*Oncology Research*, Vol. 25, pp. 537–544

0965-0407/17 $90.00 + .00
DOI: https://doi.org/10.3727/096504016X14756640150695
E-ISSN 1555-3906
www.cognizantcommunication.com

# Empty Spiracles Homeobox 2 (EMX2) Inhibits the Invasion and Tumorigenesis in Colorectal Cancer Cells

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Empty spiracles homeobox 2 (EMX2) is a homeodomain-containing transcription factor that plays an essential role in tumorigenesis. However, to the best of our knowledge, the role of EMX2 in human colorectal cancer (CRC) is still unclear. Thus, the aim of this study was to investigate the expression and role of EMX2 in CRC. Our results demonstrated that the expression of EMX2 was greatly decreased in CRC tissues and cell lines. Overexpression of EMX2 significantly inhibited the proliferation in vitro and CRC tumor growth in nude mice. In addition, EMX2 also inhibited the migration and invasion of CRC cells. Mechanically, overexpression of EMX2 downregulated the expression levels of β-catenin, cyclin D1, and c-Myc in CRC cells. Taken together, our study demonstrates that EMX2 inhibits proliferation and tumorigenesis through inactivation of the Wnt/β-catenin pathway in CRC cells. Therefore, EMX2 may be a potential therapeutic target for the treatment of CRC.

**Key words: Empty spiracles homeobox 2 (EMX2); Colorectal cancer (CRC); Invasion; Wnt/β-catenin pathway**

## INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer deaths in the world. The incidence of CRC has been increasing dramatically over the last 20 years[1]. Despite improvement in treatment strategies, the 5-year survival of patients with advanced CRC remains very low[2–4]. Therefore, a better understanding of the molecular mechanisms and identification of tumor suppressors are essential for the development of prognostic and predictive markers that aid novel effective therapies for CRC.

Empty spiracles homeobox 2 (EMX2) belongs to the homeobox gene family, which are thought to regulate morphogenesis and cell differentiation during embryogenesis[5]. EMX2 is expressed in the embryonic cortex and regulates cortical cell migration, glial development, and the formation of cortical axon projections[6,7]. EMX2 also controls mammalian reproduction by adjusting endometrial cell proliferation[7]. In addition, previous work has demonstrated that EMX2 was deregulated in a wide range of human cancers, including lung squamous cell carcinoma (SCC)[8], gastric carcinoma[9], endometrial cancer,[10] and malignant pleural mesothelioma[11]. For example, Li et al. reported that the expression of EMX2 is significantly downregulated in gastric cancer, and restoring EMX2 expression inhibits the proliferation in vitro and tumor growth in a gastric cancer xenograft model in vivo[9]. However, the function of EMX2 in CRC is still unclear. Therefore, in this study, the expression of EMX2 in cancer tissue samples from CRC patients and CRC cell lines was detected by RT-PCR and Western blotting, and the role of EMX2 on the biological properties of CRC cells was tested in vitro and in vivo. Herein we report that EMX2 deactivates the Wnt/β-catenin signaling pathway and consequently represses invasion and tumorigenesis in CRC.

## MATERIALS AND METHODS

### *Tissue Specimens*

CRC and adjacent nontumor colon tissues were collected from patients undergoing resection of CRC. None of the patients received chemotherapy prior to colectomy. Tissue samples were immediately frozen in liquid nitrogen and stored until use. Informed consent was obtained from each patient before the surgery. Experimental procedures were approved by The Second Affiliated Hospital of Medical School, Xi'an Jiaotong University.

### *Cell Culture*

The human embryonic kidney 293T cell line (HEK293T) and three human CRC cell lines (HCT116, HT29, and SW480) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA).

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All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), streptomycin (100 mg/ml), and penicillin (100 mg/ml) at 37°C in 5% $CO_2$.

