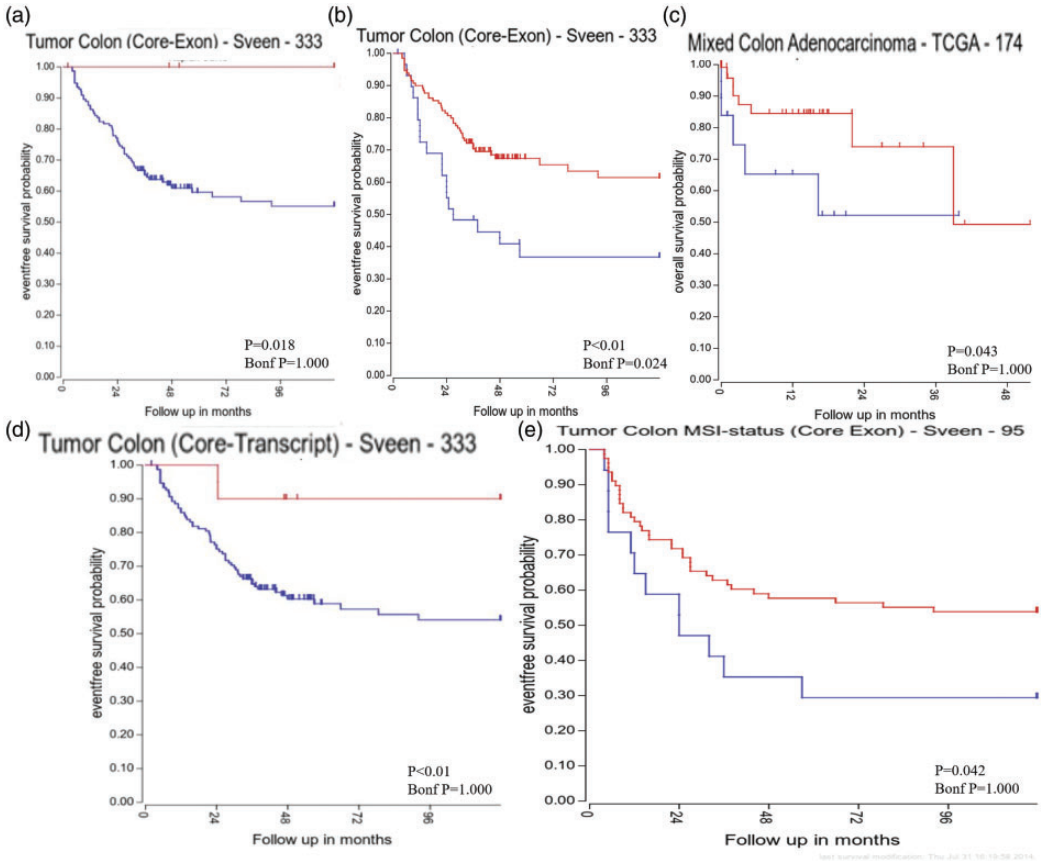

Figure 2. Validation of *FOXA1* expression in the R2 platform. (a, b, d, and e) High *FOXA1* expression was associated with significantly worse event-free survival (all $p < 0.05$). (c) High *FOXA1* expression was associated with significantly worse overall survival ($p < 0.05$). Red line represents low *FOXA1* expression; blue line represents high *FOXA1* expression.
FOXA1, Forkhead box A1.

invasion of human colon cancer cells using the Transwell assay. As shown in Figure 3d, *FOXA1* knockdown significantly inhibited HCT116 and SW480 cell migration ($p < 0.01$), with HCT116 cells showing an inhibitory rate of over 50%. *FOXA1* knockdown also significantly suppressed the invasive ability of HCT116 and SW480 cells ($p < 0.01$; Figure 3e). *FOXA1* knockdown had a greater inhibitory rate on HCT116 cell migration and invasion than that of SW480 cells.

