Knock-Down of HOXB8 Prohibits Proliferation and Migration of Colorectal Cancer Cells via Wnt/β-Catenin Signaling Pathway

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: There has been no research on the mechanism of HOXB8 action on colorectal cancer so far. This study was designed to investigate the mechanism of HOXB8 regulating colorectal cancer cell proliferation and invasion *in vivo* and *in vitro*.

Material/Methods: HOXB8 shRNA, HOXB8 overexpression, and negative control vector were designed and stably transfected into HCT116 cells. MTT assays were performed to detect cell proliferation. Western blot was utilized to detect HOXB8 expression level in HCT116 stable cells. The invasive and migration abilities were detected by Transwell assay and wound-healing assay.

Results: HOXB8 knockdown inhibited cell proliferation. The invasiveness of HCT116 cells was significantly reduced following HOXB8 depletion compared with that in the shRNA control group, whereby the rates were reduced by 67% in HOXB8 knockdown group. The wound-healing rate of HOXB8 over-expression cells was significantly increased comparing with that of cells in the blank control group (*P*<0.05). HOXB8 knockdown promotes apoptosis of HCT116 cells. The expression of E-cadherin was restrained in the HOXB8 over-expression group and increased in the HOXB8 knockdown group.

Conclusions: Knock-down of HOXB8 prohibits the proliferation and migration of colorectal cancer cells via the Wnt/β-catenin signaling pathway and the downregulation of various factors, such as MMP2, c-Myc, CyclinD1,and vimentin. Our data suggested that HOXB8 has great potential to be developed as a novel therapeutic agent for the treatment of human colorectal cancer.

MeSH Keywords: Cell Proliferation • Colorectal Neoplasms • Wnt Signaling Pathway

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Background

Colorectal cancer is known as the third most commonly diagnosed cancer and the second cause of death due to cancer worldwide. Approximately 1.4 million new cases are diagnosed yearly [1]. Numerous studies have suggested that recurrences and metastases were the major reason for death in colorectal cancer patients. Therefore, it is critical to identify the molecular mechanisms underlying the development and progression of colorectal cancer and identify potential chemoprevention and therapeutic targets. Our previous screening studies found that HOXB8 was highly expressed in colorectal cancer tissues using Affymetrix HG-U133 Plus 2.0, and HOXB8 expression was significantly correlated with the prognosis of patients [2,3]. HOXB8 gene is a member of the ANTP homeobox family and encodes a nuclear protein with a homeobox DNA-binding domain. It is included in a cluster of Homeobox B genes located on chromosome [4]. The encoded protein functions as a sequence-specific transcription factor that regulates the transcription factors during embryonic development [5]. Previous studies found that Hox genes were associated with development of various tumors, such as colorectal cancer, breast cancer, bladder cancer, and thyroid tumors [6-10]. Compared with thymic carcinoma (from ectoderm), some tumor tissues (from endoderm) with similar embryonic origins – such as colorectal cancer, prostate cancer, and lung cancer – revealed similar family-gene expression patterns of HoxA and HoxB [11]. It is reported that the overexpression of some Hox genes mediated by fusion protein could promote tumor cell proliferation [12]. Vider et al. found that HoxB8 showed upregulated expression in colorectal cancer cells [13]. And such phenomenon appeared in all periods of development of colorectal cancer including pre-cancerous polyps [12].

However, there has been almost no relevant research on HOXB8's mechanism of action on colorectal cancer so far. Thus, this study was designed to investigate the mechanism of HOXB8 regulating colorectal cancer cell proliferation and invasion *in vivo* and *in vitro*.

Material and Methods

Reagents

Anti-HOXB8 (1: 1000 dilution; cat. no. ab125727), anti-c-Myc (1: 1000 dilution; cat. no. ab32072), anti-CyclinD1 (1: 1000 dilution; cat. no. ab134175), anti-E-cadherin (1: 1000 dilution; cat. no. ab15148), anti-MMP2 (1: 1000 dilution; cat. no. ab92536), anti-vimentin (1: 1000 dilution; cat. no. ab92547), anti-Bax (1: 1000 dilution; cat. no. ab32503), anti-bcl-2 (1: 1000 dilution; cat. no. ab182858) and anti-tubulin (1: 1000 dilution, cat. no. ab6046) antibodies were obtained from Abcam (San Francisco, CA, USA). Control shRNA, and HOXB8 shRNA clones

were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1: 1000; cat no. A0208) and DAB (0.05–0.03%) horseradish peroxidase color development kits (cat no. ST033) were obtained from Beyotime Institute of Biotechnology (Haimen, China). Lipofectamine™ 2000, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). SuperSignal™ West Pico PLUS Chemiluminescent substrate was purchased from Thermo Fisher Scientific, Inc. Penicillin G and streptomycin were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China).

Cell culture

HCT116 cells and 293T cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and were maintained in DMEM containing 10% FBS, penicillin (100 U/mL) and streptomycin in (100 mg/mL). Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere incubator.