*Real-Time Quantitative RT-PCR (RT-qPCR) Analysis*

Total RNA was extracted from CRC tissues and cells using TRIzol reagent (Abcam, Cambridge, UK) according to the manufacturer's instructions. Complementary DNAs were synthesized, and real-time PCR was performed using real-time SYBR Green (Bio-Rad Laboratories, Berkeley, CA, USA). The following primer sequences were used for amplification of the following genes: EMX2, 5′-GG AAAGGAAGCAGCTGGCTCACAGTCTCAGTCTTA C-3′ (forward) and 5′-GTGGTGTGTCCCTTTTTTCTT CTGTTGAGAATCTGAGCCTTC-3′ (reverse); β-actin, 5′-TTAGTTGCGTTACACCCTTTC-3′ (forward) and 5′-ACCTTCACCGTTCCAGTTT-3′ (reverse). The relative expression levels of the target gene mRNA were calculated as the inverse log of $^{\Delta\Delta}$CT and were normalized to the reference gene β-actin[12].

*Western Blotting*

Human CRC tissues or cells were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, and 1% Triton X-100) containing protease inhibitors and phosphatase inhibitors (Sigma-Aldrich). Protein concentrations were determined by the BCA method. The samples (30 μg protein/lane) were separated on 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After blocking in TBS buffer (50 mmol/L NaCl, 10 mmol/L Tris, pH 7.4) containing 5% nonfat milk, the membranes were reacted with the antibody at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h. Subsequently, the blots were observed using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Rockford, IL, USA).

*Construction of the pcDNA3.1-EMX2 Vector and Cell Transfection*

The human EMX2 expression vector pcDNA3.1-EMX2 and empty vector were purchased from Shanghai Sangon Co., Ltd (Shanghai, P.R. China). For in vitro transfection, HCT116 cells ($5\times10^5$ cells/well) were transfected with pcDNA3.1-EMX2 or empty vector using Lipofectamine 2000 (Invitrogen, Victoria, Australia) according to the manufacturer's instructions. The transfection efficiency was examined by RT-qPCR and Western blotting.

*Cell Proliferation Assay*

Cell proliferation was evaluated using the CCK-8 assay (Sigma-Aldrich). Briefly, infected cells at a density of $1\times10^4$ cells/well were plated onto 96-well plates. Following culture for the indicated time periods (0, 24, 48, and 72 h), 10 μl of CCK-8 solution was added into each well and incubated at 37°C. After 3 h, the absorbance at 450 nm was measured with a microplate reader (Takara Biotechnology, Dalian, P.R. China).

*Cell Invasion and Migration Assays*

Cell invasion was assessed by Transwell chamber assay. The upper sides of the filters were coated with 50 μl of Matrigel (BD Biosciences, Bedford, MA, USA). Infected cells ($1\times10^4$ cells/well) in 0.5% serum containing culture medium (HyClone, Ogden, UT, USA) were plated in the upper chamber, and 500 μl of DMEM containing 5% FBS was added to the lower chamber. Following incubation at 37°C with 5% $CO_2$ for 24 h, cells on the upper side of the filter were removed, and cells that adhered to the underside of the membrane were fixed with methanol, stained with crystal violet, and then counted under a light microscope. The migration assay was done using the same procedure, except that the membrane was not coated with Matrigel.

*Tumorigenicity Assay*

Five-week-old female BALB/c nude mice were purchased from the Experimental Animal Centre of The Second Affiliated Hospital of Xi'an Jiaotong University (P.R. China) and acclimated for 2 weeks. All animal procedures were approved by the Animal Care and Use Committee of The Second Affiliated Hospital of Medical School, Xi'an Jiaotong University. HCT116 cells ($1\times10^6$ cells/0.1 ml) transfected with pcDNA3.1-EMX2 or empty vector were subcutaneously injected into the flank of BALB/c nude mice. The resulting tumors were measured once a week, and tumor volumes ($mm^3$) were calculated using the following formula: $\text{volume} = \text{width}^2 \times \text{length} \times 0.5$. Four weeks after injection, mice were euthanized, and tumors were dissected and weighed.

*Statistical Analysis*

Statistical analysis was performed using the SPSS statistical software program (Version 13.0; SPSS Inc., Chicago, IL, USA). All data were reported as means ± SD. A one-way ANOVA was used for comparison of multiple groups. A value of $p<0.05$ was considered to indicate a statistically significant difference.

