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MicroRNA-155 and FOXP3 jointly inhibit the migration and invasion of colorectal cancer cells by regulating ZEB2 expression

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Abstract. – OBJECTIVE: The study aimed to explore whether microRNA-155 and FOXP3 could regulate invasive and migratory capacities of colorectal cancer (CRC) cells by mediating Zinc finger E-box binding homeobox 2 (ZEB2) expression.

MATERIALS AND METHODS: Dual-luciferase reporter gene assay was performed to detect the binding condition between microRNA-155, FOXP3, and ZEB2. Protein and mRNA levels of ZEB2 in CRC cells were detected after overexpression of microRNA-155 and FOXP3 by Western blot and quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), respectively. *In vitro* experiments were conducted using HCT116 and SW620 cell lines. We first detected expression levels of microRNA-155, FOXP3, and ZEB2 in the normal colorectal epithelial cell line (NCM460) and CRC cell lines (HCT116 and SW620) by qRT-PCR. Protein expressions of ZEB2, E-cadherin, and vimentin in WT, LV-GFP, and LV-FOXP3 groups were detected. Wound healing assay and transwell assay were conducted to determine the regulatory effects of microRNA-155 and FOXP3 on invasive and migratory capacities of CRC cells, respectively.

RESULTS: Dual-luciferase reporter gene assay found that FOXP3 binds to the promoter and intron regions of ZEB2, and microRNA-155 binds to the 3'UTR region of wild-type ZEB2. Overexpression of FOXP3 downregulated mRNA and protein levels of ZEB2. ZEB2 was highly expressed, whereas microRNA-155 and FOXP3 were lowly expressed in HCT116 and SW620 cells than NCM460 cells. MicroRNA-155 overexpression upregulated E-cadherin and downregulated vimentin in CRC cells. Overexpression of FOXP3 and microRNA-155 inhibited invasive and migratory capacities of CRC cells.

CONCLUSIONS: MicroRNA-155 and FOXP3 can jointly regulate ZEB2 expression, thereby inhibiting the migration and invasion of colorectal cancer cells.

Key Words:
MicroRNA-155, FOXP3, ZEB2, CRC, Invasion.

Introduction

Colorectal cancer (CRC) is one of the common malignant tumors that seriously endangers human health. Its incidence ranks third in all malignant tumors worldwide, which brings a heavy economic burden to society[1]. In recent years, many studies have shown cumulative effects of genetic alterations at different stages of CRC development, such as APC, p53, VEGF, and K-RAS. Mutation or inactivation of these genes may result in the occurrence of benign or malignant tumors[2,3]. Difficulties in early diagnosis, as well as high metastatic and recurrent rates, result in the high mortality of CRC. Although some gene mutations have been found in the pathogenic progression of CRC[4], there are still no precise molecular targets for CRC treatment.

MicroRNA is a non-coding small RNA expressing in a variety of eukaryotes. It has 18-25 nucleotides in size and inhibits mRNA translation by incompletely pairing with the 3'-untranslated region (3'-UTR) of target mRNA. MicroRNAs participate in cell growth, development, proliferation, differentiation, and apoptosis[5]. MicroRNA-155, located on human chromosome 21q21, is found to be highly expressed in tumors[6]. High expression of microRNA-155 is associated with poor prognosis of CRC and pancreatic cancer[7]. Also, microRNA-155 could enhance tumor invasion and migration, and it can be served as an intermediary for epithelial-mesenchymal transition

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(EMT)[8]. These findings suggested that microRNA-155 serves as an oncogene.

FOXP3 is a member of the forkhead-like transcription factor family, which is specifically expressed in Tregs and plays a key role in the development, maturation, and immunosuppressive function of Tregs[9]. Some studies have shown that FOXP3 is expressed not only in Tregs but also in tumor cells. FOXP3 is found to be expressed in pancreatic cancer tissues, which is unable to be detected in the normal pancreatic tissues[10]. In breast cancer, FOXP3 is an X-linked inhibitory gene and a suppressor of the HER-2/ErB2[11]. In gastric cancer, chemokines of CCL17 and CCL22 are involved in the regulation of T cells by targeting FOXP3[11]. However, the biological functions of FOXP3 in CRC have been rarely reported.