Expression and methylation of FOXA1 in colorectal cancer

To evaluate whether *FOXA1* expression could be regulated by methylation, we examined the expression and methylation data of *FOXA1* between CRC and paired healthy tissues using the MethHC database. As shown in Figure 4a and b, *FOXA1* was significantly down-regulated in colon cancer tissues compared with paired healthy tissues (both $p < 0.01$). Similarly, *FOXA1*

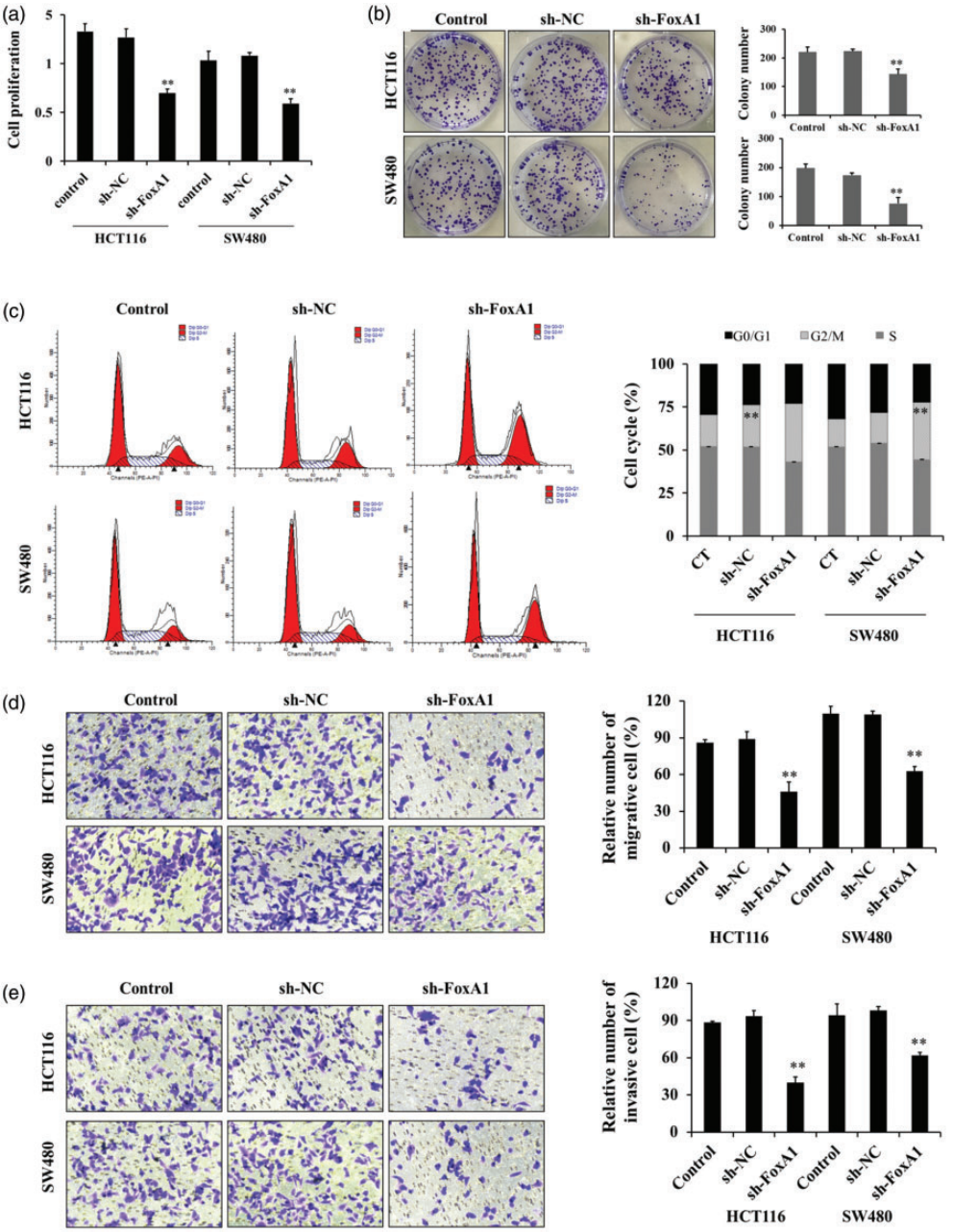

Figure 3. Effects of *FOXA1* on HCT116 and SW480 proliferation and cell cycle, and invasive and migratory abilities. (a) MTT assay for proliferation of HCT116 and SW480 cells after *FOXA1* knockdown. (b) Colony-formation assay in HCT116 and SW480 cells after *FOXA1* knockdown. (c) FACS analysis showing the cell cycle distribution after *FOXA1* knockdown. Right panel: percentage of cells in specific cell cycle phase; left panel: original images. $^{**}p < 0.01$ *vs.* control. (d) Crystal violet staining showing the inhibition of HCT116 and SW480 cell invasive abilities following *FOXA1* knockdown. Scale bars = 100 μm; quantification (right). $^{**}p < 0.01$ *vs.* control. (e) Crystal violet staining showing the inhibition of HCT116 and SW480 cell migratory abilities following *FOXA1* knockdown. Scale bars = 100 μm; quantification (right). $^{**}p < 0.01$ *vs.* control. FOXA1, Forkhead box A1; FACS, fluorescent-activated cell sorting.