## RESULTS

*EMX2 Is Lowly Expressed in CRC Tissues and Cell Lines*

We first detected the mRNA levels of EMX2 in CRC tissues and paired normal tissues using RT-qPCR. The

mRNA level of EMX2 was decreased in CRC tissues (Fig. 1A). The results of the Western blotting showed that the protein level of EMX2 in CRC tissues was also significantly reduced (Fig. 1B). In addition, EMX2 was found to be lowly expressed in three human CRC cell lines compared to the HEK293T cell line (Fig. 1C and D).

*EMX2 Inhibits the Proliferation in CRC Cells*

To illustrate the role of EMX2 in CRC, we generated a stable human CRC cell line in which EMX2 was overexpressed. After HCT116 cells were transfected with pcDNA3.1-EMX2 or empty vector, we measured the transfection efficiency of EMX2 using RT-qPCR and Western blotting. The expression of EMX2 at both mRNA and protein levels was significantly increased in HCT116 cells transfected with pcDNA3.1-EMX2, compared to cells transfected with empty vector (Fig. 2A and B). In addition, we observed that restoration of EMX2 significantly suppressed the proliferation of HCT116 cells, as demonstrated by the CCK-8 assay ($p < 0.05$) (Fig. 2C).

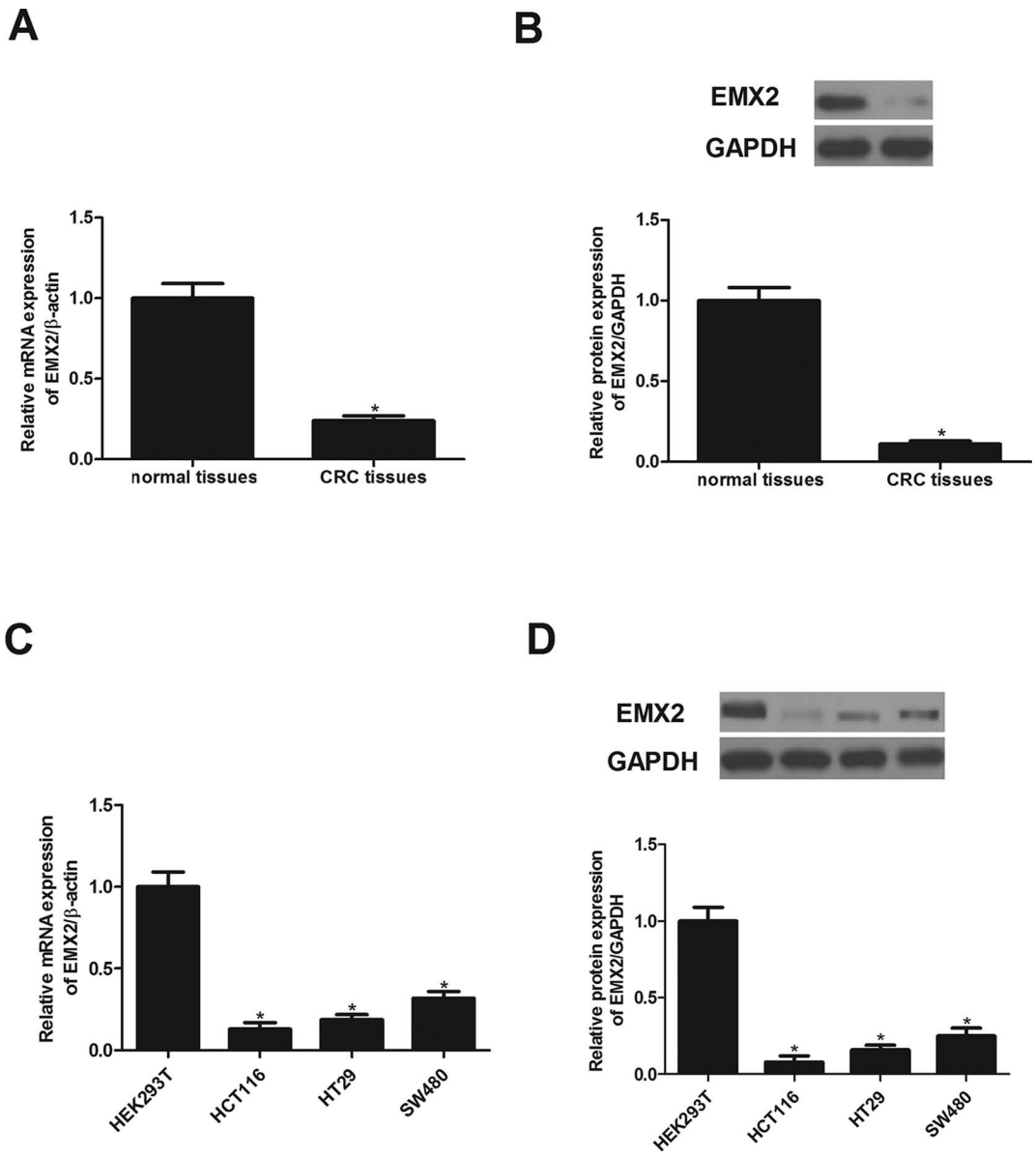


**Figure 1.** EMX2 is lowly expressed in CRC tissues and cell lines. (A) Relative EMX2 mRNA levels in CRC tissues and paired normal tissues. (B) EMX2 protein levels in CRC tissues and paired normal tissues. *$p < 0.05$ versus normal tissues. (C) Relative EMX2 mRNA levels in human CRC cell lines and the human embryonic kidney 293T cell line. (D) EMX2 protein levels in human CRC cell lines and the human embryonic kidney 293T cell line. *$p < 0.05$ versus 293T cell line.