Zinc finger E-box binding homeobox 2 (ZEB2) is a member of the ZEB family that is encoded by the ZFHX1B gene on the 2q22 chromosome. ZEB family consists of two members, ZEB1 and ZEB2, both of which contain two separate clusters of independent zinc finger structures that can independently bind to the 5'-CACCT sequence[12]. As a promoter of the downstream factors, ZEB2 could inhibit the transcription of target genes[13]. The potential functions of ZEB2 in tumor development have been widely reported[14-18]. ZEB2 not only promotes the proliferation of cancer cells, but also accelerates the invasion and metastasis of cancer cells. This study explored the roles of microRNA-155, FOXP3, and ZEB2 in CRC development, which provides new ideas for clinical treatment of CRC.

Materials and Methods

Cell Culture

Normal colorectal epithelial cell line (NCM460) and CRC cell lines (HCT116 and SW620) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and maintained in a 5% CO_2 incubator at 37°C.

Cell Transfection

Cells were pre-seeded in the 6-well plates at a dose of 2×10^5 /mL. After overnight culture, 20 μL of a solution containing LV-FOXP3 or LV-GFP was added, followed by 5 mg/L polybrene addition in each well. Culture medium was replaced the other day. GFP-positive cells were sorted by flow cytometry.

Transfection of si-NC, si-ZEB2, microRNA-155 mimic or microRNA-155 NC was performed following the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Dual-Luciferase Reporter Gene Assay

Cells were seeded in the 24-well plates and assigned into 6 groups as follows: GFP+pmiR-ZEB2-wt, FOXP3+pmiR-ZEB2-wt, microRNA-155 mimic-NC +pmiR-ZEB2-wt, microRNA-155 mimic+pmiR-ZEB2-wt, microRNA-155 mimic-NC+pmiR-ZEB2-mut, and microRNA-155 mimic+pmiR-ZEB2-mut. Cell transfection was performed based on the different treatments. Luciferase activity was finally detected according to the relative commercial kit instructions.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The TRIzol kit (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA, which was then reversely transcribed into complementary Deoxyribose Nucleic Acid (cDNA). After the cDNA was amplified, qRT-PCR was performed to detect the expressions of related genes using the SYBR Premix Ex Taq II kit (TaKaRa, Otsu, Shiga, Japan). Relative gene expression was detected using the ABI Prism 7900HT system (Applied Biosystems, Foster City, CA, USA). Primers used in the study were: GAPDH forward, 5'-AAGGTGAAGGTCGGAGTCAA-3', reverse, 5'-AATGAAGGGGTCATTGATGG-3'; MicroRNA-155 forward, 5'-CTCGTGGTAATGCTAATTGTGA-3', reverse, 5'-GTGCAGGGTCCGAGGT-3'; FOXP3 forward, 5'-AGCTTGCCGTAGCGATGATA-3', reverse, 5'-GTAGCCATGAACTGAACTGA-3'; ZEB2 forward, 5'-AGTCCTCCCCACACGTGAGCC-3', reverse, 5'-TGCGGTCTGGATCGTGGCTTC-3'.

Western Blot

Cells were lysed for protein extraction. The concentration of each protein sample was determined by a BCA (bicinchoninic acid) kit (Abcam, Cambridge, MA, USA). The protein sample was separated by gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After incubation with primary and secondary antibody, immunoreactive bands were exposed by enhanced chemiluminescence (ECL) assay.

Wound Healing Assay

Cells were seeded into 24-well plates. A sterile 10 μL micropipette tip was used to vertically scratch the cell plate until 80% of cell confluence. After removing the exfoliated cells with phosphate-buffered saline (PBS), serum-free medium was placed for 48 h-incubation. Migratory cells were observed and captured under an inverted microscope (Nikon, Tokyo, Japan), and the width of the scratch was measured and photographed.

Transwell Assay

Cells were centrifuged and resuspended in serum-free RPMI-1640 at a density of 5.0×10^4/mL. Transwell chambers pre-coated with Matrigel were placed in 24-well plates. 100 μL of cell suspension and 600 μL of medium containing 10% FBS were added in the upper and lower chamber, respectively. After cell culture for 48 h, cells were fixed with 4% paraformaldehyde for 15 min and stained with crystal violet for 15 min. Inner cells were carefully cleaned. Invasive cells in 5 randomly selected fields of each sample were captured.

