

## Original Article

# Zinc finger E-box binding homeobox 2 silencing suppresses proliferation, migration and invasion of human colon cancer cells

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**Abstract:** Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths. Investigations into the underlying molecular mechanisms of CRC development and metastasis to develop novel therapeutic targets are of great importance. Previous studies provided indirect evidence that ZEB2 regulates CRC metastasis, but the effect of ZEB2 expression on the proliferation, replication, apoptosis, migration, and invasion of CRC cells is unclear. In this study, we collected CRC and adjacent tissue samples to detect ZEB2 expression by immunohistochemistry. In addition, HT-29, CRC cell line, was cultured and ZEB2 expression was quantified by qRT-PCR and western blotting. After transfection of si-ZEB2 into HT-29 cells, ZEB2 expression was quantified by qRT-PCR and Western blotting; proliferation was evaluated by MTS, cell cycle progression and apoptosis were assessed by flow cytometry; in addition, migration and invasion were examined by transwell assay. Our results showed that ZEB2 was significantly upregulated in the CRC tissues compared to that in the adjacent tissue. In addition, ZEB2 silenced in HT-29 significantly inhibited proliferation, replication, migration, and invasion, but had no significant effect on apoptosis. In conclusion, silenced ZEB2 inhibited CRC development and metastasis. Our findings suggest that ZEB2 could be a potential therapeutic target for CRC.

**Keywords:** Colorectal cancer, ZEB2, proliferation, cell cycle, migration, invasion, apoptosis

## Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths, with an estimated 1.4 million cases and 693,900 deaths occurring in 2012 [1]. The incidence rates are higher in Europe and Northern America than in Africa and South-Central Asia. In China, the incidence of CRC is historically low, but is now increasing [2]. Studies on the mechanism of CRC development and metastasis have helped cure patients with CRC; however, the underlying mechanism is still poorly understood. Therefore, further investigation into the underlying molecular mechanisms of CRC development and metastasis to develop novel therapeutic targets is of great importance.

Zinc finger E-box binding homeobox 2 (ZEB2) belongs to the Zinc finger E-box binding protein family. Previous studies have showed that ZEB2

can directly promote epithelial-to-mesenchymal transition (EMT) and play an important role in cancer development and metastasis [3-5]. During CRC invasion, ZEB2 correlates with tumor progression and predicts cancer-specific survival in primary CRC; Therefore, it may be a potential target for the treatment of CRC [6]. MicroRNA-132 and -335 inhibit invasion and metastasis by targeting ZEB2 in CRC [7, 8]. These studies provide indirect evidence that ZEB2 regulates CRC metastasis; however, the effect of ZEB2 expression on the proliferation, cell cycle, apoptosis, migration, and invasion of CRC is unclear.

In this study, we found that ZEB2 was overexpressed in the CRC tissue. Silencing of ZEB2 could arrest the cell during transition from G1 to S phase, inhibit CRC proliferation, migration, and invasion, but it did not affect CRC cell apoptosis.

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## Materials and methods

### *Patients and tissue specimens*

Paraffin-embedded cancer and adjacent tissue samples from 28 patients with colon cancer were obtained from the Department of Pathology, Xiamen Haicang hospital, between January 2015 and June 2015. None of the patients received pre-operative anti-cancer treatment. Pathologists confirmed the diagnosis of colon cancer in all patients. The final study protocol was approved by the Ethics Committee of our hospital, and informed consent was obtained from each patient included in the study.

### *Immunohistochemistry*

Immunohistochemistry (IHC) was performed according to a previously described method [9]. After being de-waxed and re-hydrated in xylene and graded concentrations of ethanol, antigen retrieval for tissue sections were implemented using 0.01 M sodium citrate buffer (pH 6.0). Then, the tissue sections were incubated under dark conditions with anti-ZEB2 rabbit antibody (1:200, CST, Beverly, MA, USA) for 2 h at 37°C. The primary antibody was detected using an HRP-conjugated secondary antibody (Gene Tech, Shanghai, China) for 30 min at room temperature. After IHC, image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA) was used for analyzing ZEB2 expression. Integral optical density (IOD) was chosen as the total expression level of ZEB2 and mean optical density, which represents the expression level of ZEB2, is equal to IOD SUM/area.