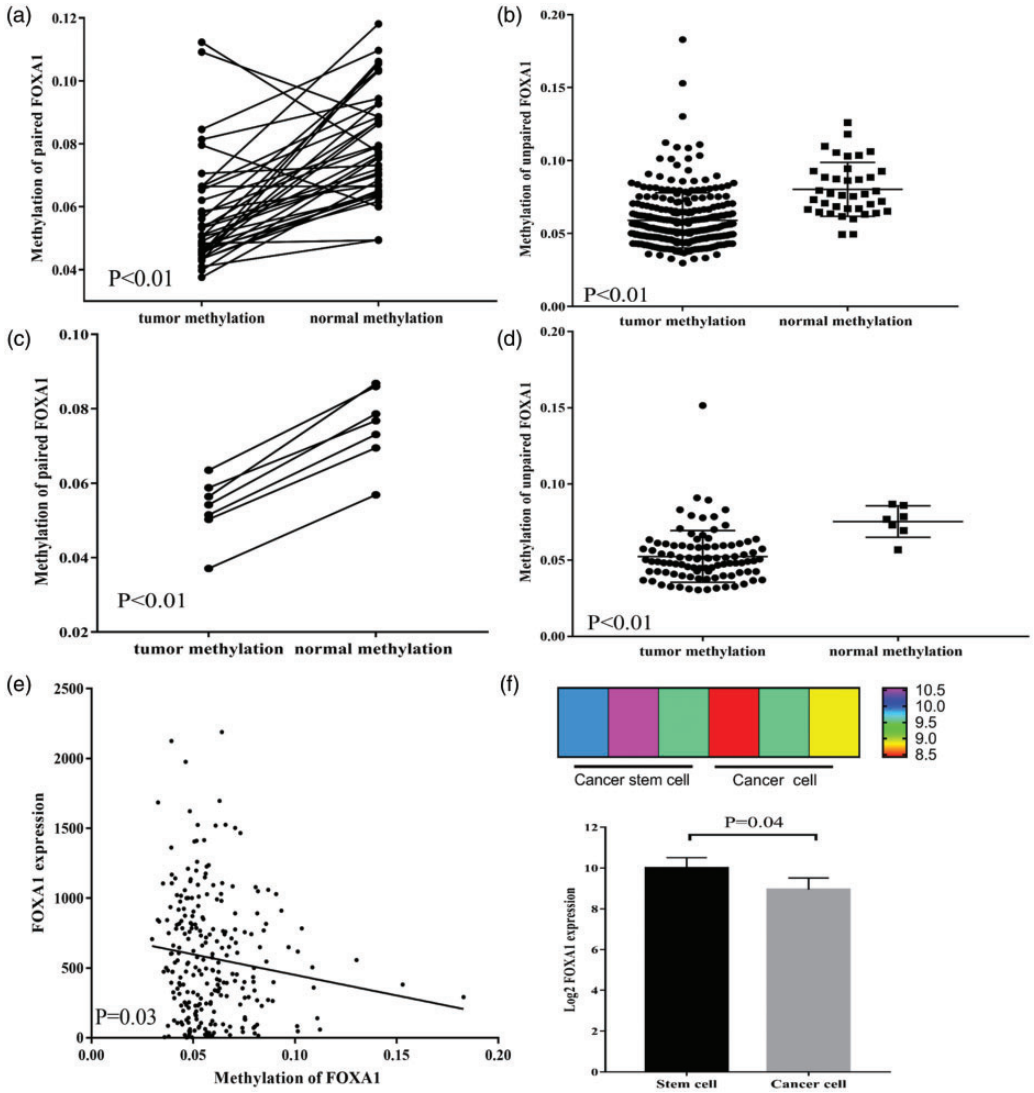

Figure 4. Analysis of *FOXA1* expression in cancer stem cells and its relationship with *FOXA1* methylation in CRC. (a) *FOXA1* expression was significantly decreased in colon cancer tissues compared with paired adjacent healthy tissues from the TCGA database ($p < 0.01$). (b) *FOXA1* expression was significantly decreased in colon cancer tissues compared with adjacent healthy tissues from the TCGA database ($p < 0.01$). (c) *FOXA1* expression was significantly reduced in rectal cancer tissues compared with paired adjacent healthy tissues from the TCGA database ($p < 0.01$). (d) *FOXA1* expression was significantly reduced in rectal cancer tissues compared with adjacent healthy tissues from the TCGA database ($p < 0.01$). (e) The degree of *FOXA1* methylation was negatively correlated with its expression in colon cancer ($p = 0.03$). (f) *FOXA1* was significantly downregulated in CD133+/CD133– colon cancer cells compared with CD133+/CD133– colon cancer stem cells ($p = 0.04$). Each block represents an independent sample and the color of the block represents the expression value.
FOXA1, Forkhead box A1; CRC, colorectal cancer.