Statistical Analysis

We used SPSS (Statistical Product and Service Solutions) 19.0 software (IBM, Armonk, NY, USA) for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). Differences between two groups were compared using the *t*-test. $p<0.05$ was considered statistically significant.

Results

FOXP3 and MicroRNA-155 Directly Regulated ZEB2

To explore whether FOXP3 and microRNA-155 can regulate ZEB2 expression, we performed dual-luciferase reporter assay at first. It is found that FOXP3 binds to the promoter and intron regions of ZEB2, and microRNA-155 binds to the 3'UTR region of wild-type ZEB2 (Figure 1A). Subsequently, FOXP3 overexpression plasmid (LV-FOXP3) was transfected into HCT116 cells. QRT-PCR data showed that mRNA level of ZEB2 remarkably decreases after FOXP3 overexpression (Figure 1B). Similarly, Western blot results indicated that the protein level of ZEB2 is significantly lower in the LV-FOXP3 group than WT and LV-GFP group (Figure 1C). These results elucidated that FOXP3 and microRNA-155 can negatively regulate the mRNA and protein expression levels of ZEB2.

Expressions of ZEB2, MicroRNA-155, and FOXP3 in CRC Cell Line

Expressions of ZEB2, microRNA-155, and FOXP3 in the normal colorectal epithelial cell line (NCM460) and CRC cell lines (HCT116 and SW620) were detected by qRT-PCR. ZEB2 was highly expressed in HCT116 and SW620 cells than NCM460 cells (Figure 2A). However, microRNA-155 and FOXP3 were lowly expressed in HCT116 and SW620 cells (Figure 2B and 2C).

MicroRNA-155 and FOXP3 Inhibited EMT by Downregulating ZEB2

ZEB2 is a crucial transcription factor in EMT induction [19]. To explore the potential effects of microRNA-155 and FOXP3 on EMT of CRC cells, protein expressions of ZEB2, E-cadherin, and vimentin were detected by Western blot. Overexpression of microRNA-155 could downregulate protein expression of ZEB2 (Figure 3A). Besides, microRNA-155 overexpression upregulated E-cadherin and downregulated vimentin (Figure 3B). It is suggested that microRNA-155 and FOXP3 inhibit EMT by downregulating ZEB2.

Overexpression of FOXP3 and MicroRNA-155 Inhibited Invasive and Migratory Capacities of CRC Cells

Wound healing assay was conducted to explore the roles of microRNA-155 and FOXP3 in regulating invasive capacities of CRC cells. Overexpression of microRNA-155 could inhibit the invasive capacity of HCT116 and SW620 cells (Figure 4A). ZEB2 knockdown in HCT116 cells obtained the similar result as the inhibited invasive capacity (Figure 4C). Furthermore, the transwell assay was carried out to determine the migratory capacity of CRC cells. Overexpression of microRNA-155 and FOXP3 inhibited the migratory capacity of HCT116 cells (Figure 4B).

Discussion

Localized invasion is one of the key steps in tumor metastasis. Many epithelial cell phenotypes of tumor cells are lost, allowing the capacities of motion and invasion in the epithelial layer, that is, EMT. The main features of EMT include decreased expressions of cell adhesion molecules,