### *Cell culture and siRNA transfection*

The CRC cell line, HT-29, was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL) (Gibco, NY, USA) at 37°C in 5% CO<sub>2</sub> humidified atmosphere. When HT-29 cells reached 50% confluence, they were transfected with si-ZEB2 (Santa Cruz Biotechnology, Santa Cruz, USA), or si-NC (5'-UUUGUACUACACAAAAGUACUG-3', GenPharma, Shanghai, China), or not transfected (control) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA,

USA) according to the manufacturer's instructions.

### *Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)*

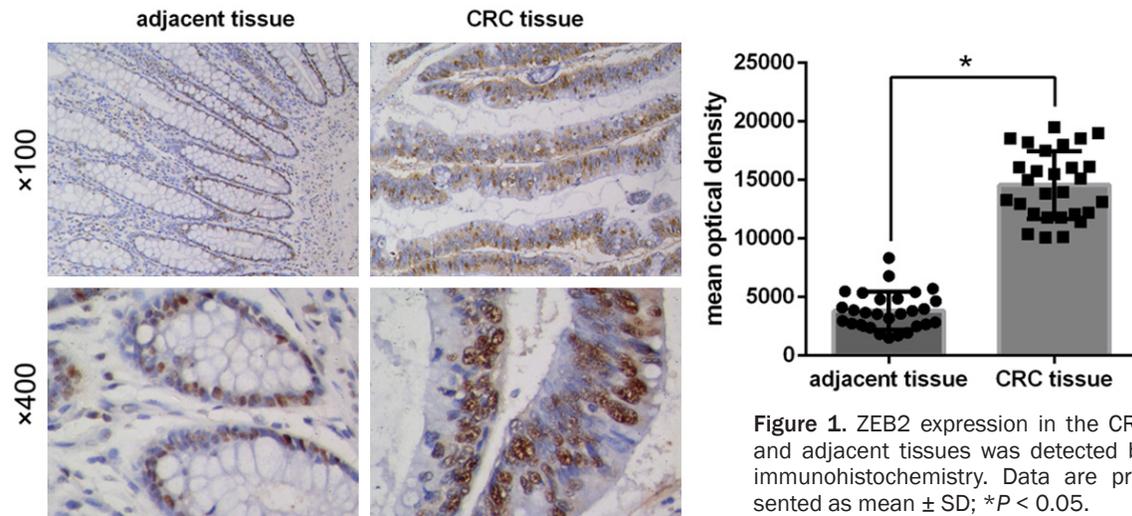
After culturing for 24 h, HT-29 cells were harvested and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The total RNA was reverse transcribed into cDNA using PrimeScript RT reagent kit with cDNA Eraser (Takara Bio, Dalian, China). qPCR was performed using SYBR Green master mix system (Applied Biosystem, Carlsbad, CA, USA). QRT-PCR was performed on an Applied Biosystems 7500 system (Applied Biosystems, Warrington, UK). *GAPDH* was used as an internal control. Gene expression was measured in triplicate, quantified using the 2<sup>-ΔΔCT</sup> method, and normalized to the control. For *ZEB2*, the forward and reverse primers were 5'-AAATGCACAGAGTGTGGCAAGG-3' and 5'-CTGCTGATGTGCGAACTGTAGGA-3', respectively. For *GAPDH*, the forward and reverse primers were 5'-ACACCCACTCCTCCACCTTT-3' and 5'-TTACTCCTTGGAGGCCATGT-3', respectively.

### *Western blotting*

After culturing for 48 h, HT-29 cells were harvested and lysed using RIPA buffer (Takara, Dalian, China). Total protein concentration was estimated using the BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA). Proteins were separated using 12% SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with rabbit anti-ZEB2 (1:1000, CST, Beverly, MA, USA), followed by incubation with HRP-conjugated goat anti-rabbit IgG H&L secondary antibody (1:10000, Southern Biotech, Birmingham, AL, USA) for 40 min. The membranes were also incubated with HRP-conjugated monoclonal mouse anti-GAPDH (1:10000, KangChen, Shanghai, China). Protein bands were visualized using ECL (Thermo Scientific Pierce ECL Plus, Thermo Scientific, Rockford, IL, USA). The expression levels of the proteins of interest were normalized against the expression level of GAPDH.