was significantly down-regulated in rectal cancer tissues compared with paired healthy tissue (both $p < 0.01$, Figure 4c and d). Additionally, the extent of *FOXA1* methylation was negatively correlated with *FOXA1* expression in colon cancer ($p = 0.03$, Figure 4e). We also demonstrated that *FOXA1* was significantly down-regulated in CD133– colon cancer cells compared with CD133+ colon cancer stem cells ($p = 0.04$).

FOXA1 knockdown activated the PTEN/Akt signaling pathway in HCT116 and SW480 cells

To elucidate the underlying mechanism of *FOXA1*, we used the TCGA data set to explore gene co-expression and found that Akt was co-expressed with *FOXA1* (Figure 5a). We therefore investigated the PTEN/Akt pathway by RT-qPCR and Western blotting. Significantly higher PTEN mRNA and protein expression was detected in sh-FoxA1-transfected HCT116 and SW480 cells compared with the sh-NC groups ($p < 0.05$ vs. sh-NC), as shown in Figure 5b and c. Meanwhile, *FOXA1* knockdown significantly decreased Akt mRNA expression and Akt phosphorylation in both HCT116 and SW480 cells compared with the sh-NC group (Figure 5b and c, $p < 0.05$).

EMT plays a vital role in the progression of colon cancer.[17] To evaluate the effects of *FOXA1* knockdown on EMT, we detected the expression of epithelial and mesenchymal markers using RT-qPCR and western blotting. As shown in Figure 5d and 5e, E-cadherin was significantly upregulated in both HCT116 and SW480 cells compared with the sh-NC group, while in contrast