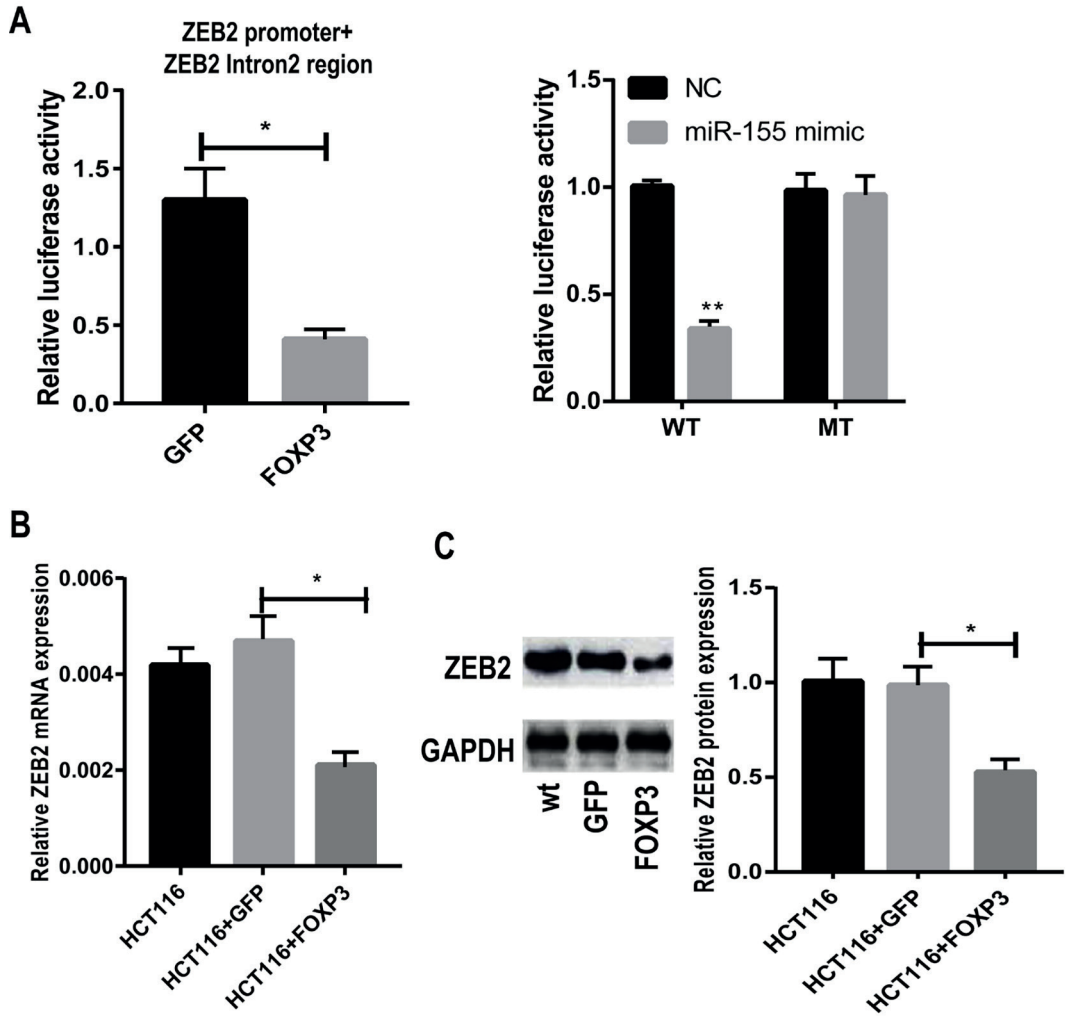

Figure 1. FOXP3 and microRNA-155 directly regulated ZEB2. ***A,*** Dual-luciferase reporter assay found that FOXP3 binds to the promoter and intron regions of ZEB2, and microRNA-155 binds to the 3'UTR region of wild-type ZEB2. ***B,*** QRT-PCR data showed that mRNA level of ZEB2 remarkably decreases after FOXP3 overexpression. ***C,*** Western blot results indicated that protein level of ZEB2 is significantly lower in the LV-FOXP3 group than WT and LV-GFP group.

transformation of cytoskeletal cytoskeleton into vimentin-based cytoskeleton, and morphologies of mesenchymal cells[20-22]. Tumor tissues and cells acquire strong interstitial phenotypes through the EMT process, such as anti-apoptosis, extracellular matrix degradation, migration, and invasion. In recent years, the relationship between microRNAs and EMT has been well studied[23]. MicroRNA-155 is believed to be involved in EMT. It is reported that microRNA-155 promotes macroscopic tumor formation yet inhibits tumor dissemination from mammary fat pads to the lung by preventing EMT[24]. MicroRNA-155 induces EMT in lung cancer cells through downregulating Ubiquilin 1[25]. Upregulation of microRNA-155 reverses the EGF-induced EMT process and increases cisplatin chemosensitivity of human cervical cancer cells[26]. We found that overexpression of microRNA-155 in CRC cells inhibits the EMT process, cell migration, and invasion, indicating the inhibitory effect of microRNA-155 on CRC progression.