Preparation of viruses and cell infection

Lentiviral vectors were stored in our laboratory. Total RNA of RKO was extracted using TRIzol (Invitrogen). We measured its 260 nm/280 nm absorbance value on an Enzyme standard instrument, calculated the concentration and selected 260 nm/280 nm absorbance value between 1.8~2.1 as the template to get HOXB8 full-length cDNA by RT-PCR using Superscript First-Strand Synthesis Kit (Promega, USA) with specific designed primers(HOXB8-F-EcoR I: 5'-CGA ATT CGC CAC CAT GAG CTC TTA TTT CG TCA AC-3, HOXB-R-Sac II: 5'-CCG CGG CTA CTT CTT GTC GCC CTT CTG-3'). The PCR products were purified and then inserted into the EcoR I/Sac II sites of lentiviral vector. The inserted fragment was confirmed by sequencing. Two short-hairpins RNA (shRNA) were designed targeting HOXB8: shHOXB8-1 (5'-GCTCTTATTTCGTCAACTCACTGTTCTCC-3') and shHOXB8-2 (5'-GAGCTGGAGAAGGAGTTCCTATTTAATCC-3'). Briefly, lenti-shRNA vector construction was done as follow. The DNA fragments containing CCAA were synthesized as the loop for shRNA, and then we cloned the shRNA into human pBluescript SK (+) plasmid (pU6) with U6 promoter and inserted the U6-shRNA cassettes into an appropriate lentiviral vector. Similarly, we selected a control: lentiviral vector carrying shRNA targeting firefly luciferase (shLuc: 5'-TGC GCT GCT GGT GCC AAC CCT ATT CT-3'). The transfer vector and the other 3 packaging vectors (pMD2.G, pMDL-G/P-RRE and pRSV-REV) were co-transfected into 293T cells to produce the viral particles. The concentration of each vector was added as follow: pMD2.G 3.5 mg/10 cm, pMDL-G/P-RRE 6.5 mg/10 cm, pRSV-REV 3.5 mg/10 cm and transfer vector 12 mg/10 cm. After 48 hours, the supernatant was collected and subsequently purified using ultracentrifugation to get high-quality viral particles. The

first day we seeded 5×10^4 cells in 24-well plates, and the next day transduced with lentivirus (MOI=5) supplemented with 8 mg/mL of polybrene (Sigma-Aldrich Chemie, The Netherlands). Transfection efficiency was verified by western blot.

A total of 1×10^5 HCT116 cells were plated into each well of a 6-well plate and infected with a total volume of 100 µl lentiviruses for 24 hours. Cells that stably expressed lentiviral shRNAs were obtained by selecting the infected cells with 1 mg/mL puromycin in for a period of 3 to 4 weeks at 37°C in a humidified 5% CO_2 atmosphere incubator

Cell viability

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed according to the protocol outlined in Mosmann [14], with slight modifications. Cells were seeded in 96-well plates at a density of 6×10^3 cells/well and incubated in a CO_2 incubator for up to 4 days at 37°C overnight. At the end of each experiment, 20 µL of MTT solution (5 mg/mL) was added to each well and the cells were incubated for an additional 3 hours. Afterwards, the solution was discarded and 100 µL of dimethyl sulfoxide was added to each well to solubilize the crystals. The plates were then measured using an enzyme-linked immunosorbent assay (ELISA) plate reader (BioTek Instruments, Winooski, Vermont, USA) at 570 nm. Cell viability assay was performed with 3 independent experiments. The data were then plotted as time points on the x-axis and A570 optical density numbers on the y-axis.

Cell migration assay

After cells had grown to 100% confluence in 6-well culture plates, an artificial wound was created by scratching the cell monolayer with the tip of a 10-µL pipette. The wound area was inspected after 24 hours and 48 hours using an inverted phase-contrast microscope with a digital camera (magnification, 400×). The wound healing speed was calculated as the percentage of the initial wound at different time points (36 hours and 48 hours) until total wound closure.

Invasion assays

To examine the effect of HOXB8 on cell invasion, 100 µL of Matrigel (BD, 356234; 0.35 mg/mL; 1: 30 dilution in serum-free DMEM) was added to each Transwell polycarbonate filter (6-mm diameter; 8-µm pore size) and incubated with the filters at 37°C for 6 hours. HCT116 were trypsinized and washed 3 times with DMEM containing 1% FBS. The cells were resuspended in DMEM containing 1% FBS at a density of 5×10^5 cells/mL. The cell suspensions (100 µL) were seeded into the upper chambers and 600 µL of DMEM medium containing 10% FBS was added to the lower chambers. The cells were allowed to invade

for 12 hours in a CO_2 incubator, then fixed, stained and quantified as described previously [15].

Annexin V/fluorescein isothiocyanate (FITC) flow cytometric assay

The flow cytometric assay was performed with the Annexin V kit (BD Pharmingen; BD Biosciences, San Jose, CA, USA). Cells were seeded in 6-well plates at a density of 1×10^5 cells/well and incubated at 37°C in a humidified 5% CO_2 atmosphere incubator. After 12 hours, cells were harvested and pelleted via centrifugation (1000×g for 5 minutes) at 4°C, immediately resuspended in binding buffer and subsequently stained with 5 µL FITC Annexin V or 5 µL propidium iodide (PI), according to the kit protocol. The mixture was placed on ice (4°C) in the dark and analyzed using a FACS (fluorescence-activated cell sorting) system with BD Accuri C6 Software (BD 1.0.264.21; BD Biosciences).