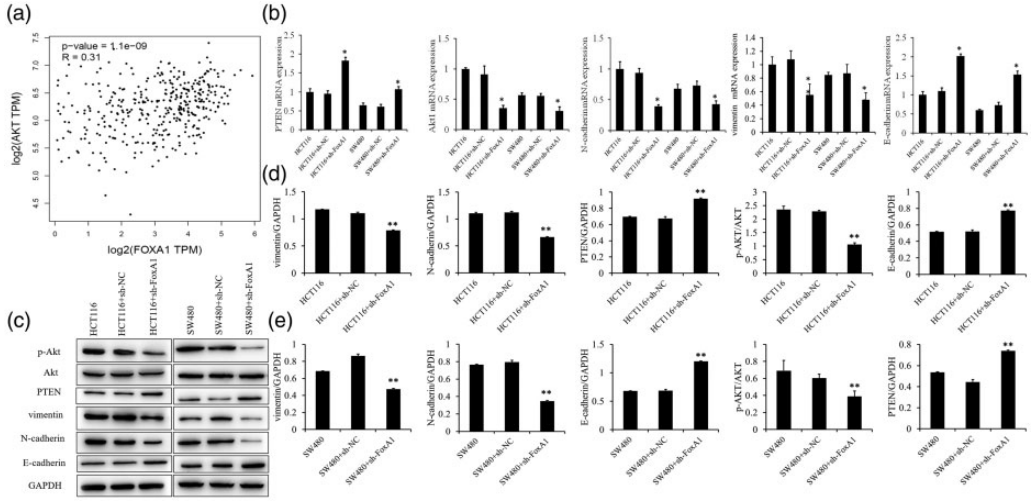

Figure 5. Knockdown of *FOXA1* regulated the PTEN/Akt signal transduction pathway in HCT116 and SW480 cells. (a) Using the TCGA data set, PTEN was found to be co-expressed with *FOXA1*. (b) RT-qPCR analyses of mRNA expression of PTEN/Akt signal transduction pathway-related molecules, Akt phosphorylation, and EMT molecules in HCT116 and SW480 cells. * $p < 0.05$ *vs.* control. (c) Western blotting of the expression of PTEN/Akt signal transduction pathway-related proteins, Akt phosphorylation, and EMT molecules in HCT116 and SW480 cells. (d) Histogram showing relative band intensity ratios generated from western blotting experiments in HCT116 cells. (e) Histogram showing relative band intensity ratios generated from western blotting experiments in SW480 cells. ** $p < 0.01$ *vs.* control.
FOXA1, Forkhead box A1; PTEN, phosphatase and tensin homolog; EMT, epithelial–mesenchymal transition.

N-cadherin and vimentin expression was significantly reduced after *FOXA1* knockdown compared with the sh-NC group ($p < 0.01$ vs. sh-NC). Taken together, these findings suggest that *FOXA1* knockdown had an inhibitory effect on EMT in colon cancer cells.

Discussion

FOXA1 is a transcriptional factor that plays an important role in tumor development and progression in various types of cancers.[7–9,11] However, its function in colon cancer has not been fully evaluated. In this study, we found increased expression of *FOXA1* in human colon cancer tissues and human colon cancer cells, and a negative correlation between *FOXA1* methylation and expression in colon cancer. Knockdown of *FOXA1* suppressed the proliferation, invasion, and migration of HCT116 and SW480 cells by inhibiting EMT through suppressing the PTEN/Akt signaling pathway. Our findings suggest that *FOXA1* may function as an oncogene in colon cancer.

Previous studies have demonstrated that *FOXA1* could act as an oncogene or tumor suppressor gene depending on the type of tumor. To evaluate its role in colon cancer, we first used immunohistochemistry and revealed higher FOXA1 expression in colon cancer tissues than in adjacent noncancerous colon tissues. *FOXA1* mRNA and protein expression was also increased in HCT116, HT-29, SW480, and LOVO cells compared with CCD-18Co cell lines. Similarly, GEPIA analysis revealed higher *FOXA1* expression in CRC tissues compared with healthy tissues, and this was associated with worse prognosis in the R2 database. Taken together, these results highlight the clinical relevance of *FOXA1* in colon cancer progression.