FOXP3 function in tumors has been a hotspot in tumor research nowadays. FOXP3 was initially found to be expressed in pancreatic cancer ductal carcinoma cells and tumor tissues, revealing a new mechanism for immune escape in pancreatic cancer[27]. With the in-depth studies, FOXP3 is found to be expressed in 25 types of tumor cells[28]. Tumor Cyclooxygenase-2/Prostaglandin E2 is de-

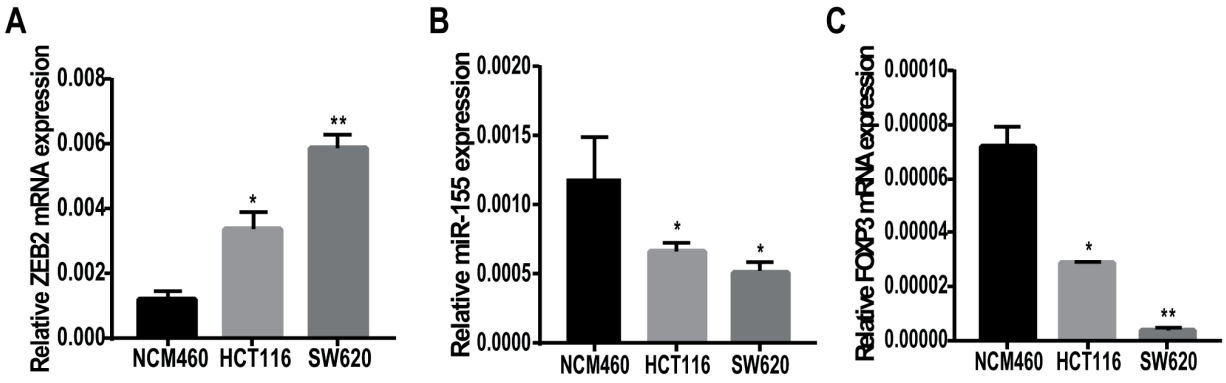

Figure 2. Expressions of ZEB2, microRNA-155 and FOXP3 in CRC cell line. ***A,*** ZEB2 was highly expressed in HCT116 and SW620 cells than NCM460 cells. ***B-C,*** MicroRNA-155 and FOXP3 were lowly expressed in HCT116 and SW620 cells.

