Am J Transl Res 2018;10(2):605-617
www.ajtr.org /ISSN:1943-8141/AJTR0068299

Original Article

Interplay between long noncoding RNA ZEB1-AS1 and miR-101/ZEB1 axis regulates proliferation and migration of colorectal cancer cells

Wan-Cheng Xiong[1], Na Han[2], Nan Wu[3], Ke-Lei Zhao[4], Chen Han[3], Hui-Xin Wang[3], Guan-Fang Ping[3], Peng-Fei Zheng[1], Hailong Feng[1], Lei Qin[5], Peng He[1]

[1]Department of General Surgery, First Affiliated Hospital of Xinxiang Medical University, Weihui 453100, Henan, China; [2]Department of Central Laboratory, First Affiliated Hospital of Xinxiang Medical University, Weihui 453100, Henan, China; [3]Department of Pharmacy, First Affiliated Hospital of Xinxiang Medical University, Weihui 453100, Henan, China; [4]Department of Oncology, First Affiliated Hospital of Xinxiang Medical University, Weihui 453100, Henan, China; [5]Department of Gastroenterology, First Affiliated Hospital of Xinxiang Medical University, Weihui 453100, Henan, China

Received October 31, 2017; Accepted January 22, 2018; Epub February 15, 2018; Published February 28, 2018

Abstract: Long noncoding RNAs (lncRNAs) are dysregulated in many diseases. MicroRNA-101 (miR-101) functions as a tumor suppressor by directly targeting ZEB1 in various cancers. However, the potential mechanism of lncRNA ZEB1-AS1 and miR-101/ZEB1 axis in CRC remains unknown. In this study, we further investigated the potential interplay between miR-101/ZEB1 axis and lncRNA ZEB1-AS1 in colorectal cancer (CRC). Results showed that ZEB1-AS1 was upregulated in CRC tissues and cells. MiR-101 was downregulated in CRC tissues and negatively correlated with ZEB1-AS1 and ZEB1 expression levels in CRC. Functional experiments showed that, consistent with ZEB1-AS1 depletion, miR-101 overexpression and ZEB1 depletion inhibited the proliferation and migration of CRC cells. Overexpression of miR-101 partially abolished the effects of ZEB1-AS1 on the proliferation and migration of these cells. Moreover, combined ZEB1-AS1 depletion and miR-101 overexpression significantly inhibited cell proliferation and migration of the CRC cells. Hence, ZEB1-AS1 functioned as a molecular sponge for miR-101 and relieved the inhibition of ZEB1 caused by miR-101. This study revealed a novel regulatory mechanism between ZEB1-AS1 and miR-101/ZEB1 axis. The interplay between ZEB1-AS1 and miR-101/ZEB1 axis contributed to the proliferation and migration of CRC cells, and targeting this interplay could be a promising strategy for CRC treatment.

Keywords: lncRNA ZEB1-AS1, miR-101, ZEB1, proliferation, migration

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide according to the latest statistics [1, 2]. Despite recent advances in CRC treatments, which include combined surgery and adjuvant therapy [3], the overall prognosis of this malignancy remains poor because of tumor recurrence and metastases [4, 5]. Therefore, new therapeutic strategies aimed at decreasing these malignant events are urgently needed.

Long noncoding RNAs (LncRNAs) are noncoding RNAs (ncRNAs) with more than 200 nucleotides in length [6, 7]. Emerging evidence indicates that specific lncRNAs have critical roles in the regulation of multiple biological processes [8]. The expression of lncRNA is frequently elevated or decreased in tumor, and several lncRNAs participate in the development, metastasis, recurrence, and poor prognosis in various cancers, including CRC [9-15]. These molecules may provide new biomarkers for the diagnosis and prognosis of CRC.

ZEB1-AS1 is a noncoding antisense transcript generated from ZEB1 promoters and located in physical contiguity with ZEB1 [16]. Emerging studies showed that overexpression of ZEB1-AS1 increased ZEB1 levels and promoted tumor progression in different kinds of malignancies [16-20]. However, the functions and mechanisms of ZEB1-AS1 in CRC remain largely unknown. Thus, further exploration of the functions of lncRNA in CRC is crucial. Thus, we spe-

culated the effects of ZEB1-AS1 on miR-101/ZEB1 axis on CRC cells and detected the underlying mechanisms.