Western blot analysis

A total of 2×10^6 cells (HCT116 or HCT116 HOXB8 knockdown cells or HCT116 HOXB8 overexpression) were seeded overnight in 6-well plates, cells were washed with ice-cold PBS and harvested using RIPA buffer (cat no. P0013C; Beyotime Institute of Biotechnology). The protein concentration was determined using the BCA method (cat No. P0011; Beyotime Institute of Biotechnology) and 20 µg of total protein was separated using 12% SDS-PAGE prior to western blot analysis. The proteins were transferred to a nitrocellulose membrane that was blocked in 5% milk for 1 hour at room temperature. The expression levels of HOXB8, c-Myc, CyclinD1, E-cadherin, MMP2, vimentin, Bax, bcl-2, and tubulin were determined by incubating the membrane with the specific aforementioned primary antibodies (1: 1000 dilution in 5% milk) overnight at 4°C. This was followed by incubation with HRP-conjugated secondary antibody (Beyotime Institute of Biotechnology) for 1 hour at room temperature prior to development using SuperSignal West Pico PLUS Chemiluminescent substrate. Tubulin was used as the loading control. All protein expressions levels were quantified using ImageJ software (ImageJ 1.43u/Java 1.6.0-10; National Institutes of Health, Bethesda, MD, USA).

Xenografts and animal experiments

Animal experiments were performed following a protocol approved by the Institutional Animal Committee of Wenzhou Medical University. Twenty BALB/c nude mice (5 to 6 weeks old) were injected subcutaneously with 1×10^6 HCT116 cells per animal. Mice body weight and tumor diameters were measured 3 times per week. Tumor size was measured daily or every other day with calipers, tumor volumes were calculated using the formula: volume=(width)^2×length/2. Mice were euthanized 4 weeks later, and tumors were weighed.
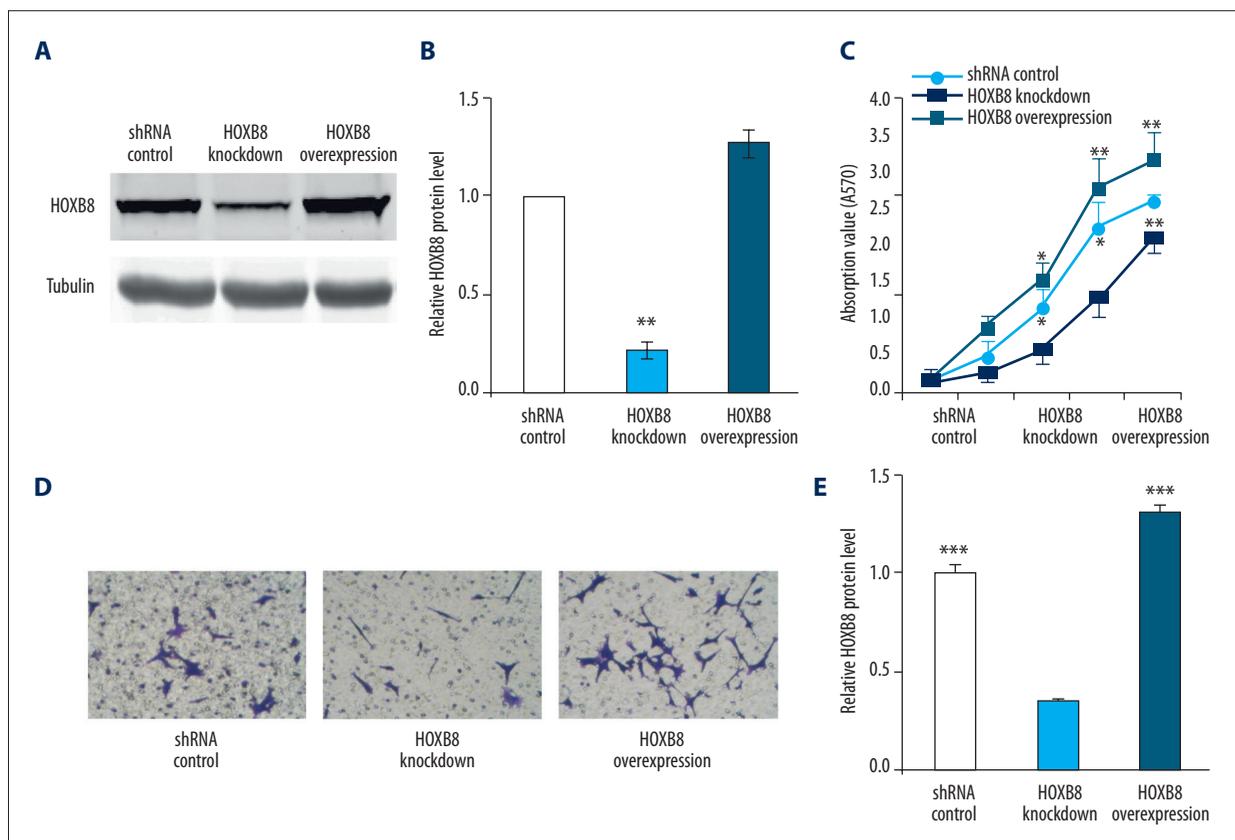
Figure 1. HOXB8 knockdown inhibits the proliferation and invasion of HCT116 cells. (**A**) The levels of HOXB8 were measured by western blot in HOXB8-depleted and HOXB8 over-expression HCT116 cells. (**B**) The relative protein expression levels of HOXB8 in HOXB8-depleted and HOXB8 over-expression HCT116 cells. ** $P<0.01$ is compared to the control. (**C**) Depletion of endogenous HOXB8 inhibited the proliferation of HCT116 cells. * $P<0.05$ and ** $P<0.01$ compared to the control. (**D**) Depletion of endogenous HOXB8 inhibited the invasion of HCT116 cells. (**E**) Quantification of invasion assays. The data are expressed as means ±SEM. from at least 3 independent experiments. *** $P<0.001$ compared to the control. SEM – standard error of the mean.

