FOXR2 Promotes the Proliferation, Invasion, and Epithelial–Mesenchymal Transition in Human Colorectal Cancer Cells

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Forkhead box R2 (FOXR2), a member of the FOX gene family, has not been very well investigated for its role in cancer. A recent study has shown that FOXR2 is highly expressed in breast cancer samples and is associated with poor prognosis. In addition, FOXR2 was identified as an oncogene in medulloblastoma. Nevertheless, whether FOXR2 plays a role in colorectal cancer (CRC) remains unclear. In the present study, we conducted several in vitro and in vivo studies to investigate the expression and effect of FOXR2 in CRC. The study results demonstrated that FOXR2 was upregulated in CRC tissues and cells. Downregulation of FOXR2 inhibited CRC cell proliferation, invasion, and the epithelial–mesenchymal transition (EMT) phenotype in vitro and also suppressed CRC cell growth and metastasis in vivo. Furthermore, downregulation of FOXR2 remarkably reduced the protein expression of Shh, Gli1, and Ptch1 in SW480 cells. Taken together, our data suggested that FOXR2 significantly promoted proliferation, invasion, and EMT of CRC cells. All these findings provided evidence for the role of FOXR2 as an oncogene in CRC development.

Key words: Forkhead box R2 (FOXR2); Proliferation; Invasion;
Epithelial–mesenchymal transition (EMT); Colorectal cancer (CRC)

INTRODUCTION

Colorectal cancer (CRC), a common gastrointestinal tract malignancy, is characterized by an increasing incidence and a high mortality[1–3]. The occurrence of CRC has been found to be associated with several factors such as genetics, habit, ethnicity, and diet[4–8]. According to statistics, about 950,000 cases are newly diagnosed every year, making CRC a global public health problem[9]. So far, the diagnostic methods available for CRC include colonoscopy and fecal occult blood test[10]. With improvements in clinical practice, CRC could be treated via modified surgical techniques and chemotherapy in combination with radiation therapy[11]. However, about 50% of CRC patients succumb to the disease because of distant metastasis or multidrug resistance[12]. Therefore, it is urgent to identify a novel effective target for CRC treatment.

The forkhead box (FOX) gene family consists of 43 members from FOXA1 to FOXQ1[13]. All the members are involved in a series of biological processes such as proliferation, apoptosis, differentiation, transformation, and metabolic homeostasis[14–16]. A growing number of studies have indicated the role of the FOX gene family in carcinogenesis, either as a suppresser or a promoter of cancer progression. For instance, FOXD3 has been found to be downregulated in melanoma and is important in suppressing cancer development[17]. In contrast, FOXM1 has been reported to be upregulated in a wide range of neoplasms and functions as an oncogene[18,19].

FOXR2, another member of the FOX gene family, has not been investigated much for its role in cancer. A recent study has shown that FOXR2 is highly expressed in breast cancer and is associated with poor prognosis[20]. In addition, FOXR2 was identified as an oncogene in medulloblastoma[21]. Nevertheless, whether FOXR2 plays a role in CRC remains unclear.

In the present study, we conducted several functional studies to investigate the expression and effect of FOXR2

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in CRC. The study results demonstrated that FOXR2 was upregulated in CRC tissues and cells. In addition, downregulation of FOXR2 inhibited CRC cell proliferation, invasion, and epithelial–mesenchymal transition (EMT) and also reduced the protein expression of Shh, Gli1, and Ptch1 in CRC cells, suggesting an oncogenic role of FOXR2 in CRC.

MATERIALS AND METHODS

Tissue Specimens

Human CRC tissues and matching adjacent noncancerous tissues were obtained from 36 patients at the Hubei Cancer Hospital (P.R. China). All patients provided written consent prior to participation in the study and received no therapy before tissue samples were collected. All specimens were handled in accordance with ethical and legal standards. The study was carried out with the approval of the ethics committee of the Hubei Cancer Hospital.