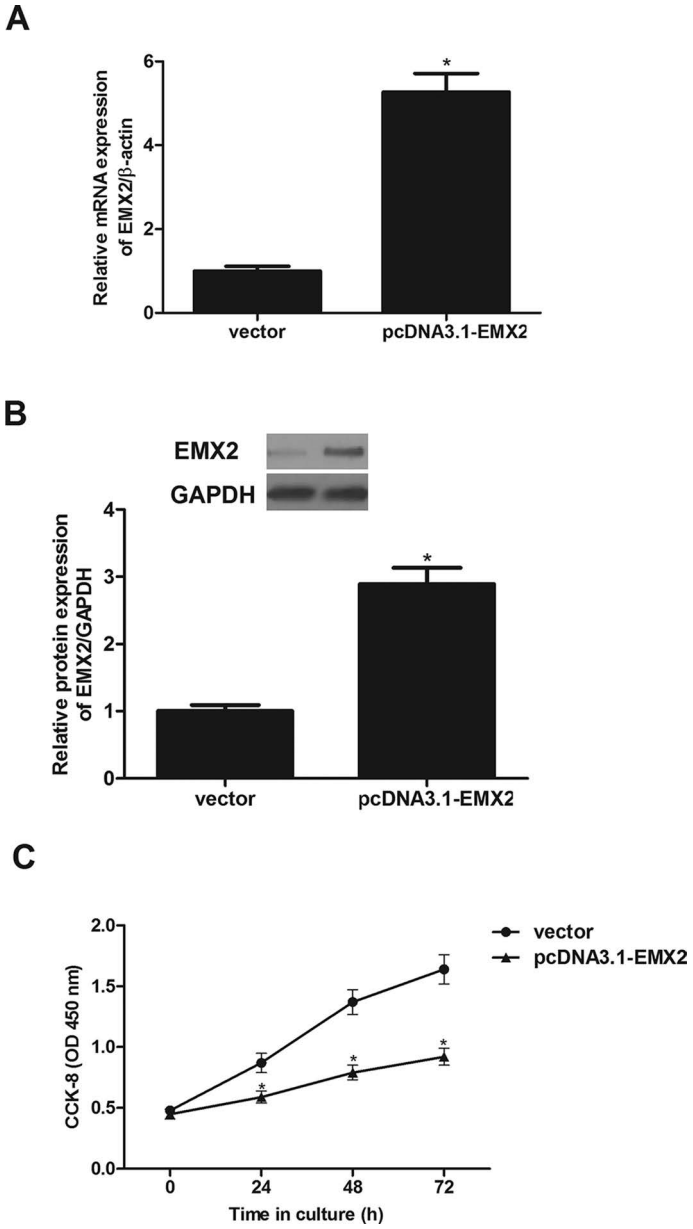


**Figure 2.** EMX2 inhibits proliferation in CRC cells. HCT116 cells were transfected with pcDNA3.1-EMX2 or empty vector for 24 h. (A) EMX2 mRNA expression was determined using RT-qPCR analysis. (B) EMX2 protein expression was determined using Western blot analysis. (C) Cell proliferation was evaluated by the CCK-8 assay. *$p < 0.05$ versus vector group.

*EMX2 Inhibits the Tumorigenicity of CRC Cells In Vivo*

To examine whether EMX2 affects the tumorigenicity of CRC cells in vivo, we generated tumor xenografts by subcutaneously injecting $1 \times 10^6$ HCT116 cells transfected with pcDNA3.1-EMX2 or empty vector. Restoration of EMX2 significantly reduced the average volume of tumors, compared with the empty vector control group (Fig. 3A). In addition, the average final tumor weight in the EMX2-transfected group (0.21 g) was dramatically less than that in the empty vector control group (0.67 g) upon termination of the experiment (Fig. 3B).

*EMX2 Inhibits the Migration and Invasion of CRC Cells*

Then we examined the effects of EMX2 on the migration and invasion in HCT116 cells. Overexpression of EMX2 substantially inhibited the migration of HCT116 cells in the Transwell chamber assay, compared to the empty vector control-transfected cells (Fig. 4A). Consistent with this result, the Transwell Matrigel invasion assay