In this study, ZEB1-AS1 post-transcriptionally enhanced ZEB1 mRNA stability and upregulated ZEB1 expression in CRC cells. Moreover, miR-101 was downregulated in CRC tissues than in adjacent normal tissues, and miR-101 expression levels were negatively correlated with ZEB1 or ZEB1-AS1 expression in the same CRC tissues. Additionally, miR-101 overexpression in vivo inhibited the proliferation and migration of CRC cells by targeting ZEB1. Moreover, this overexpression partially abolished the effects of ZEB1-AS1 on the proliferation and migration of CRC cells. Combined ZEB1-AS1 depletion and miR-101 overexpression significantly inhibited the cell proliferation and migration of the CRC cells. These data support the inter-regulation among ZEB1-AS1, miR-101, and ZEB1. In addition, the loss of balance between ZEB1-AS1 and miR-101/ZEB1 axis in CRC may contribute to the progression of this disease.

Materials and methods

Ethics statement

Experimental procedures were approved by the Institutional Review Board and Ethics Committee of the First Affiliated Hospital of Xinxiang Medical University (Xinxiang, China).

Patients and tissue samples

Samples were collected from patients admitted to the First Affiliated Hospital of Xinxiang Medical University between September 2014 and April 2017. Written informed consent and approval were obtained from each patient or family. A total of 50 primary CRC samples were obtained from patients who were diagnosed histopathologically and subjected to surgical resection. No patients received radiotherapy, chemotherapy, or other anticancer treatment before surgery. Clinical staging was performed according to the American Joint Committee on Cancer Staging Manual. All tissue samples were collected at surgery, immediately frozen in liquid nitrogen, and stored at -80°C until RNA or protein extraction.

Cell culture

Five human CRC cell lines (SW480, DLD-1, HCT116, SW620, and HT29) and a normal colonic cell line (NCM460) were obtained from the Shanghai Institute for Biological Sciences (Shanghai, China) and American Type Culture Collection (Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). The cells were routinely grown to 80% confluence at 37°C in a humidified atmosphere containing 5% CO_2, and the cells from passages 2 to 4 were used in the experiments and expended no later than 6 months after receipt.

Vector construction and cell transfection

The effects of ZEB1-AS1, miR-101, and ZEB1 on cell activity and miR-101 mimics were investigated using shZEB1-AS1 (to knockdown the ZEB1-AS1 expression) and ZEB1-siRNAs, which were obtained from Gene Pharma (Shanghai, China). The lncRNA-ZEB1-AS1 cDNA plasmid was constructed by introducing the cDNA sequence of ZEB1 into the pEX3 expression vector (Gene Pharma).

Cells were plated in individual wells of 6-well plates. SW480 and HCT116 (2×10^5 cells) were transfected with miRNA mimics, siZEB1, or shZEB1-AS1 at a final concentration of 25 nmol/L using Lipofectamine 2000 Reagent (Life Technologies, Carlsbad, CA, USA). After 48 h, the cells were harvested for the assays described below. Cells were transfected with pcDNA-lncRNA-ZEB1-AS1 constructs at a final concentration of 1 μg/μL according the manufacturer's protocol. The empty pEX3 vector and scrambled sequences of the miRNA mimics or siRNAs were used as negative controls.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA of fresh tissues and cell lines was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed to generate the first-strand cDNA using M-MLV Reverse Transcriptase (Invitrogen). The mRNA expression was detected with SYBR Premix Ex Taq II (TaKaRa, Dalian, China) through qRT-PCR. The total RNA was reverse-transcribed with a miScript reverse transcription kit (Qiagen) and then amplified with SYBR Premix Ex Taq™ (TaKaRa) to quantify the miRs. $2^{-\Delta\Delta CT}$ method was applied to determine the relative gene expression, and the expression lev-