Cell Lines and Cell Culture

Human CRC cell lines (SW480, HT29, and LOVO) and the normal colonic epithelial cell line NCM460 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were maintained in RPMI-1640 (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco) and cultured at 37°C in a humidified incubator with 5% CO_2.

Quantitative Real-Time PCR

Total RNA was extracted from tissue samples or cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription of RNA into cDNA was performed using the PrimeScript RT Reagent Kit (Takara Biotechnology, Dalian, P.R. China) according to the manufacturer's recommendations. The cyclical conditions of PCR were 35 cycles of denaturation at 94°C for 2 min, annealing at 56°C for 30 s, and extension at 72°C for 10 min. The primers for cDNA amplification were as follows: FOXR2, 5′-TCAGTGTGCAGGAGATCTAC-3′ (forward) and 5′-TACCAAGATCAAAGAGAGAGGTCAAC-3′ (reverse); β-actin, 5′-CCTGGCACCCAGCACAATG-3′ (forward) and 5′-GGGCCGGACTCGTCATACT-3′ (reverse). The relative mRNA expression levels were normalized to β-actin and measured using the comparative CT method $(2^{-\Delta\Delta CT})$[22].

Western Blot

Tissues or cells were lysed in RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA). Lysates were electrophoresed in 10% SDS-PAGE (Sigma-Aldrich) and then transferred to PVDF membranes (Sigma-Aldrich). After blocking for 1 h in 5% nonfat milk, the membranes were incubated overnight at 4°C with the primary antibodies against FOXR2, E-cadherin, vimentin, N-cadherin, Shh, Ptch1, Gli1, or β-actin. Then the membranes were washed three times with TBST and incubated at room temperature for 1 h with secondary HRP-conjugated antibodies. All antibodies for experiments were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein bands were visualized by a chemiluminescence system (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. The band intensities were quantified using analysis software (Life Technologies, Gaithersburg, MD, USA).

Small Interfering RNA (siRNA) Transfection

Human FOXR2 siRNA (siFOXR2) and negative control siRNA (siNC) were purchased from Ambion (Grand Island, NY, USA). The sense sequences of siFOXR2 and siNC were 5′-GCUCCCUAGAUGAGAUACAdTdT-3′ and 5′-UUCUCCGAACGUGUCACGUdTdT-3′, respectively. SW480 and HT29 cells were transfected with siFOXR2 or siNC using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's protocol. After 24 h of transfection, the cells were harvested for additional experiments. Western blot analysis was performed to confirm the transfection efficiency.

Cell Proliferation Assay In Vitro

After transfection, cells were seeded into 96-well plates at a density of 3×10^3 cells/well and incubated for 24, 48, 72, and 96 h, respectively. Then 20 µl of MTT (5 mg/ml; Sigma-Aldrich) was added to each well, and the cells were further incubated for 4 h at 37°C. Next, the culture medium was removed, and 150 µl of DMSO (Sigma) was added. The absorbance was measured at a wavelength of 570 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

Cell Invasion Assay In Vitro

Transwell chambers (Millipore, Boston, MA, USA) with Matrigel were used to perform the invasion assay. Transfected cells (3×10^4 cells/well) were seeded in the upper chamber coated with Matrigel. Serum-free medium (200 µl) was added to the upper chamber, and 800 µl of culture medium containing 20% FBS was added to the lower chamber. After 24 h of incubation, cells remaining on the upper surface of the filter were scraped off with a cotton swab and those invading the lower surface of the filter were fixed and stained with 0.1% crystal violet. The invading cells were counted in four random visual fields under a microscope (200×).