showed that the mean number of invasive cells (mean number = 114) was significantly fewer in the HCT116 cells stably transfected with EMX2 expression group than that in the empty vector (mean number = 256) control group (Fig. 4B). In order to investigate whether EMX2 decreased CRC cell invasion by inhibiting EMT, we analyzed the level of several EMT markers in HCT116 cells transfected with EMX2. The results of the Western blotting analysis showed that EMX2 overexpression accelerated E-cadherin upregulation, and N-cadherin and vimentin downregulation, compared to control vector-transfected cells (Fig. 4C).

### *EMX2 Inhibits Activation of the Wnt/β-Catenin Pathway in CRC Cells*

In order to explore the underlying molecular mechanisms responsible for the functions of EMX2 in CRC, we examined the effect of EMX2 on the expression of β-catenin, cyclin D1, and c-Myc in HCT116 cells. Western blotting showed that restoration of EMX2 obviously downregulated the protein expression levels of β-catenin, cyclin D1, and c-Myc in HCT116 cells, compared with the empty vector group (Fig. 5).

## DISCUSSION

In the present study, we show for the first time that EMX2 is downregulated in CRC tissues and cell lines at both the mRNA and protein levels. Restoration of EMX2 significantly inhibited the proliferation in vitro and tumorigenesis in vivo. Moreover, overexpression of EMX2 significantly inhibited the migration, invasion, and EMT phenotype in CRC cells. Mechanistically, restoration of EMX2 obviously downregulated the protein expression levels of β-catenin, cyclin D1, and c-Myc in HCT116 cells.