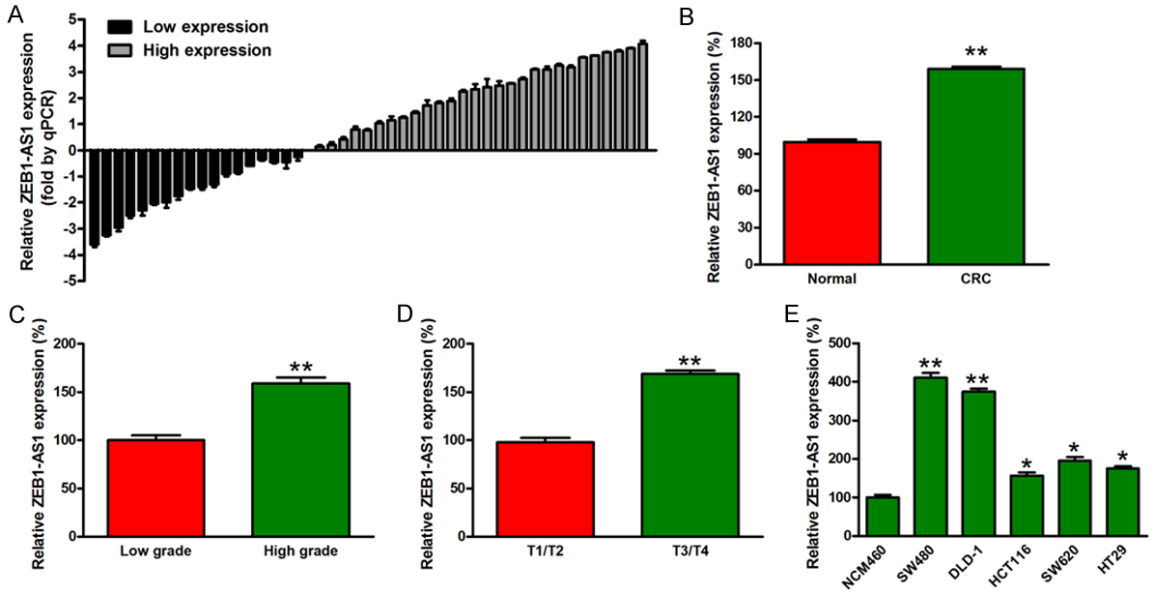

Figure 1. Overexpression of ZEB1-AS1 in CRC and its association with poor prognosis of CRC patients. The relative expression of ZEB1-AS1 was measured by qRT-PCR. A: The height of the column represents the fold change (log 2-transformed) in ZEB1-AS1 expression in a cohort of 50 patients with CRC. B: ZEB1-AS1 expression was higher in CRC tissues than in paired noncancerous tissues. C: ZEB1-AS1 expression was higher in patients with high histological grade than in patients with low histological grade. D: ZEB1-AS1 expression was higher in patients with advanced tumor stage T (T3/T4) than in T1/T2. E: ZEB1-AS1 expression levels in CRC cell lines and human normal colonic cell line. Data are shown as mean ± SD based on at least three independent experiments. Normal: noncancerous tissues; CRC: colorectal cancer tissues. **P*<0.05, ***P*<0.01.

els of mRNAs and miRs were normalized to tHCT116e of GAPDH and U6, respectively. The primers used in this experiment were as follows: ZEB1-AS1, 5'-CCGTGGGCACTGCTGAAT-3' (forward) and 5'-CTGCTGGCAAGCGGAACT-3' (reverse); ZEB1, 5'-A-CTCTGATTCTACA CCGC-3' (forward) and 5'-TGTCACATTGATAGGGCTT-3' (reverse); miR-101, 5'-CGGCGGTACAGTACTGTGATAA-3' (forward) and 5'-CTGGTGTCGTGGA GTCGGCAATTC-3' (reverse); U6, 5'-CTCGCTTCGGCAGCACA-3' (forward) and 5'-AACGCTTCACGAATTTGCGT-3' (reverse); and GAPDH, 5'-GCACCG TCAAGGCTGAGAAC-3' (forward) and 5'-TGGTGAAGACGCCAGTGGA-3' (reverse).

Dual-luciferase reporter assay

Plasmid pGL3-ZEB1-3'-UTR-WT or pGL3-ZEB1-3'-UTR-MUT was cotransfected with 100 ng of miR-101 mimics and miR-NC in the cells using Lipofectamine 2000 (Invitrogen Life Technologies). After 48 h of transfection, a luciferase reporter gene assay was conducted in the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Renilla luciferase was cotransfected as a control for normalization.