Xenograft Tumor Model In Vivo

Male BALB/c nude mice (4 to 6 weeks old) were obtained from the Animal Center of the Chinese Academy of Science (Shanghai, P.R. China). All animal studies

were performed with the approval of the Institutional Animal Care and Use Committee of the Hubei Cancer Hospital. For the tumor growth assay, 200 μl of suspension of transfected cells at a concentration of 1×10^7 cells/ml was subcutaneously inoculated into nude mice (six mice per group). Tumor size was measured every week.
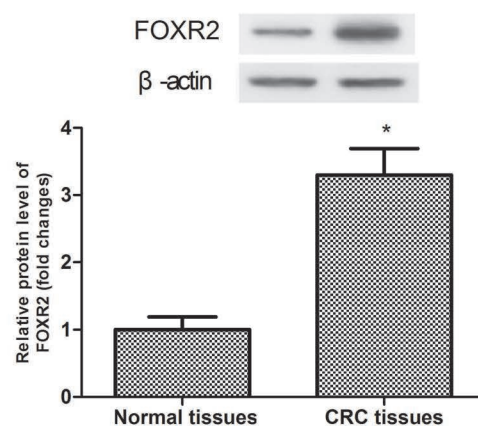
Four weeks later, all mice were sacrificed, and tumors were weighed. Tumor volume (cm³) was determined by the following formula: length×width²/2.

For the tumor metastasis assay, 200 μl of suspension of transfected cells at a concentration of 1×10^7 cells/ml was intravenously injected into the tails of nude mice
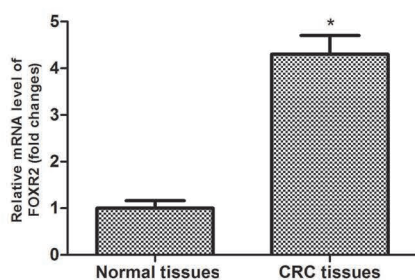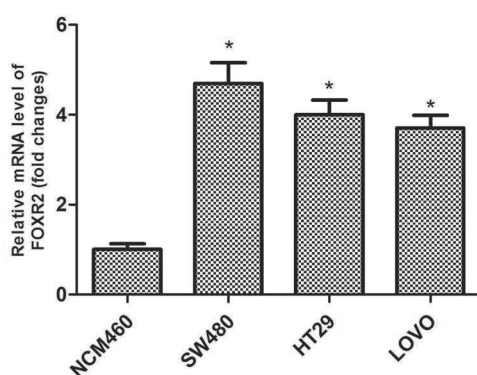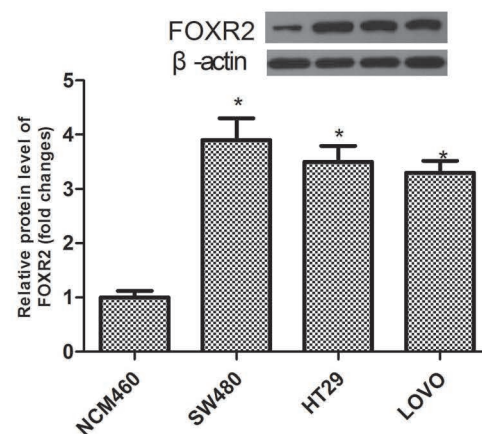