Statistical analysis

All data were presented as the mean ± standard deviation and analyzed using single-factor analysis of variance (one-way ANOVA) for comparison between groups. Multiple comparisons were performed among groups using ANOVA followed by the least significant difference test. The software package SPSS 19.0 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. The results are representative of 3 independent experiments. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HOXB8 regulates cancer cell proliferation and invasion

To establish the stable HOXB8 knockdown and HOXB8 over-expression cell lines, HOXB8 recombinant lentivirus infected

HCT116 cells were screened with puromycin for 3 to 4 weeks and then amplified. The protein expression levels of HCT116 in the knockdown cell lines were significantly lower than that in the HCT116 shRNA control group. Moreover, the protein expression levels of HCT116 in the over-expression cell lines were higher than HCT116 shRNA control group (Figures 1A, 1B). The results revealed successful construction of stable HOXB8 knockdown and HOXB8 over-expression cell lines, which laid the foundation for the subsequent experiments.

The MTT assay manifested that HOXB8 knockdown inhibited cell proliferation obviously at 48 hours. The antiproliferation effect of HOXB8 knockdown was more obvious with the increase of culture time. On the contrary, HOXB8 over-expression promotes cell proliferation (Figure 1C). To examine the role of HOXB8 in HCT116 cell invasion, HCT116 cells were infected with lentiviruses that expressed HOXB8 knockdown, HOXB8 over-expression or shRNA control. The invasive growth potential of these cells was measured by examining the functional
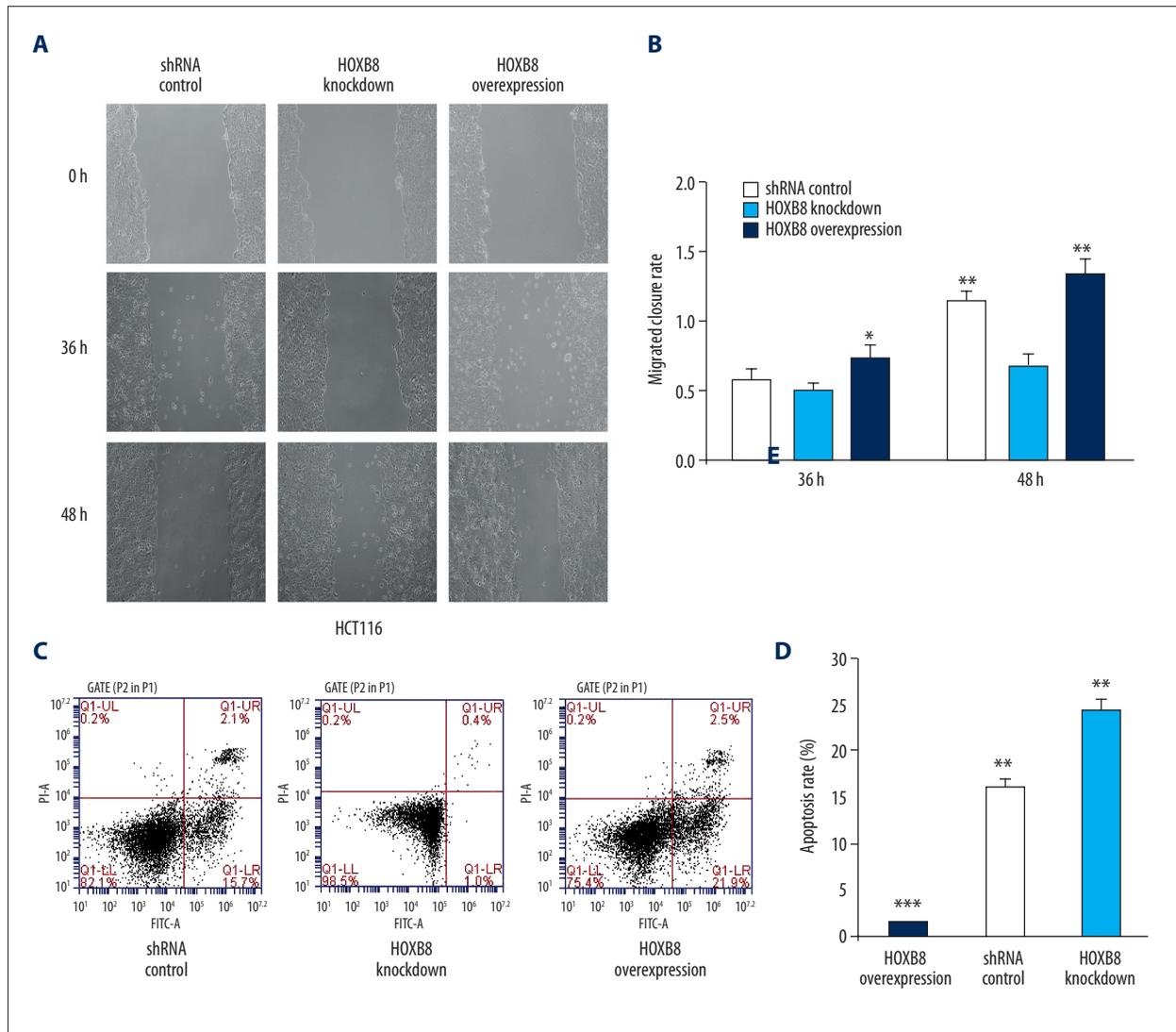
Figure 2. HOXB8 knockdown inhibited migration and induces apoptosis of HCT116 cells. (**A**) Wound-healing assays of shRNA control group, HOXB8-depleted and HOXB8 over-expression (400×) group. (**B**) Quantification of the wound-healing assays. The data are expressed as means ± SEM from at least 3 independent experiments. * *P*<0.05 and ** *P*<0.01 compared to the control. (**C**) The apoptotic rate of HCT116 cells among shRNA control, HOXB8-depleted, and HOXB8 over-expression HCT116 cells subjected to FACS. (**D**) Quantification of FACS. *** *P*<0.001, HOXB8 knockdown versus shRNA control. SEM – standard error of the mean; FACS – fluorescence-activated cell sorting.