### *Cell proliferation assay*

Cell proliferation was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (MTS) (Promega, Madison, WI, USA). Cells were seeded into 96-well plates



**Figure 1.** ZEB2 expression in the CRC and adjacent tissues was detected by immunohistochemistry. Data are presented as mean  $\pm$  SD; \* $P < 0.05$ .

at a density of  $5 \times 10^3$  per well in triplicate. After culturing for 0, 24, 48, and 72 h, 10  $\mu$ L MTS was added in each well and cells were incubated for 4 h at 37°C. Absorbance at 490 nm was measured with a microplate reader (Thermo Scientific, Vantaa, Finland).

#### Cell cycle and apoptosis assays

At 48 h post transfection, HT-29 cells were digested with trypsin and centrifuged at  $2,000 \times g$  for 5 min to form a pellet. For cell cycle assay, pelleted HT-29 cells were fixed with 70% ethanol, which was pre-cooled at 4°C overnight, and incubated with 200  $\mu$ g/mL ribonuclease A at 37°C for 30 min. Then, 100  $\mu$ L propidium iodide (PI) was added and incubated under dark conditions at 4°C for 30 min. For apoptosis assay, the cell pellet ( $5 \times 10^3$  cells) was resuspended in 500  $\mu$ L binding buffer. Then, 5  $\mu$ L Annexin V-FITC and 5  $\mu$ L PI was added and mixed at room temperature and incubated under dark conditions for 15 min. Apoptosis and cell cycle progression were assessed by flow cytometry (BD Biosciences, San Jose, CA, USA). Each experiment was repeated three times.

#### Transwell migration and invasion assays

Cell migration and invasion were evaluated using a Transwell assay. For the migration assay, HT-29 cells were resuspended at a density of  $5 \times 10^4$  cells per 200  $\mu$ L in medium containing 0.1% serum, and placed into the upper chamber (8  $\mu$ m pore size) (BD Biosciences, San Diego, CA, USA). The lower chamber was filled with medium containing 10% FBS (600

$\mu$ L). The chamber was incubated at 37°C and 5% CO<sub>2</sub> for 48 h. For the invasion assay,  $5 \times 10^3$  cells were resuspended in 250  $\mu$ L serum-free medium, placed into the upper chamber that was pre-coated with 20  $\mu$ L 5 mg/mL Matrigel (BD Biosciences, San Diego, CA, USA). The lower chamber was filled with medium containing 10% FBS (600  $\mu$ L). The chamber was incubated at 37°C and 5% CO<sub>2</sub> for 48 h. The cells in the lower chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution for 2 h at room temperature. The migrating and invading cells in five randomly selected fields were counted using a LEICA microscope at 200  $\times$  magnification and average of the counts was calculated.