Western blot analysis

Total protein was extracted from the cells or tissues using a RIPA lysis buffer (Beyotime, Jiangsu, China), separated through 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred onto nitrocellulose membranes (Millipore, Burlington, MA, USA). After blocking with 5% nonfat dry milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.1% Tween 20, the membranes were incubated with primary antibody overnight at 4°C. All primary antibodies were purchased from Santa Cruz. The blots were then washed and incubated with goat anti-mouse IgG conjugated to horseradish peroxidase for 1 h at room temperature. Protein bands were detected through enhanced chemiluminescence (ECL) by utilizing a Pierce ECL Western blot substrate (Thermo Fisher Scientific) and exposed to an X-ray film using an ECL detection system (Thermo Fisher Scientific). GAPDH served as a control.

Cell viability detection

Cell proliferation was analyzed using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories,

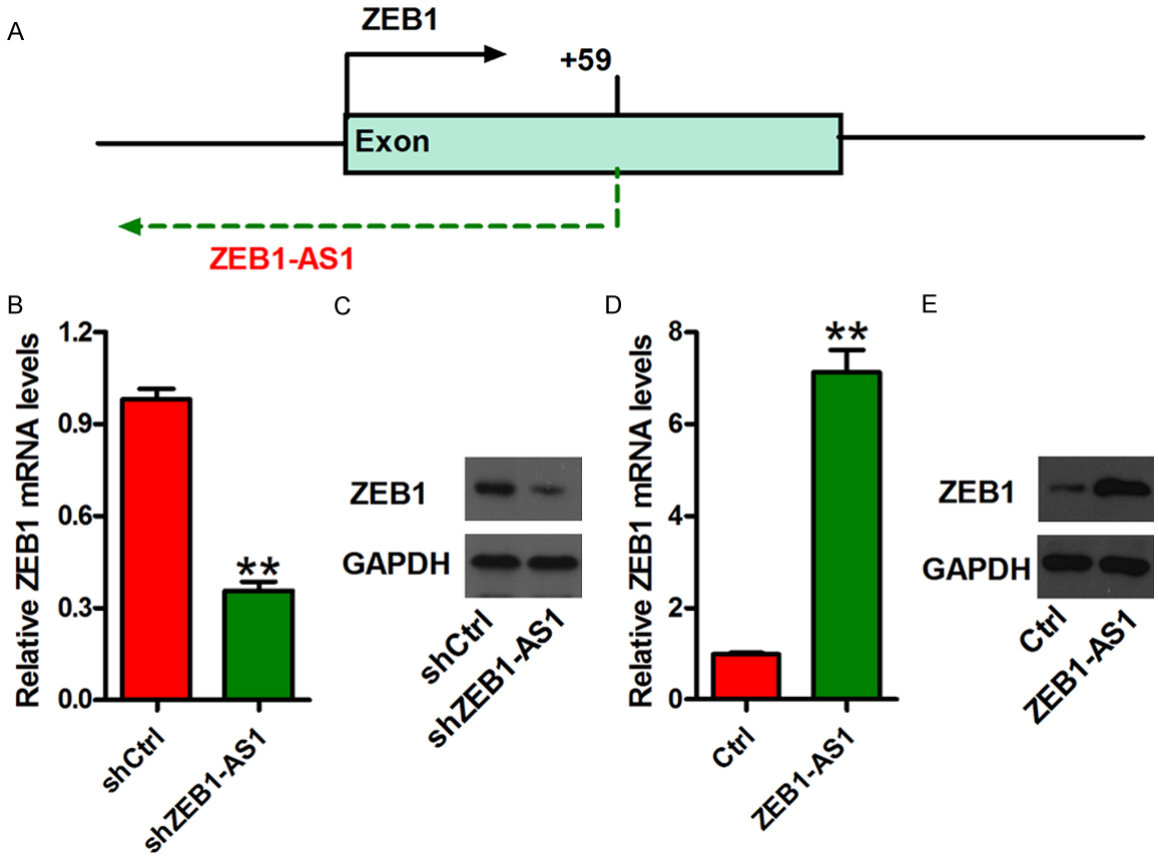

Figure 2. ZEB1-AS1 upregulates ZEB1 expression. A: Schematic of the human ZEB1-AS1 locus. Arrows mark transcription start sites. B: The relative expression of ZEB1 was measured by qRT-PCR. ZEB1 mRNA levels are inhibited in shZEB1-AS1 group than in shNC group in SW480 cells. C: Western blot was performed to analyze the expression of ZEB1 protein levels. The ZEB1 protein level in SW480 cells was decreased in the shZEB1-AS1 group than in the shNC group. D: Real-time RT-PCR analysis of ZEB1 mRNA levels in ZEB1-AS1 stably overexpression and control cells in HCT116. ZEB1 mRNA levels in HCT116 cells were enhanced in ZEB1-AS1 group than in the control group. E: Western blot assay showed