EMX2 is aberrantly expressed in some human cancers and has been implicated in the progression of some malignancies. Okamoto et al. reported that EMX2 was

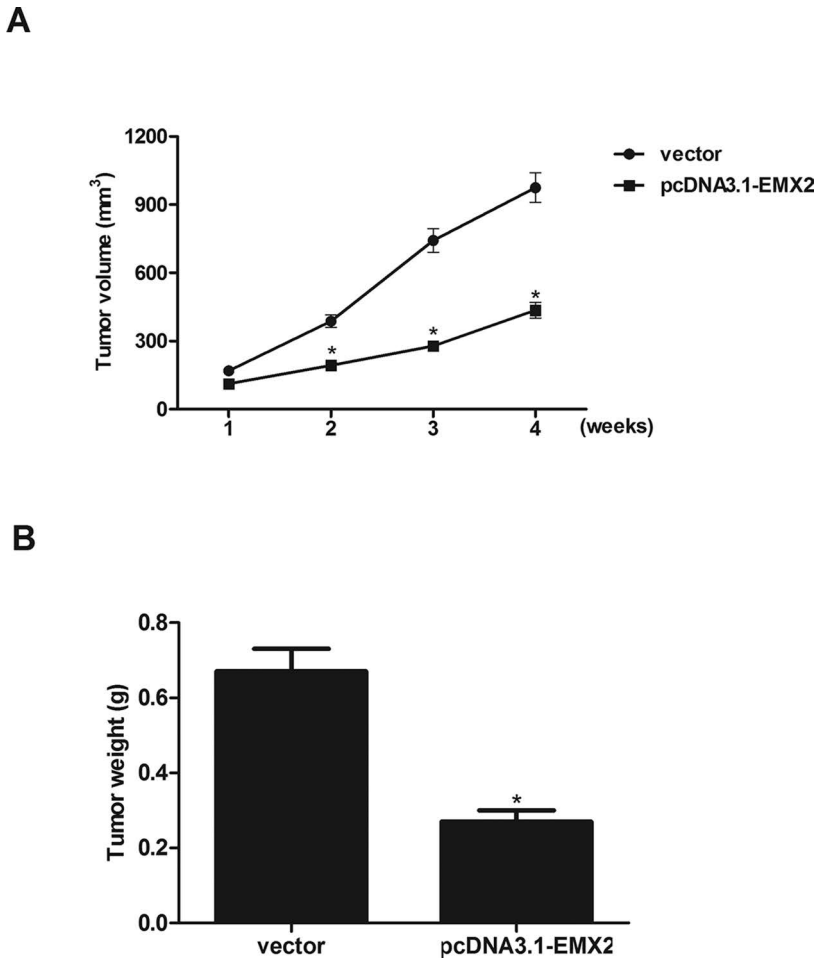


**Figure 3.** EMX2 inhibits the tumorigenicity of CRC cells in vivo. HCT116 cells ($1 \times 10^6$ cells/0.1 ml) transfected with pcDNA3.1-EMX2 or empty vector were subcutaneously injected into the flank of BALB/c nude mice. (A) Tumor volume was monitored every week. (B) Four weeks after injection, mice were euthanized, and tumors were dissected and weighed. *$p < 0.05$ versus vector group.