#### Statistical analysis

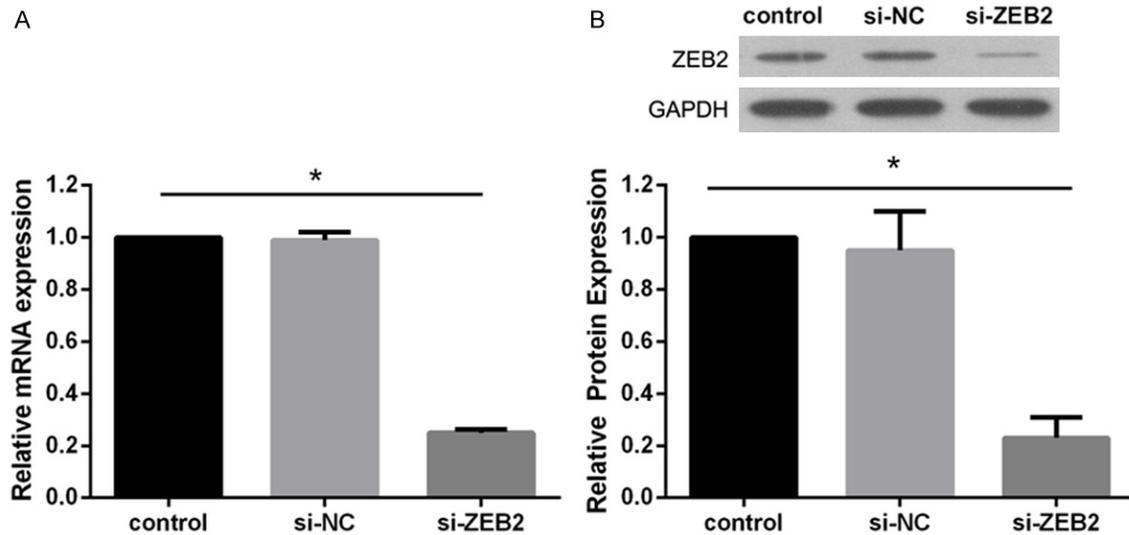
SPSS 19.0 software (IBM Inc., Chicago, IL, USA) was used to perform all statistical analyses. Continuous variables are presented as mean  $\pm$  standard deviation (SD). The data were analyzed using one-way ANOVA followed by a post-hoc LSD test;  $P$  values  $< 0.05$  were considered statistically significant.

## Results

### ZEB2 is upregulated in the CRC tissue

To study the localization and expression of ZEB2, IHC analysis was performed (**Figure 1**). In all the samples, ZEB2 expression was predominantly observed in the cytoplasm, whereas nuclear localization was observed only in a few cells. ZEB2 was weakly expressed in the

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**Figure 2.** ZEB2 expression quantified by (A) qRT-PCR and (B) Western blotting. Data are presented as mean  $\pm$  SD; \* $P < 0.05$ .

adjacent tissues, while it was strongly expressed in the CRC tissues. Compared to that in the adjacent tissues, ZEB2 expression in the CRC tissues was significantly increased. Due to the overexpression of ZEB2 in CRC tissues, we hypothesized that ZEB2 regulates CRC development and metastasis. To verify that, ZEB2 was silenced to determine its effect on HT-29 cell proliferation, cell cycle, apoptosis, migration, and invasion.

*Transfection with si-ZEB2 could effectively decrease ZEB2 expression level*

After transfection, ZEB2 expression was quantified by qRT-PCR and Western blotting. The results indicated that ZEB2 expression was significantly lower in si-ZEB2 group than in si-NC and control groups ( $P < 0.05$ , **Figure 2**).

*ZEB2 silencing suppressed HT-29 proliferation and cell cycle*

After transfection, proliferation, cell cycle, and apoptosis were assessed to evaluate the effect of ZEB2 expression on CRC development. The results are shown in **Figure 3**. The results indicated that the proliferation of HT-29 cells at 24, 48, and 72 h in the si-ZEB2 group was significantly suppressed compared to that in the si-NC and control groups ( $P < 0.05$ , **Figure 3A**). In addition, compared to the si-NC and control groups, si-ZEB2 group, with suppressed ZEB2 expression, induced G1-phase arrest, signifi-

cantly decreased the percentage of cells in the S-phase, and prevented HT-29 cell replication (**Figure 3B** and **3D**). Furthermore, the results indicated that the extent of apoptosis was not significantly different between si-ZEB2, si-NC, and control groups ( $P > 0.05$ , **Figure 3C** and **3E**).