that ZEB1 protein levels in ZEB1-AS1 stably overexpression and control groups in HCT116 cells. ZEB1 protein level in HCT116 cells was increased in ZEB1-AS1 group than in the control group. Data are shown as mean ± SD based on at least three independent experiments. shNC: shRNA negative control; shZEB1-AS1: ZEB1-AS1 stably deleted cells. Ctrl: negative control; ZEB1-AS1: ZEB1-AS1 stably overexpression cells. $^{**}P<0.01$ by Student's t-test.

Kumamoto, Japan) assays and ethynyl deoxyuridine (EdU) incorporation assays. CCK-8 was used to detect cell viability. A total of approximately 1×10^3 cells/well was plated in a 96-well plate. Cell viability was detected in 6 wells per group and in blank controls. Afterward, 10 μL of the CCK-8 solution was added at 0, 24, 48, 72, and 96 h, and the resulting solution was incubated for 3 h at 37°C. The absorbance at 450 nm of each well was measured. Cell growth curves were plotted using the absorbance at each time point. EdU incorporation assays were performed with an EdU kit (RiboBio, Guangzhou, China), as described previously [21]. Six random fields were selected to observe each well and photographed under an inverted fluorescent microscope (Carl-Zeiss, Berlin, Germany).

Migration and invasion assay

Cell motility was assessed using Transwell chambers. For the migration assays, 1×10^4 cells plated in a serum-free medium were added to the upper chamber of 8 μm pore size Transwells (BD Biosciences, Franklin Lakes, NJ, USA). DMEM containing 10% FBS was then added to the lower chamber as a chemoattractant. For the invasion assays, 5×10^4 cells were added to the upper chamber of 8 μm pore size Transwell precoated with 30 μL of Matrigel (BD Biosciences) at 200 μg/mL. After the prep-