capacity of the cells to penetrate through Transwell membranes coated with 0.35 mg/mL Matrigel. The results indicated that the invasiveness of HCT116 cells was significantly dropped following HOXB8 depletion as compared with that in the shRNA control group, whereby the rates were reduced by 67% in HOXB8 knockdown (Figure 1D, 1E).

116 cells

To determine whether HOXB8 regulates the migration of HCT116 cells, an artificial wound was created by scratching the cell monolayer of HCT116 cells with the tip of a 10-µL

pipette. The wound area was examined after 24 hours and 48 hours using an inverted phase-contrast microscope with a digital camera. The effect of HOXB8 on the migratory ability of cells is shown in Figure 2A and 2B. At 36 hours, the wound healing rate of HOXB8 over-expression cells was significantly increased compared with that of cells in the blank control group (*P*<0.05). At 48 hours, the migratory ability of HOXB8 knockdown cells was 0.59 times that of the control cells and HOXB8 over-expression is 1.12 times. These results indicated that HOXB8 had a significant promotive effect on the migratory ability of HCT116 cells.
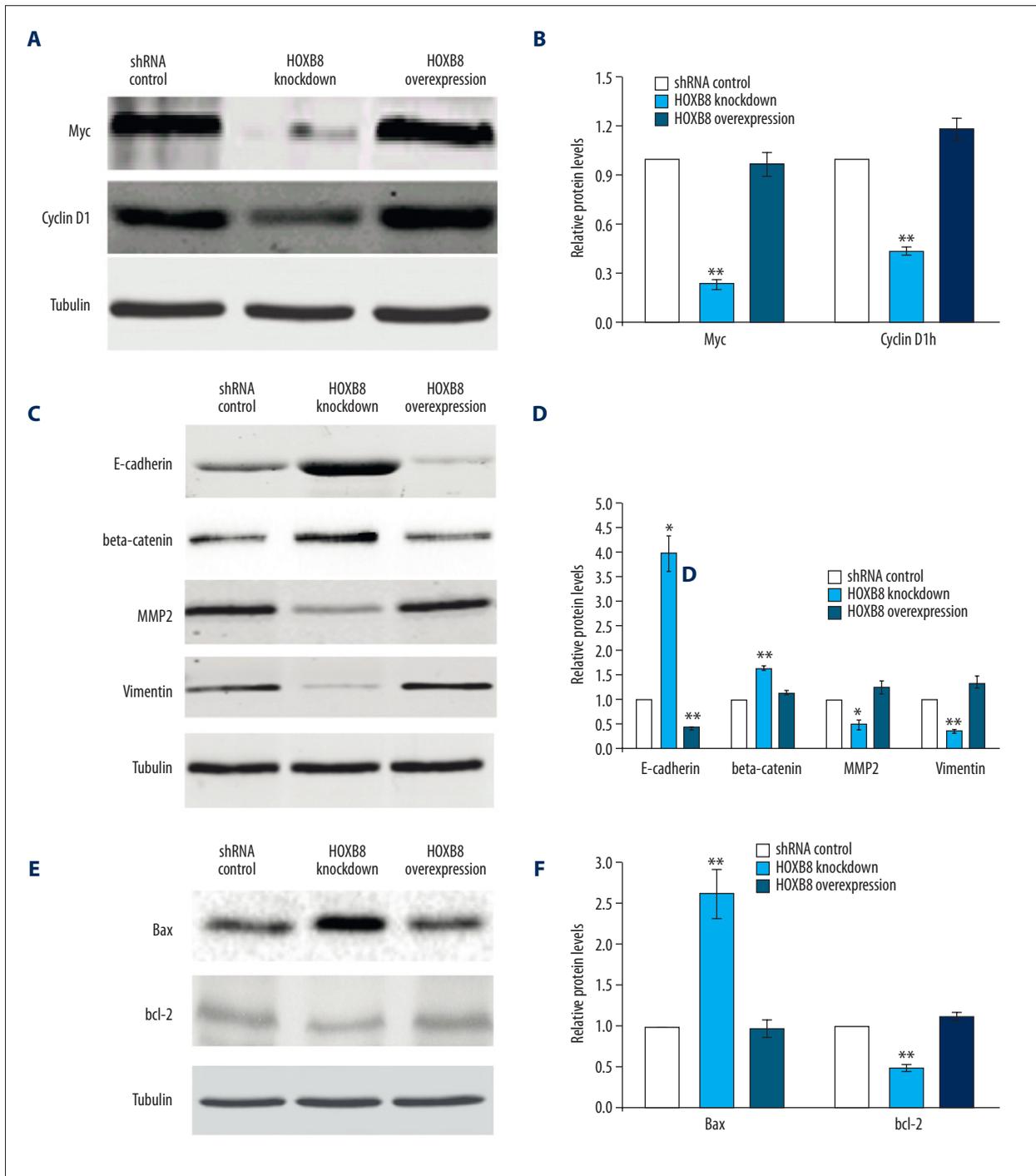