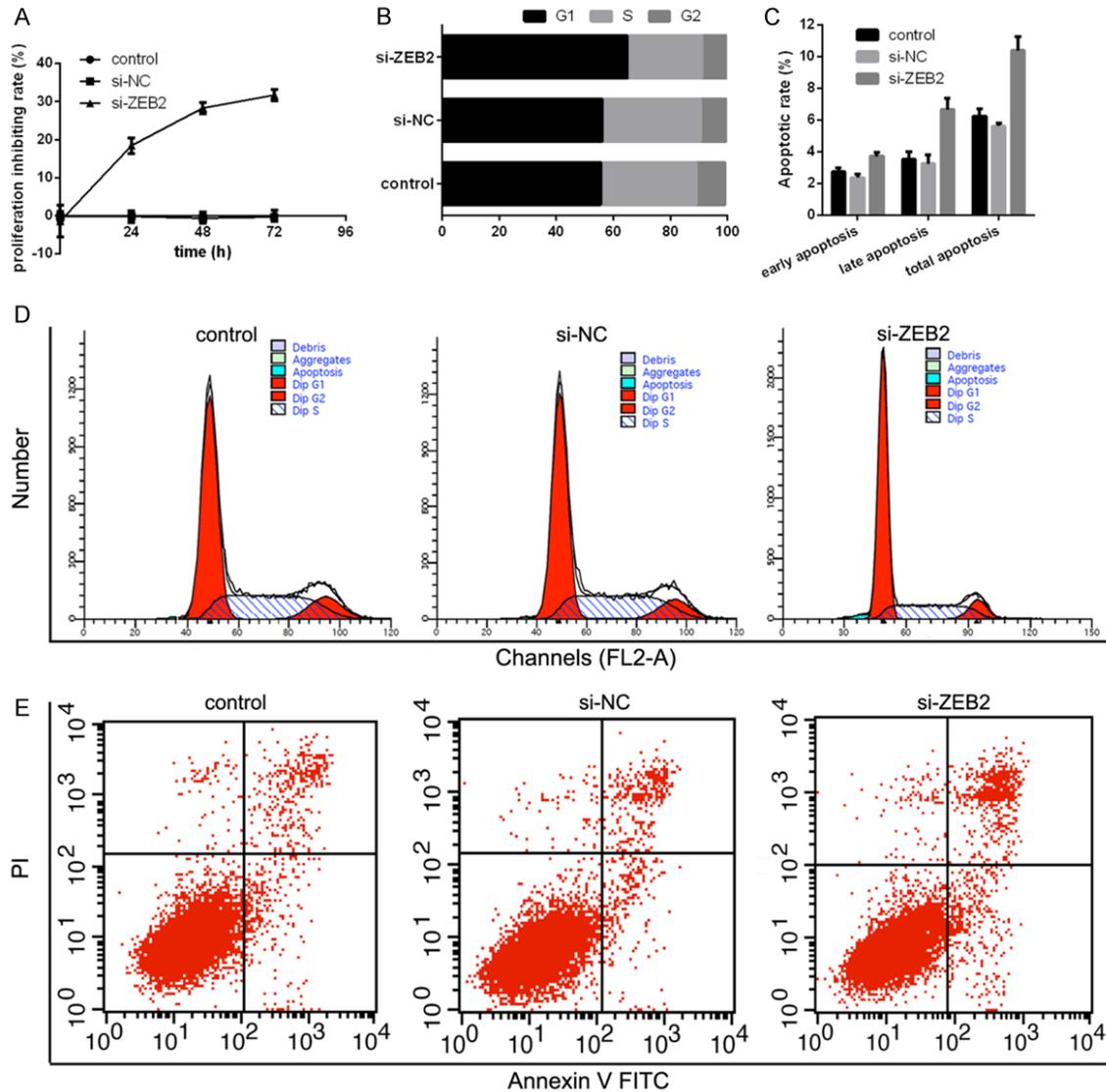
*ZEB2 silencing suppresses HT-29 migration and invasion*

To demonstrate the role of ZEB2 in regulation of HT-29 cell migration and invasion, a transwell assay was performed 48 h after transfection. The number of migrating and invading cells was significantly lower in the si-ZEB2 group than in the si-NC and control groups ( $P < 0.05$ , **Figure 4**).

### Discussion

ZEB2, belonging to the ZEB family of proteins that are zinc-finger and homeobox domain-containing transcription factors, contain two separate zinc-finger domains and a homeodomain [10]. One of the functions of ZEB2 is suppression of E-cadherin expression [3]. Downregulation of E-cadherin is one of the hallmarks of EMT, which malignant epithelial tumors follow for undergoing metastasis [11]. ZEB2 mutations are associated with tumor malignancy. Previous studies reported that ZEB2 expression was upregulated in hepatocellular carcinoma and renal cell carcinomas, melanoma, and head and neck and other cancers [6,

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**Figure 3.** ZEB2 silencing suppresses HT-29 (A) proliferation and (B) cell cycle progression, and does not affect (C) apoptosis. Representative images showing HT-29 (D) cell cycle progression and (E) apoptosis in control, si-NC, and si-ZEB2 groups ( $\times 200$ ). Data are presented as mean  $\pm$  SD.