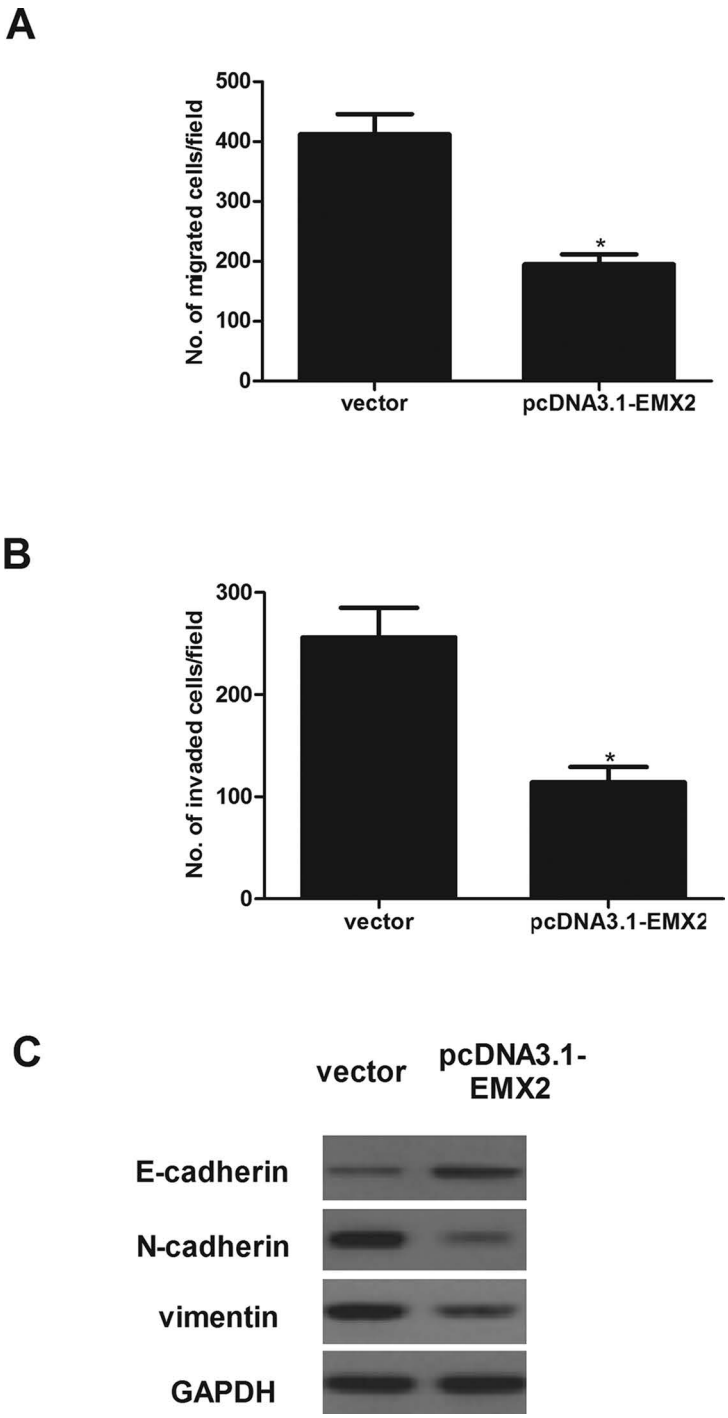


**Figure 4.** EMX2 inhibits the migration and invasion of CRC cells. HCT116 cells were transfected with pcDNA3.1-EMX2 or empty vector for 24 h. (A) Cell migration assay was detected using the Transwell chamber assay. (B) Cell invasion assay was detected using the Transwell Matrigel invasion assay. (C) The protein levels of E-cadherin, N-cadherin, and vimentin were determined by Western blotting. $*p < 0.05$ versus vector group.