Previous studies have shown that *FOXA1* promotes tumor progression in prostate[7,18] and breast[19] cancers. To further explore the effects of *FOXA1* on the tumorigenesis and progression of colon cancer, HCT116 and SW480 cells showing high *FOXA1* expression were selected as cell models for *FOXA1* knockdown. We observed inhibitory effects of *FOXA1* knockdown on the proliferation, colony formation, migration, and invasion of HCT116 and SW480 cells, which was in accordance with findings from breast[19] and prostate cancers.[18] Together, these findings indicate the inhibitory effects of *FOXA1* on colon cancer progression, consistent with results from previous studies in CRC.[12,13]

A recent study has reported that *FOXA1* methylation altered gene expression in estrogen receptor-negative breast cancer.[20] Only two studies[12,13] have reported the oncogenic role of FOXA1 in human CRC; however, the exact mechanism remains to be fully elucidated. To evaluate whether *FOXA1* expression could be regulated by methylation in colon cancer, we compared expression and methylation data between CRC and paired healthy tissues using the MethHC database. Unlike previous studies, we demonstrated for the first time that *FOXA1* methylation was significantly downregulated in colon and rectal cancer tissues compared with paired healthy tissues. Moreover, the extent of *FOXA1* methylation was negatively correlated with its expression in colon cancer, suggesting that *FOXA1* demethylation could increase its expression in CRC. We also found that *FOXA1* was significantly downregulated in CD133– colon cancer cells compared with CD133+ colon cancer stem cells, indicating that *FOXA1* might be associated with stemness in colon cancer.

We then further investigated the underlying mechanism of *FOXA1* in colon cancer. PTEN is an anti-cancer gene closely associated with cell proliferation, differentiation, and apoptosis.[21] We demonstrated

that *FOXA1* knockdown increased PTEN expression in HCT116 and SW480 cells. Akt regulates various cellular processes, including apoptosis, survival, and angiogenesis, and pAkt expression is inhibited by PTEN.[22] In the present study, we showed that Akt was co-expressed with *FOXA1* using the TCGA data set, and that *FOXA1* knockdown downregulated Akt mRNA expression and reduced Akt phosphorylation Akt in HCT116 and SW480 cells.

EMT plays an important role in tumor invasion and metastasis in several cancers.[23,24] Previous studies showed that *FOXA1* knockdown suppressed tumor invasion by inhibiting EMT in lung cancer,[11,25] and we similarly found that *FOXA1* knockdown significantly inhibited migration and invasion in HCT116 and SW480 cells. As indicated by the observed E-cadherin up-regulation and down-regulation of N-cadherin and vimentin, our study also indicated that *FOXA1* knockdown inhibited EMT, in line with previous findings.

Conclusion

We found increased expression of *FOXA1* in human colon cancer tissues and cell lines. Knockdown of *FOXA1* suppressed proliferation, colony formation, invasion, and migration, and induced G2/M phase arrest in HCT116 and SW480 cells. Additionally, *FOXA1* methylation correlated negatively with its expression in colon cancer, while *FOXA1* knockdown inhibited EMT by suppressing the Akt/PTEN signaling pathway. Our findings suggest that *FOXA1* functions as an oncogene in colon cancer tumorigenesis and development. Further experiments are necessary to elucidate its role in colon cancer progression and to elucidate the underlying mechanisms both *in vitro* and *in vivo*.

Acknowledgements

We thank all the staff in the Department of Medical Oncology, Fujian Medical University Union Hospital, Fuzhou, Fujian, P.R. China.

Author contributions

JP, ZBX, MFX, and MXL designed the study, evaluated immunohistochemistry staining, participated in data analysis, and drafted the manuscript. XYL and BQL participated in data analysis and provided tissue specimens. All authors reviewed the manuscript for important intellectual content. All authors read and approved the final manuscript.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Science and Technology Innovation Project in Fujian Province (Nos. 2016Y9026, 2017Y9035, and 2019Y9081), the Talent Cultivation Fund in the Fujian Province Health Department (Nos. 2016-ZQN-30 and 2017-ZQN-35), and the Fujian Province Science Foundation of China (No. 2018J05133).

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