Figure 1. Expression of FOXR2 is upregulated in CRC tissues and cell lines. The mRNA (A) and protein (B) expression levels of FOXR2 were significantly higher in CRC tissues than in the corresponding noncancerous tissues. The mRNA (C) and protein (D) expression levels of FOXR2 were remarkably upregulated in three CRC cell lines (SW480, HT29, and LOVO) when compared to the normal colonic epithelial cell line NCM460. *$p < 0.05$.
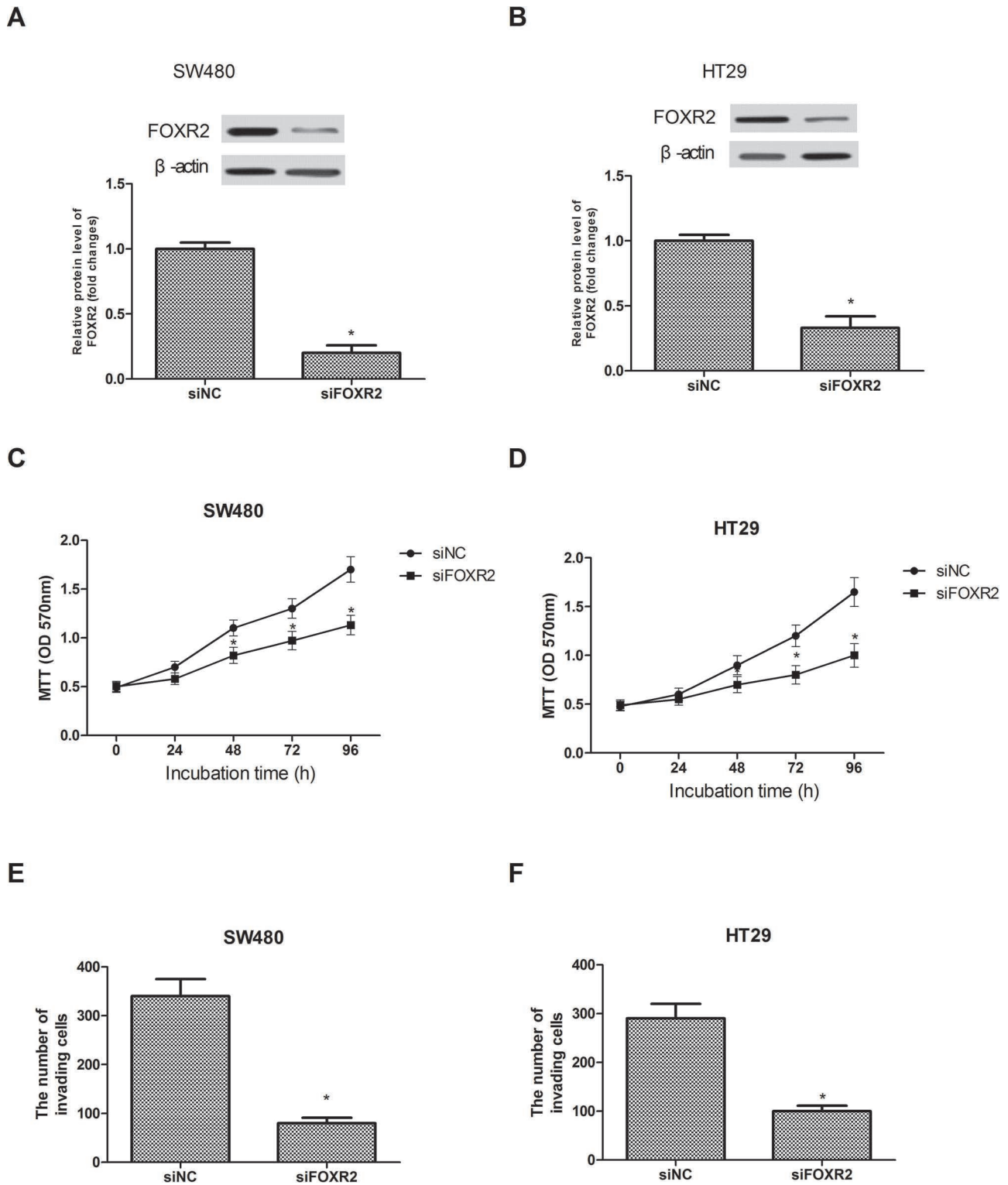
Figure 2. Downregulation of FOXR2 inhibits CRC cell proliferation and invasion in vitro. Western blot assay showed decreased FOXR2 protein expression after siRNA-mediated downregulation in SW480 (A) and HT29 (B) cells. Downregulation of FOXR2 remarkably inhibited the proliferation rate of SW480 (C) and HT29 (D) cells as measured by the MTT assay. Downregulation of FOXR2 significantly inhibited the invasive capability of SW480 (E) and HT29 (F) cells as measured by the Transwell assay. *$p < 0.05$.