Figure 3. HOXB8 knockdown effects on expression levels of epithelial-mesenchymal transition (EMT) signaling pathway associated proteins in HCT116 cell lines. (**A**) The expression levels of c-Myc and CyclinD1 were measured by western blot in HCT116 cells. (**B**) The relative protein expression levels of c-Myc and CyclinD1 in HCT116 cells. * $P<0.05$ and ** $P<0.01$ compared to the control. (**C**) The expression levels of E-cadherin, MMP2, and vimentin were measured by western blot in HCT116 cells. (**D**) The relative protein expression levels of E-cadherin, MMP2, and vimentin in HCT116 cells. * $P<0.05$ and ** $P<0.01$ compared to the control. (**E**) The expression levels of BAX and bcl-2 were measured by western blot in HCT116 cells. (**F**) The relative protein expression levels of BAX and bcl-2 in HCT116 cells. * $P<0.05$ and ** $P<0.01$ compared to the control.
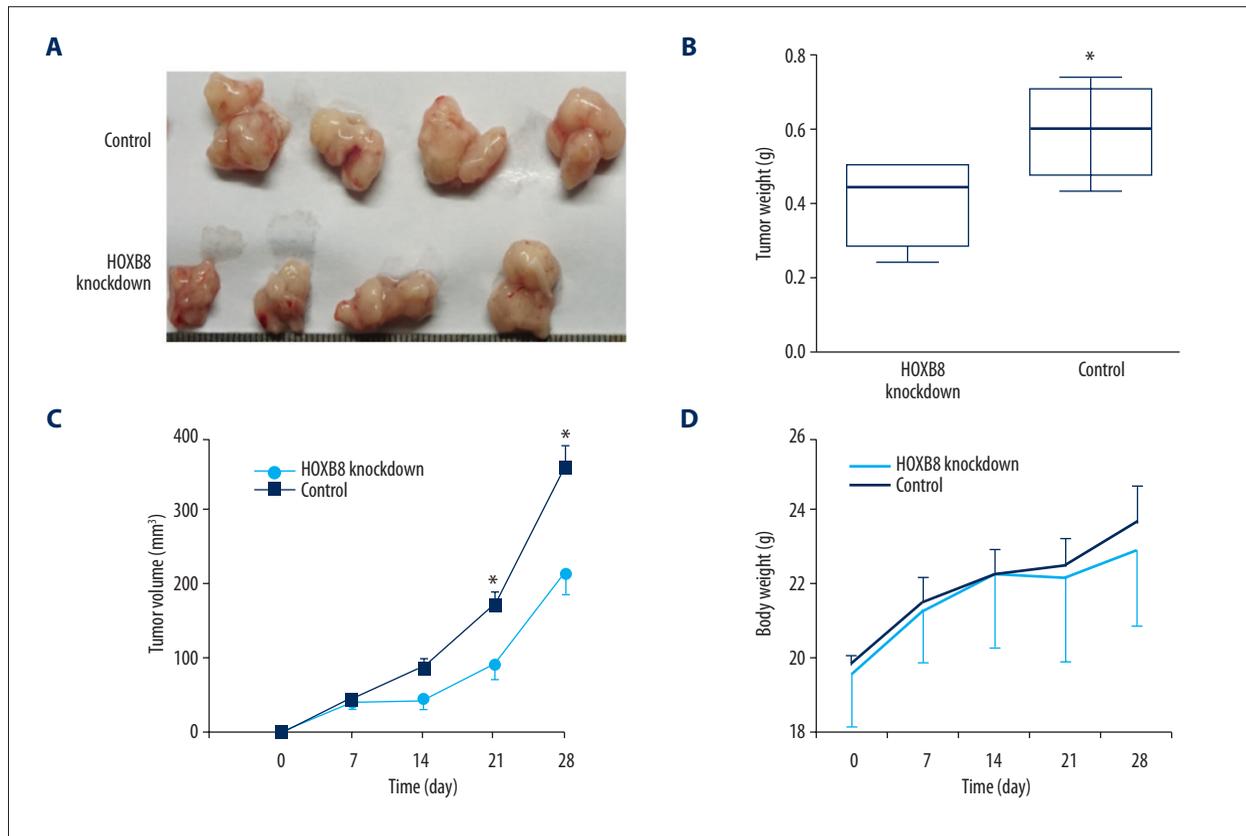
Figure 4. HOXB8 knockdown suppressed tumor growth in a xenograft model. BALB/c nude mice (5 to 6 weeks old) were injected subcutaneously with 1×10^6 shRNA control, HOXB8-depleted HCT116 cells. Tumor size was measured daily or every other day with calipers, and tumor volumes were calculated using the formula: volume=(width)$^2 \times$length/2. HOXB8 knockdown inhibited tumor growth (**A**), reduced tumor weight (**B**), and tumor volumes (**C**) but did not decrease mouse body weights (**D**). * $P<0.05$ is compared to the control.