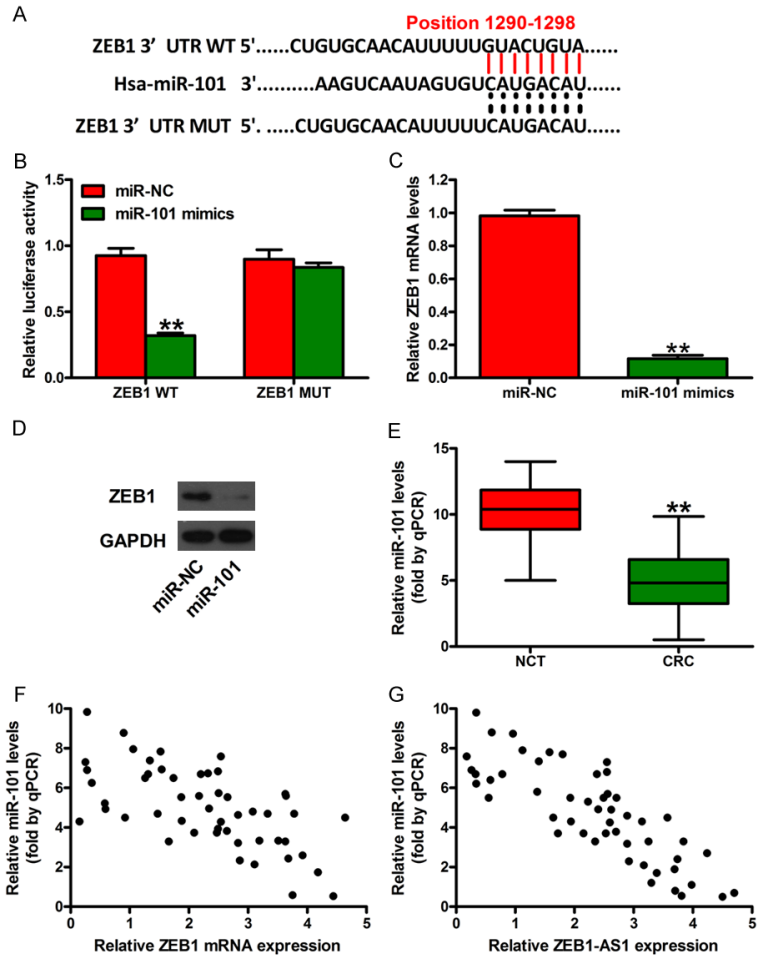

Figure 3. Identification of ZEB1 as a direct target of miR-101. A: Predicted binding sites of miR-101 in the WT and MUT 3'-UTR of ZEB1. B: Dual-luciferase reporter assays were performed 24 h after cotransfection of SW480 cells with miR-NC or miR-101 mimics and a pGL3 construct containing WT or MUT 3'-UTR of ZEB1. Data were normalized to those from cells cotransfected with miR-NC and pGL3 plasmid. ***P*<0.01, miR-101 mimics versus miR-NC in ZEB1wt group. C: The mRNA levels of ZEB1 decreased significantly in SW480 cells transfected with miR-101 mimics than those transfected with miR-NC, as measured by qPCR. GAPDH was used as internal control. ***P*<0.01, miR-101 mimics versus miR-NC. D: The protein levels of ZEB1 were attenuated in SW480 cells transfected with miR-101 mimics than those transfected with miR-NC, as detected by Western blot assays. GAPDH was used as internal controls. E: The relative expression of miR-101 in 50 pairs of CRC tissues and adjacent normal tissues were quantified by qPCR. MiR-101 was more highly expressed in adjacent normal tissues than in CRC tissues. U6 served as internal control. ***P*<0.01, CRC versus NCT group. NCT: noncancerous tissue; CRC: colorectal cancer tissue. F: Negative correlation between miR-101 and ZEB1 expression levels in these CRC tissues (r=-0.6391, ***P*<0.01). G: Negative correlation between miR-101 and ZEB1-AS1 expression levels in these CRC tissues (r=-0.8236, ***P*<0.01). All data are shown as mean ± SD of three separate experiments.

arations were incubated at 37°C for 24 h, cells that did not migrate or invade through the pores were carefully removed with a cotton swab. The filters were then fixed in methanol and stained with crystal violet. Three invasion chambers were utilized per condition, and five random fields were counted per chamber under an inverted microscope (Carl-Zeiss).

Statistical analysis

Data were statistically analyzed in SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Data from three independent experiments were presented as mean ± standard deviation (SD). One-way ANOVA among groups and two-tailed Student's t-test were performed to compare between groups. P< 0.05 was considered statistically significant.

Results

ZEB1-AS1 was upregulated in CRC tissues and cells

The ZEB1-AS1 expression level was determined using qRT-PCR analyses. **Figure 1A** and **1B** show that the expression level of ZEB1-AS1 was significantly higher in the CRC tissues than in the corresponding nontumor tissues. Then, the ZEB1-AS1 levels in the tissue samples from patients of different TNM stages were examined. Our findings revealed that ZEB1-AS1 was upregulated with advanced stage of CRC (**Figure 1C** and **1D**). In addition, we measured ZEB1-AS1 expression in NCM460, SW480, DLD-1, HCT116, SW-620, and HT29. The results showed that ZEB1-AS1 expression level was significantly higher in the CRC cell lines than that in the normal colonic cell line (**Figure 1E**).

ZEB1-AS1 upregulated ZEB1 expression

ZEB1-AS1 is an antisense transcript along the ZEB1 locus, comprising promoter and part of