(six mice per group). Four weeks later, all mice were sacrificed, and all organs were checked for macroscopic metastasis. Tumor metastasis was mainly found in the lungs.

Statistical Analysis

Data were obtained from at least three independent experiments and expressed as the means ± standard deviation (SD). The SPSS 16.0 software was applied for statistical analysis, and the GraphPad Prism 5 software was used for graphical representation. Differences between different groups were analyzed by Student's *t*-tests. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Expression of FOXR2 Is Upregulated in CRC Tissues and Cell Lines

We conducted RT-PCR and Western blot analysis to measure the expression levels of FOXR2 in CRC tissues and matching adjacent noncancerous tissues. We observed significantly increased mRNA and protein expression levels of FOXR2 in CRC tissues compared to corresponding noncancerous tissues (Fig. 1A and B). We also detected the expression levels of FOXR2 in three CRC cell lines (SW480, HT29, and LOVO) and the normal colonic epithelial cell line NCM460. FOXR2 was highly expressed in three CRC cell lines but lowly expressed in NCM460 cells (Fig. 1C and D).

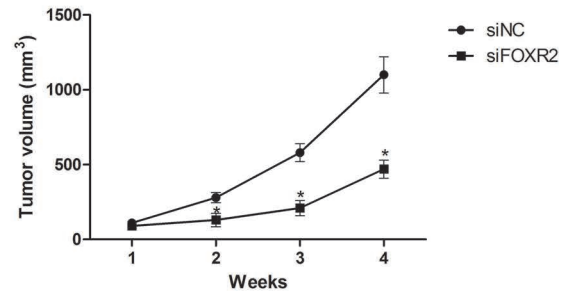*Downregulation of FOXR2 Inhibits CRC Cell Proliferation and Invasion In Vitro*

We downregulated the expression of FOXR2 in SW480 and HT29 cells by transfecting siFOXR2. Western blot analysis was performed to confirm the transfection efficiency. siFOXR2 transfection dramatically decreased the expression of FOXR2 in the SW480 (Fig. 2A) and HT29 (Fig. 2B) cell lines at the protein levels.

We performed MTT assay to examine the effect of FOXR2 downregulation on cell proliferation. The assay indicated that downregulation of FOXR2 remarkably inhibited the proliferation rate of the SW480 (Fig. 2C) and HT29 (Fig. 2D) cells compared to the control group. In addition, we determined the invasion rate. The assay results showed that downregulation of FOXR2 significantly inhibited the invasive capability of the SW480 (Fig. 2E) and HT29 (Fig. 2F) cells compared to the control group.
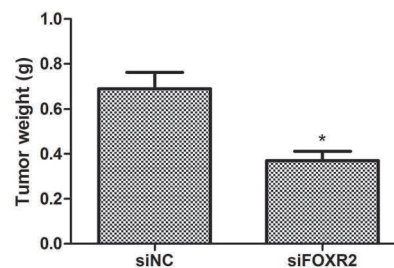
Downregulation of FOXR2 Inhibits CRC Cell Growth and Metastasis In Vivo

We further investigated the effect of FOXR2 downregulation on the growth and metastasis of CRC cells in vivo. SW480 cells transfected with siFOXR2 or siNC
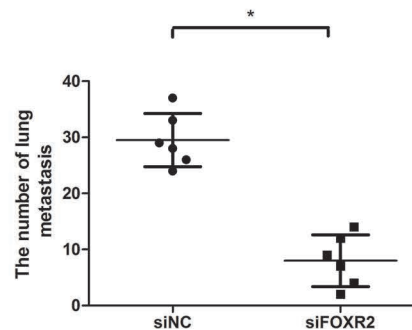

Figure 3. Downregulation of FOXR2 inhibits CRC cell growth and metastasis in vivo. Tumor volume (A) and weight (B) were significantly decreased in SW480 cells, compared to the control group. (C) The number of lung metastasis was quantified and shown by each data point. $n = 6$; *$p < 0.05$.

were subcutaneously inoculated into nude mice, and tumor size was measured every week. Four weeks later, all mice were sacrificed, and tumors were weighed. Tumor volume (Fig. 3A) and weight (Fig. 3B) were distinctly decreased in the siFOXR2 group compared with the control group.