The development of tumors is associated with the disruption of homeostasis between cell proliferation and apoptosis. In order to analyze the effect of HOXB8 on the apoptosis of HCT116 cells, the cell apoptotic rate was detected by FACS analysis with AnnexinV-FITC and PI double labeling. As shown in Figure 2C and 2D, the apoptotic rate of HCT116 cells was 1.53±0.088% and HOXB8 over-expression were 1.43±0.265%, while the apoptotic rates of HOXB8 knockdown cells were 4.13±0.218%. The difference between the HOXB8 knockdown groups and the normal control group were statistically significant (both $P<0.01$). These data suggest that HOXB8 knockdown promotes apoptosis of HCT116 cells.

HOXB8 knockdown effects on expression levels of epithelial-mesenchymal transition (EMT) signaling pathway associated proteins

Tumor cell invasion and metastasis are the main cause of death in cancer patients. In order to further reveal the mechanism that HOXB8 have effects on tumor invasion and migration, we examined the expression levels of related proteins in the epithelial-mesenchymal transition (EMT) signaling pathway. As opposed to control group, the expression levels of MMP and vimentin in HOXB8 knockdown group dramatically declined and HOXB8 over-expression group did not increase markedly. Meanwhile, it was also found that the expression of E-cadherin was restrained in HOXB8 over-expression group and apparently increased in HOXB8 knockdown group (Figure 3C, 3D). These results demonstrated that HOXB8 knockdown inhibited the tumor invasion, probably through the EMT signal pathway. In order to analyze the mechanism that HOXB8 regulates cancer cell proliferation, the protein expression levels of c-Myc and CyclinD1 were detected. As illustrated in Figure 3A, 3B, the expression levels of c-Myc and CyclinD1 in the HOXB8 knockdown group dramatically declined while the HOXB8 over-expression group did not show a significant increase.

To further analyze the cause of HOXB8-induced apoptosis, the effect of HOXB8 on the tumor apoptosis signaling pathway was determined using western blot analysis. The protein expression levels of BAX and bcl-2 were detected. The expression level of BAX and bcl-2 are shown in Figure 3E and 3F. The

level of BAX in HOXB8 knockdown cells was substantially higher than that in the shRNA control-transfected group and the HOXB8 over-expression group. Moreover, the expression level of bcl-2 in the HOXB8 knockdown cells was significantly lower than that in the shRNA control-transfected group and the HOXB8 over-expression group, which is indicative of the possibility that HOXB8 regulates apoptosis through mitochondrial pathways. However, the expression levels of BAX and bcl-2 in the HOXB8 over-expression group did not change significantly compared with that in the shRNA control.

HOXB8 knockdown suppressed tumor growth

To evaluate HOXB8 therapeutic potential, we determined whether HOXB8 knockdown inhibit tumor xenograft growth in nude mice. BALB/c nude mice (5 to 6 weeks old) were injected subcutaneously with 1×10^6 HCT116 shRNA control or HOXB8 knockdown cells per animal and mice were euthanized 4 weeks later. HOXB8 knockdown inhibited tumor growth, resulting in significantly reduced tumor volumes (Figure 4C). Indeed, the tumors in HOXB8 knockdown-treated mice were significantly smaller (Figure 4A), and HOXB8 knockdown decreased the tumor mass by 31.43% (Figure 4B). Interestingly, HOXB8 knockdown did not decrease mouse body weights (Figure 4D). Taken together, the study demonstrated that HOXB8 knockdown inhibited tumor growth in *vivo*.

Discussion

The research on transcription factors and signaling pathways related with cancer has gradually become a "hot spot" in the field of cancer research. It is a promising therapeutic approach to regulate the epigenetic characteristics of cells by controlling the expression levels of certain transcription factors or some key points in signaling pathways.

In this study, we found that in the HCT116 cells, HOXB8 knockdown inhibited the proliferation, invasion, and migration and induced apoptosis *in vitro*. The data on beta-catenin in Figure 3C shows that over-expression of HOXB8 had no significant effect on the beta-catenin level, and knock-down of HOXB8 would moderately increase beta-catenin levels. This was not consistent with the simple model that HOXB8 would activate the Wnt pathway by upregulation of the beta-catenin level. The reason is that there are 2 forms of beta-catenin in cells. The cytoplasmic form is the co-activator in the Wnt pathway. The membrane-bound form is in a complex with E-cadherin, and it is not involved in gene regulation. There is an equilibrium between these 2 forms, and the balance between them would be changed by E-cadherin level. Knock-down of HOXB8 leads to high level expression of E-cadherin. This could lead to the acumination of membrane-bound beta-catenin, which is seen