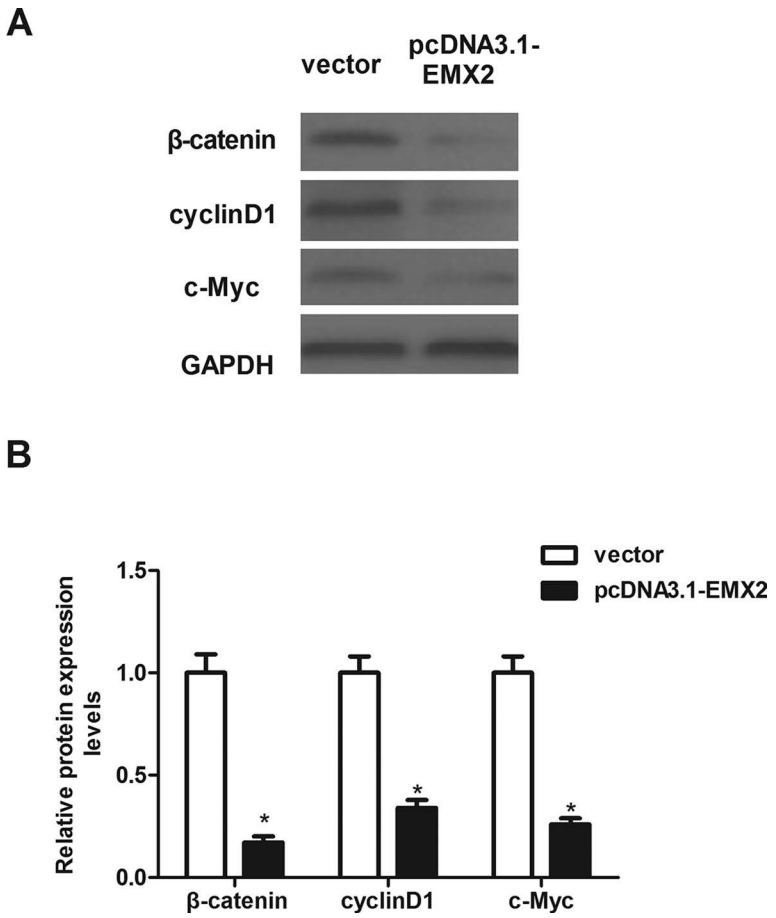


**Figure 5.** EMX2 inhibits the activation of the Wnt/β-catenin pathway in CRC cells. HCT116 cells were transfected with pcDNA3.1-EMX2 or empty vector for 24 h. (A) β-Catenin, cyclin D1, and c-Myc expression levels were detected by Western blot. (B) The relative protein expression levels of β-catenin, cyclin D1, and c-Myc were quantified using Image-Pro Plus 6.0 software and normalized to GAPDH. $*p<0.05$ versus vector group.

dramatically downregulated in lung cancer tissue samples, and restoration of EMX2 expression suppressed cell proliferation in lung cancer cells[13]. Downregulation of EMX2 was also found in endometrial cancer tissues[10]. Consistent with previous findings, we found that EMX2 is downregulated in CRC tissues and cell lines. Restoration of EMX2 significantly inhibited the proliferation in vitro and tumorigenesis in vivo. All of these data suggest that EMX2 may serve as a tumor suppressor in the development and progression of CRC.

Metastasis remains the major cause of death in CRC[14]. EMT is a pathological process whereby epithelial cells lose their cell polarity and adhesion characteristics while acquiring a mesenchymal phenotype[15]. It is essential for the initial and overall rate-limiting steps of CRC invasion and metastasis[16]. A recent study showed that knockdown of EMX2 expression in lung SCC cells promoted cell migration and downregulated the expression of E-cadherin[8]. In this study, we observed that overexpression of EMX2 significantly inhibited migration and invasion in HCT116 cells, which was associated with a significant increase in the expression of E-cadherin, but a reduced expression of N-cadherin and vimentin. These results suggest that EMX2 greatly induced a decrease in HCT116 cell migration and invasion by suppressing the EMT phenotype.

The Wnt/β-catenin signaling pathway is one of the most important signaling pathways identified as being implicated in tumorigenesis[17–19]. The key effector protein in the canonical Wnt/β-catenin pathway is the transcriptional coactivator β-catenin. When Wnt signaling is activated, β-catenin is discharged from the degradation of complex resulting in the translocation of β-catenin into the nucleus, where it associates with the T-cell factor/lymphoid-enhancer factor (TCF/LEF) family of transcription factors to activate the Wnt downstream genes[20].

Previous studies showed that the level of β-catenin is significantly increased in advanced CRC[21–23]. In addition, c-Myc expression is thought to be an indicator of Wnt/β-catenin activity, and overexpression of c-Myc induced EMT in mammary epithelial cells[24]. The Wnt/β-catenin signaling pathway has been shown to promote EMT in CRC cells. For example, Lee et al. reported that IWR-1, one of the inhibitors of Wnt response (IWR), inhibits the EMT of CRC cells by suppressing the Wnt/β-catenin signaling pathway[25]. Our results showed that ectopic EMX2 expression significantly downregulated the expression of β-catenin and of the well-established target proteins of Wnt/β-catenin signaling, namely, c-Myc and cyclin D1 in HCT116 cells. These data suggest that EMX2 inhibits the proliferation and tumorigenesis in CRC cells through the Wnt/β-catenin signaling pathway.

In conclusion, EMX2 plays an important role in inhibiting the invasion and tumorigenesis in CRC cells through the Wnt/β-catenin signaling pathway. Therefore, EMX2 is expected to provide a more powerful potential to suppress CRC invasion and metastasis, representing a promising strategy for the treatment of CRC.

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