To explore the role of FOXR2 in CRC metastasis, SW480 cells transfected with siFOXR2 or siNC were intravenously injected into the tails of nude mice. Four weeks later, all mice were sacrificed, and all main organs were checked for tumor metastasis. Downregulation of FOXR2 remarkably decreased metastatic foci in the lungs (Fig. 3C).

Downregulation of FOXR2 Inhibits the EMT Process in CRC Cells

Involvement of EMT in cancer cell invasion and metastasis is well known[23–25], so we tested the effect of FOXR2 downregulation on the EMT process by detecting the protein expression of EMT-associated markers in SW480 cells. Downregulation of FOXR2 significantly increased the protein expression of the epithelial marker E-cadherin and decreased that of the mesenchymal markers vimentin and N-cadherin in SW480 cells, compared to the control group (Fig. 4).
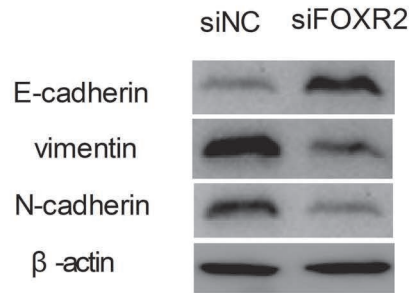
Downregulation of FOXR2 Inhibits the Activation of the Shh Signaling Pathway

It has been found that the Shh signaling pathway plays a key role in the pathogenesis of CRC[26]. Therefore, we tested the effect of FOXR2 downregulation on the Shh signaling pathway by detecting the protein expression levels of its several important components Shh, Gli1, and Ptch1 in SW480 cells. Downregulation of FOXR2 remarkably reduced the protein expression of Shh, Gli1, and Ptch1 in SW480 cells in comparison with the control group (Fig. 5).

DISCUSSION

CRC has drawn worldwide attention for its increasing incidence and unacceptably high mortality[2,3]. The outcome for CRC patients remains poor due to metastasis or drug resistance, despite improvements in clinical practice and therapies[11,12]. Therefore, there is an urgent
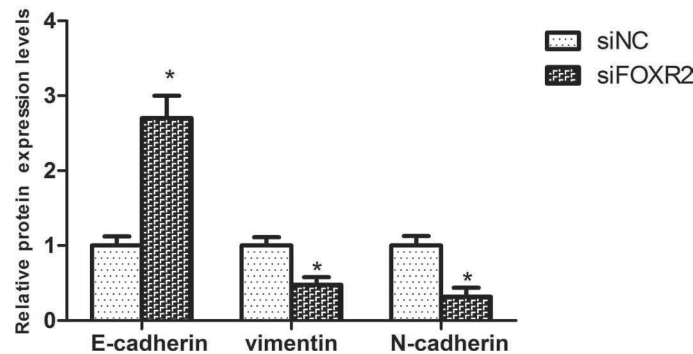

Figure 4. Downregulation of FOXR2 inhibits the EMT process in CRC cells. (A) Western blot analysis was performed to detect the protein expression levels of E-cadherin, vimentin, and N-cadherin in SW480 cells. (B) Relative protein expression levels of E-cadherin, vimentin, and N-cadherin in SW480 cells were measured using analysis software. *$p < 0.05$.

need to identify a novel therapeutic target and to better understand the underlying molecular mechanism.