in Figure 3C. However, the cytoplasmic form of beta-catenin could be depleted, leading to reduced Wnt pathway activity. Overexpression of HOXB8 leads to reduced E-cadherin level. Although the overall beta-catenin level did not chance, the cytoplasmic form of beta-catenin could be increased, leading to activation of Wnt pathway [16]. HOXB8 knockdown reduced tumor growth and tumor weight in nude mice *in vivo*. The results were in stark contrast to those in the control group and the over-expression group. We further found that the expression levels of c-Myc and CyclinD1 in HOXB8 knockdown group dramatically declined and HOXB8 over-expression group increased. Previous studies found c-Myc proto-oncogene (MYC) is necessary to tumorigenesis in mouse models of colorectal cancers [17–20]. c-Myc has a number of putative targets, including genes involved in cell cycle control, apoptosis, DNA metabolism and dynamics along with energy metabolism and macromolecular synthesis[15]. CyclinD1 is responsible for cell cycle progression in the transition from G0/G1 to S phase and is overexpressed in various cancers such as cervical cancer [21]. The C-MYC and CyclinD1 were also identified as target genes in Wnt/β-catenin signaling conducted in the human HT29 colorectal cancer cell line harboring mutant APC alleles using a differential RNA expression screen [22]. Approximately 90% of sporadic colorectal cancers contain mutations in components of the Wnt/β-catenin signaling pathway [9]. These mutations are observed in the earliest neoplasms, suggesting that this pathway serves as a critical gatekeeper to prevent colorectal carcinogenesis [23]. When aberrantly activated, this signaling pathway leads to the accumulation of β-catenin in the cytoplasm, translocation of β-catenin to the nucleus to trigger theβ-catenin/T-cell factor/lymphoid enhancer factor (TCF/LEF) transcriptional machinery, and upregulation of target genes, such as those encoding CyclinD1, c-myc and matrix metalloproteinase (MMP)-7 [24].These mutations lead to inappropriate expression of genes controlled by Wnt responsive DNA elements (WREs). T-cell factor/lymphoid enhancer factor transcription factors bind WREs and recruit the β-catenin transcriptional co-activator to activate target gene expression. We then assessed the protein expression of 2 downstream products of β-catenin-TCF/LEF-driven transcription – c-Myc and CyclinD1 – and found that the expression levels of Myc and CyclinD1 dramatically declined in HOXB8 Knockdown group and increased in overexpression group. Accordingly, β-catenin-TCF/LEF-driven transcriptional activity was positively correlated with C-Myc and CyclinD1 protein expression. As a result, we deduced that HOXB8 gene might regulate the proliferation and migration of colorectal cancer cells via Wnt/β-catenin signaling.

Several studies show that Wnt/β-catenin signaling plays a crucial role in epithelial-mesenchymal transition (EMT) [25–27]. Downregulation of E-cadherin, which releases free β-catenin, induces EMT in colon epithelial cells [28–30]. During the EMT procedure, tumor cells accumulate nuclear β-catenin by the

progressive loss of E-cadherin and the acquisition of mesenchymal markers such as vimentin, MMP2 and N-cadherin [31,32]. EMT also plays a crucial role in cancer migration and metastasis [33]. Thus, Wnt/β-catenin signaling and EMT might act synergistically during carcinogenesis. To further illustrate that HOXB8 gene might regulate the migration and metastasis of colorectal cancer cells via Wnt/β-catenin signaling, we also examined 2 downstream EMT markers and found that the level of E-cadherin in HOXB8 knockdown cells was significantly higher than that in shRNA control-transfected and HOXB8 over-expression groups. Moreover, the expression level of MMP2 and vimentin in HOXB8 knockdown cells was significantly lower compared with that in shRNA control-transfected and HOXB8 over-expression groups. Therefore, we consider that HOXB8 gene could regulate the proliferation and migration of colorectal cancer cells via Wnt/β-catenin signaling. HOXB8 gene not only regulates the level of CyclinD1 and c-Myc but also regulates EMT procedure via Wnt/β-catenin signaling.

Cellular damage is not only the consequence of mitochondrial bioenergetic failure, given that these organelles also play a crucial role in apoptosis, when in response to several stimuli they release cytochrome c and other pro-apoptotic proteins that execute cell demise. Mitochondrial permeabilization during apoptosis is controlled by members of the Bcl-2 family and is accompanied by both morphological and ultrastructural changes of the organelle [34–36]. The pro-apoptotic BCL2 gene family member, BAX, plays a pivotal role in the intrinsic apoptotic pathway and executes the switch to cell death. Cellular stressors activate BH3-only proteins like tBID, BIM, and PUMA, which drive a cell toward apoptosis by both activating BAX and by neutralizing anti-apoptotic proteins [37–39].

Conclusions

The current study reveals that knock-down of HOXB8 prohibits the proliferation and migration of colorectal cancer cells via the Wnt/β-catenin signaling pathway and the downregulation of various factors, such as MMP2, c-Myc, CyclinD1, and vimentin. Targeting Wnt/β-catenin signaling pathway has been considered as a potential therapeutic strategy for colorectal cancer. Therefore, our data suggest that HOXB8 has great potential to be developed as a novel therapeutic agent for the treatment of human colorectal cancer.

We examined 2 downstream apoptosis markers and found that the level of BAX in HOXB8 knockdown cells was significantly higher compared with that in shRNA control-transfected and HOXB8 over-expression groups. Moreover, the expression level of bcl-2 in HOXB8 knockdown cells was significantly lower than that in shRNA control-transfected and HOXB8 over-expression groups, indicating that HOXB8 may regulate apoptosis through mitochondrial pathway. This specific action mechanism needs further study.

Conflicts of interest

None.

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