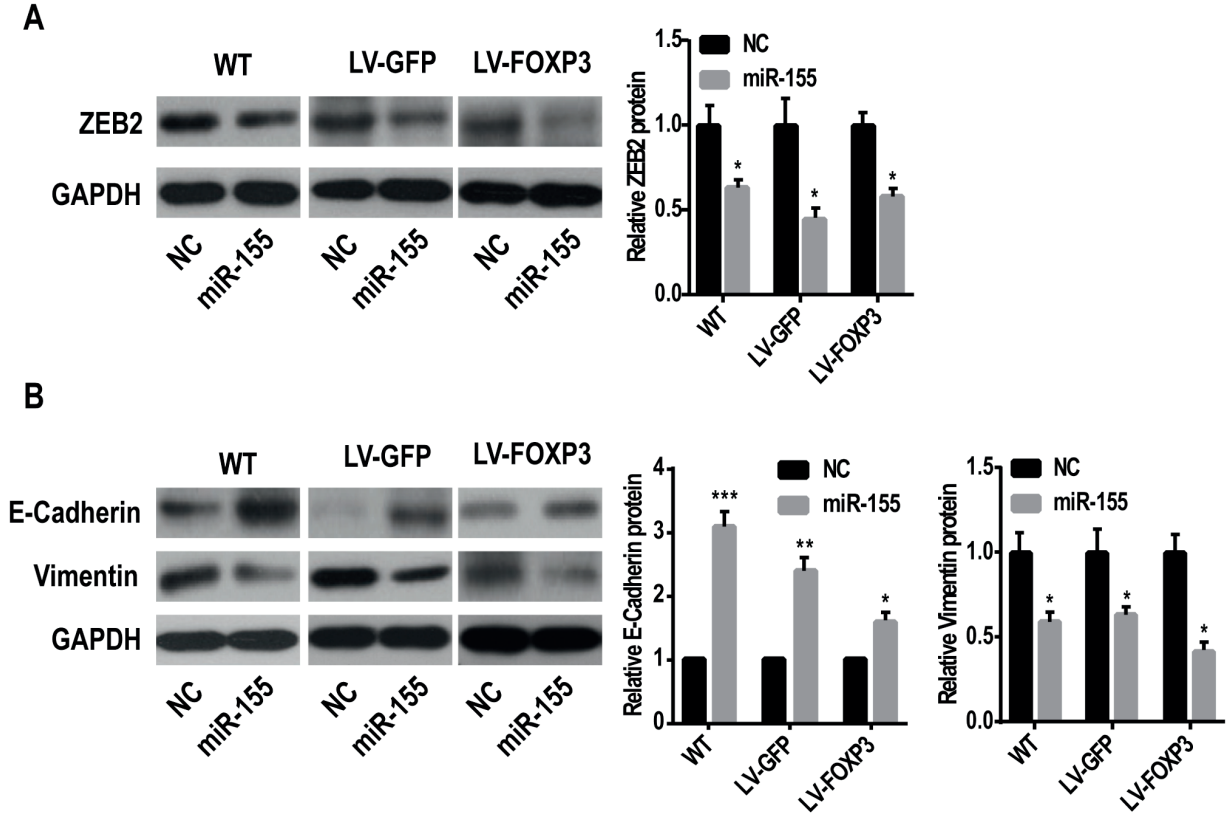

Figure 3. MicroRNA-155 and FOXP3 inhibited EMT by downregulating ZEB2. ***A,*** Overexpression of microRNA-155 could downregulate protein expression of ZEB2in CRC cells. ***B,*** MicroRNA-155 overexpression upregulated E-cadherin and downregulated vimentin in CRC cells.

pendent on FOXP3 expression and activities of $CD4^+CD5^+$T cells in lung cancer[29]. T cell-specific FOXP3 expression is associated with poor prognosis of ovarian cancer[30]. In the present study, we found that FOXP3 is lowly expressed in CRC, suggesting the potential role of FOXP3 in CRC development.

ZEB2 is an essential transcription factor in the embryonic development and exerts a crucial role in the early development of embryo and nervous system[31]. ZEB2 has been transcriptionally activated during neuroblastogenesis[32]. Many studies have shown the potential effect of ZEB2 in tumorigenesis and tumor development. ZEB2 is involved