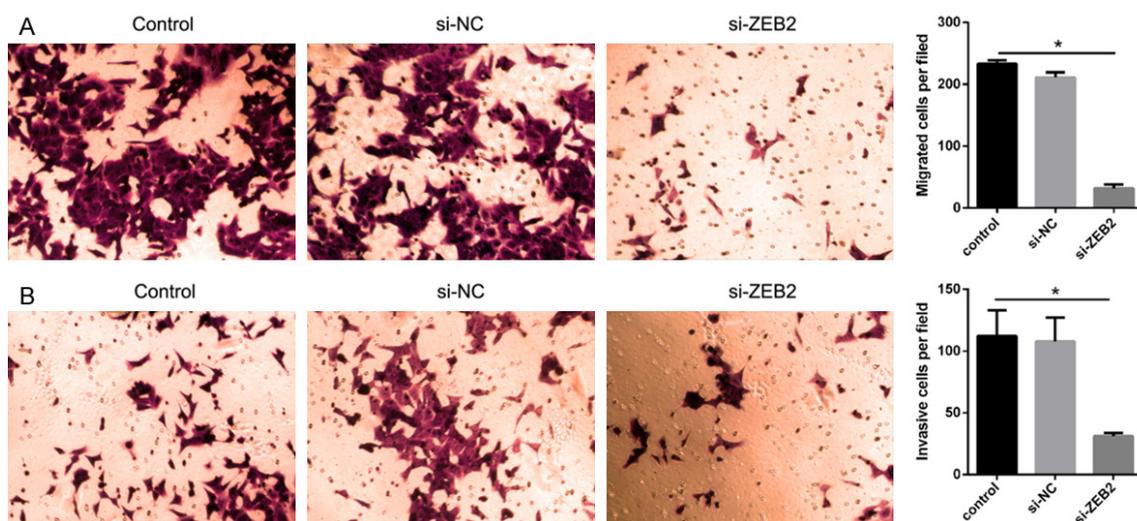
12-14]. Suppression of ZEB2 expression by microRNA could inhibit cancer migration and invasion via EMT, and prevent cancer development and metastasis [7, 11, 15].

In this study, we found that ZEB2 expression was significantly higher in the CRC tissues than in the adjacent tissues. In addition, ZEB2 expression was significantly higher in patients with metastatic CRC than in patients with non-metastatic CRC. The results showed that ZEB2 expression was associated with development and metastasis in clinical CRC samples. These results are similar to those described in the

CRC study by Kahlert et al. [6], which indicated that overexpression of ZEB2 during invasion correlates with tumor progression and predicts cancer-specific survival in primary CRC.

Proliferation and apoptotic dysfunction play key roles in CRC development, and inhibited proliferation and promoted apoptosis could suppress CRC development. In this study, one of our significant finding is the correlation between ZEB2 expression and CRC proliferation, which showed that silenced ZEB2 inhibited HT-29 cell proliferation and cell cycle transition from G1 to S phase, but had no significant effect on cell

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**Figure 4.** ZEB2 silencing suppressed HT-29 migration and invasion. Representative images of HT-29 (A) cell migration and (B) cell invasion ( $\times 200$ ). Data are presented as mean  $\pm$  SD;  $*P < 0.05$ .

apoptosis. We also found that silenced *ZEB2* inhibited HT-29 cell migration and invasion. A previous study demonstrated that *ZEB2* expression inhibited by miR-132 could suppress EMT and markedly attenuate invasion of CRC cell [7], which provides an indirect evidence for *ZEB2* to regulate the CRC metastasis, which is consistent with our study.

In conclusion, silenced *ZEB2* inhibited CRC cell replication, proliferation, migration, and invasion. Our findings suggest that *ZEB2* could be a potential therapeutic target for CRC.

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### Disclosure of conflict of interest

None.

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