The FOX gene family has frequently been studied and is reported to play an important role in cancer development[27]. In addition, many of the family members function as oncogenes in CRC. For example, FOXM1 was reported to promote CRC cell migration and invasion[28]. Similarly, Kaneda et al. found the tumor-promoting role of FOXQ1 in CRC[29]. However, another member of the FOX gene family, FOXR2, has been scarcely investigated in tumorigenesis. In this study, we explored the effect of FOXR2 on CRC cells for the first time. The results of our study showed that FOXR2 was upregulated in CRC tissues and cells, and downregulation of FOXR2 exerted an inhibitory effect on the proliferation and invasion of CRC cells. Similar results were found in hepatocellular cancer, breast cancer, and medulloblastoma[20,21,30]. All the

evidence has strongly suggested FOXR2 as an oncogene that plays a significant role in CRC development, which was further proved by our functional studies.

Since proliferation and invasion are important biological features during tumor formation, we performed corresponding experiments to test the effect of FOXR2 on these biological processes. The MTT and Transwell assays indicated that downregulation of FOXR2 decreased CRC cell proliferation and invasion, respectively. To validate the conclusions drawn from our in vitro experiments, we designed xenograft models of CRC cells in nude mice. In the in vivo tumor growth and metastasis assays, CRC cells transfected with siFOXR2 were injected into nude mice. The assay results showed that downregulation of FOXR2 remarkably decreased tumor volume and weight as well as metastases, which were consistent with the results of our in vitro experiments. Increasing evidence
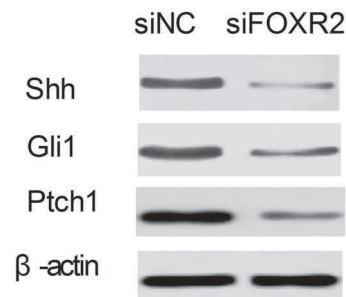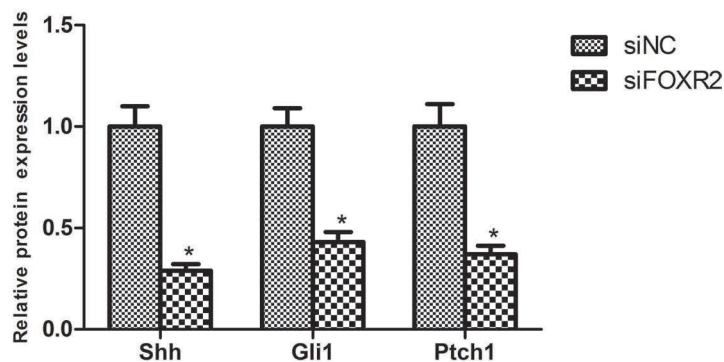

Figure 5. Downregulation of FOXR2 inhibits the activation of the Shh signaling pathway. (A) As shown by the Western blot assay, downregulation of FOXR2 remarkably decreased the protein expression of Shh, Gli1, and Ptch1 in SW480 cells. (B) Relative protein expression levels of Shh, Gli1, and Ptch1 in SW480 cells were measured using analysis software. $*p<0.05$.

has demonstrated the critical role of EMT in cancer progression, so we investigated the effect of FOXR2 on the EMT process in CRC cells by measuring the protein expression of EMT-associated markers. As expected, downregulation of FOXR2 significantly inhibited the EMT process in CRC cells by increasing the protein expression of the epithelial marker E-cadherin and decreasing that of the mesenchymal markers vimentin and N-cadherin.

Given that the Shh signaling pathway is strongly correlated with the pathogenesis of CRC[26,31], we investigated the effect of FOXR2 downregulation on the Shh signaling pathway via detecting the protein expression levels of its several important components Shh, Gli1, and Ptch1 in CRC cells. The results showed that downregulation of FOXR2 remarkably reduced the protein expression of Shh, Gli1, and Ptch1 in CRC cells.

In conclusion, we demonstrated that FOXR2 was upregulated in CRC tissues and cells. Downregulation of FOXR2 significantly inhibited proliferation, invasion, and EMT of CRC cells. Furthermore, downregulation of FOXR2 remarkably reduced the protein expression of Shh, Gli1, and Ptch1 in CRC cells. These findings provide evidence for the role of FOXR2 as an oncogene in CRC development.

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