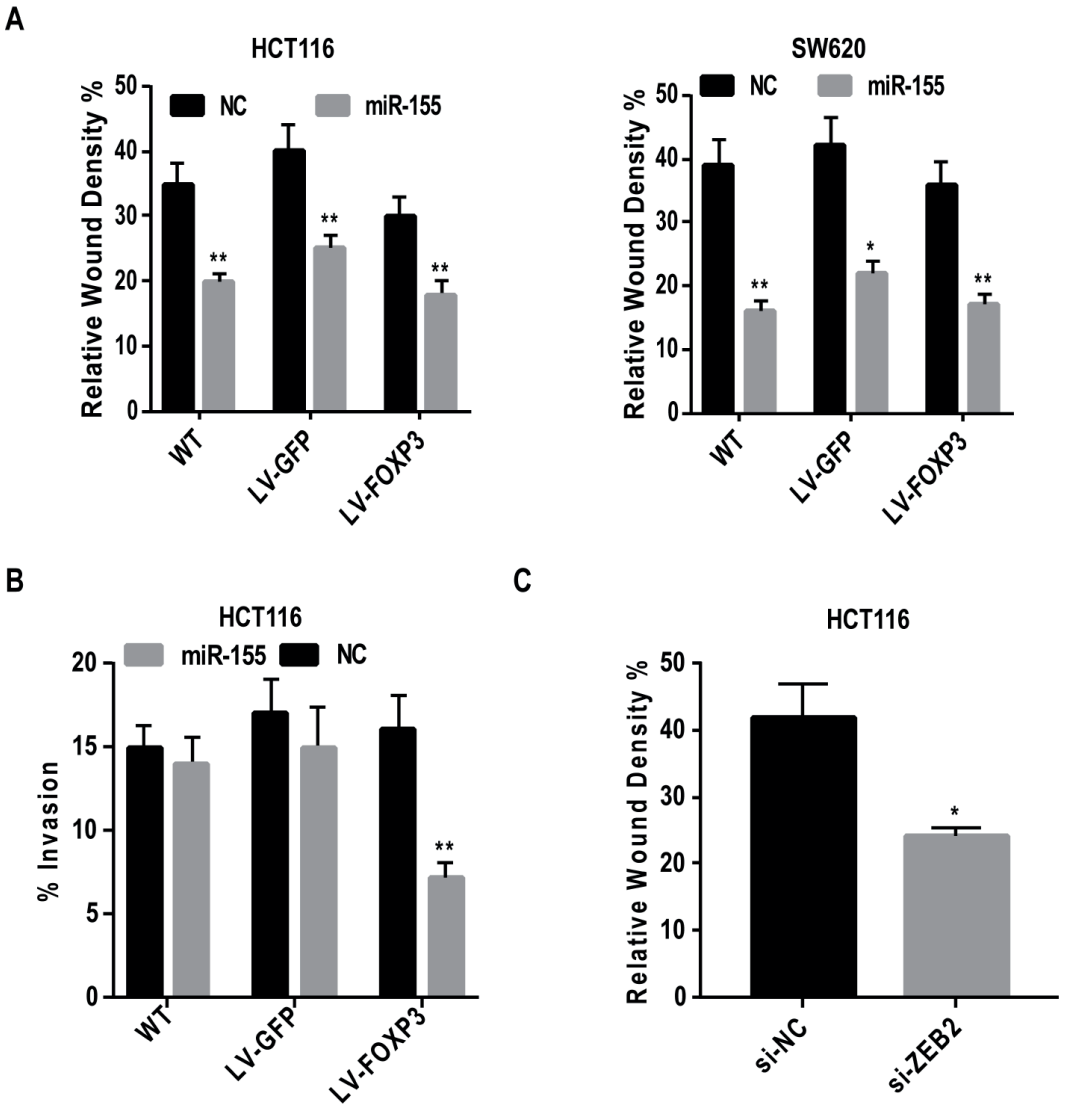

Figure 4. Overexpression of FOXP3 and microRNA-155 inhibited invasive and migratory capacities of CRC cells. ***A,*** Overexpression of microRNA-155 could inhibit invasive capacity of HCT116 and SW620 cells. ***B,*** Overexpression of microRNA-155 and FOXP3 inhibited migratory capacity of HCT116 cells. ***C,*** ZEB2 knockdown in HCT116 cells inhibited invasive capacity of HCT116 cells.

in the regulation of cell cycle and proliferation of tumor cells. As a transcription factor, ZEB2 can directly bind to the promoter sequence of Cyclin D1, further phosphorylating Rb and arresting the cell cycle in G1 phase[33]. E-cadherin is a marker of epithelial cells, and its expression reduction is a hallmark of EMT progression[34]. ZEB2 could bind to the 5'-CACCT(G) sequence on the E2-box in the promoter region of E-cadherin, thereby inhibiting E-cadherin transcription, inducing EMT transformation, enhancing cell invasion, and metastasis[35]. It is suggested that inhibition of ZEB2 expression can inhibit EMT process, migration, and invasion of tumor cells.

Our study showed that ZEB2 is highly expressed, whereas microRNA-155 and FOXP3 are lowly expressed in CRC. We speculated that ZEB2 may serve as an oncogene in CRC. Dual-luciferase reporter gene assay showed that ZEB2 may be the target gene of FOXP3 and microRNA-155. Protein expression of ZEB2 in CRC cells was downregulated after overexpression of microRNA-155 and FOXP3. Additionally, overexpression of microRNA-155 and FOXP3 inhib-

ited EMT, manifesting as upregulated E-cadherin and downregulated vimentin. Invasive and migratory capacities of CRC cells were inhibited by overexpressed microRNA-155 and FOXP3. We concluded that FOXP3 and microRNA-155 could regulate CRC development by inhibiting ZEB2 expression.

Conclusions

We found that microRNA-155 and FOXP3 can jointly regulate ZEB2 expression, thereby inhibiting the migration and invasion of colorectal cancer cells.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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