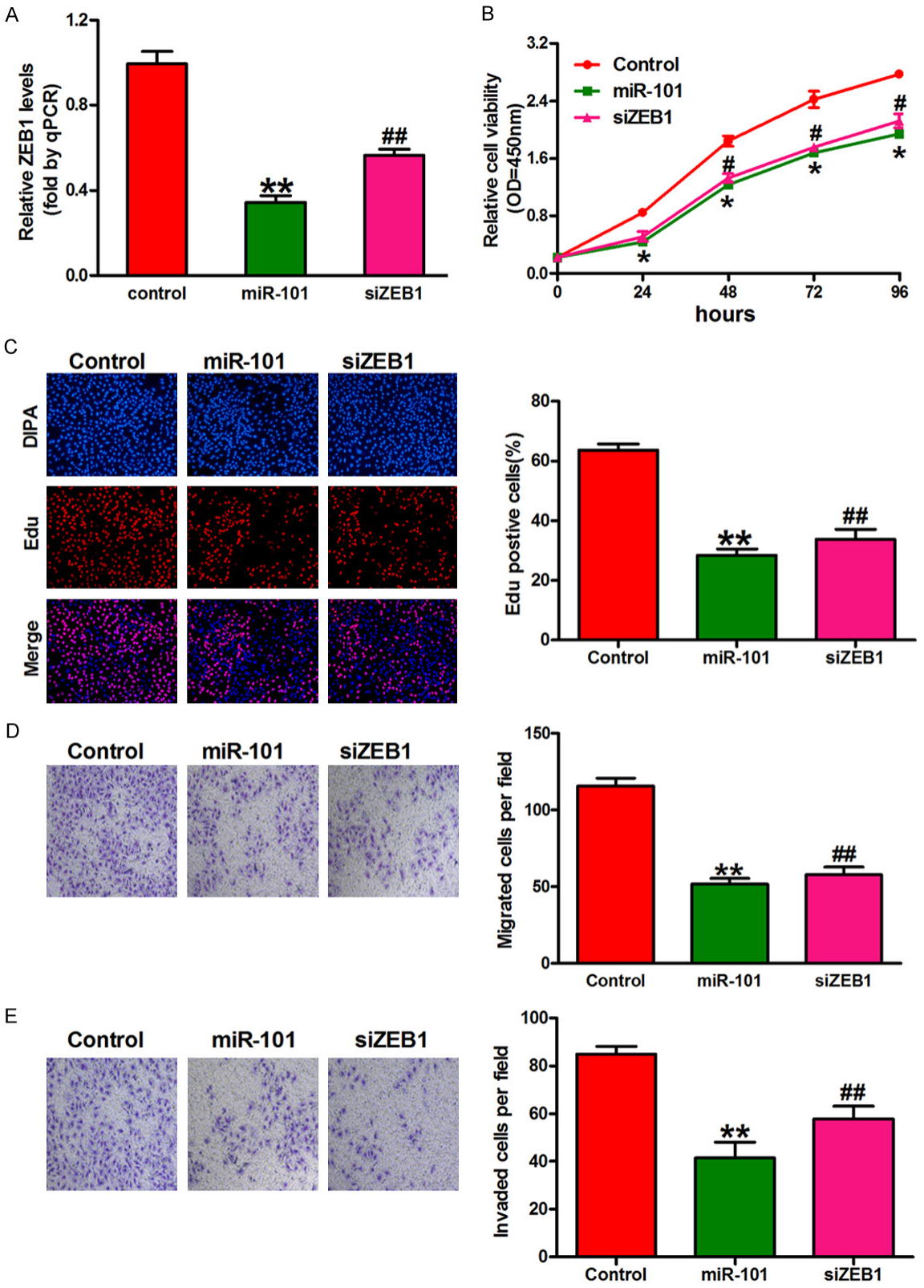

Figure 4. MiR-101 overexpression or ZEB1 depletion inhibited the cell proliferation and migration of CRC cells. A: ZEB1 expression level in SW480 cells transfected with miR-101 mimics or ZEB1 specific shRNAs was significantly decreased compared with that in the control group, as measured by qRT-PCR. GAPDH was used as internal control. B: The effects of miR-101 overexpression or ZEB1 depletion on SW480 cell proliferation were assessed using CCK-8 assays. The proliferation ability of SW480 cell overexpressed miR-101 or ZEB1 depletion was decreased compared

with that of the control group. C: The effects of miR-101 overexpression or ZEB1 depletion on SW480 cell proliferation were determined by EdU incorporation assays. The red color indicates EdU-+ nuclei. These results were consistent with the results from the CCK-8 assays. D: The effects of miR-101 overexpression or ZEB1 depletion on SW480 cell migration were determined by Transwell assays. The migration ability of SW480 was significantly inhibited after transfection with miR-101 mimics or ZEB1 specific shRNAs. E: The invasion ability of SW480 was significantly inhibited after transfection with miR-101 mimics or ZEB1 specific shRNAs, as determined by Transwell assays. Total magnification of all images is 100×. Data are shown as mean ± SD based on at least three independent experiments. **P*<0.05, ***P*<0.01, miR-101 versus control, #*P*<0.05, ##*P*<0.01, siZEB1 versus control.

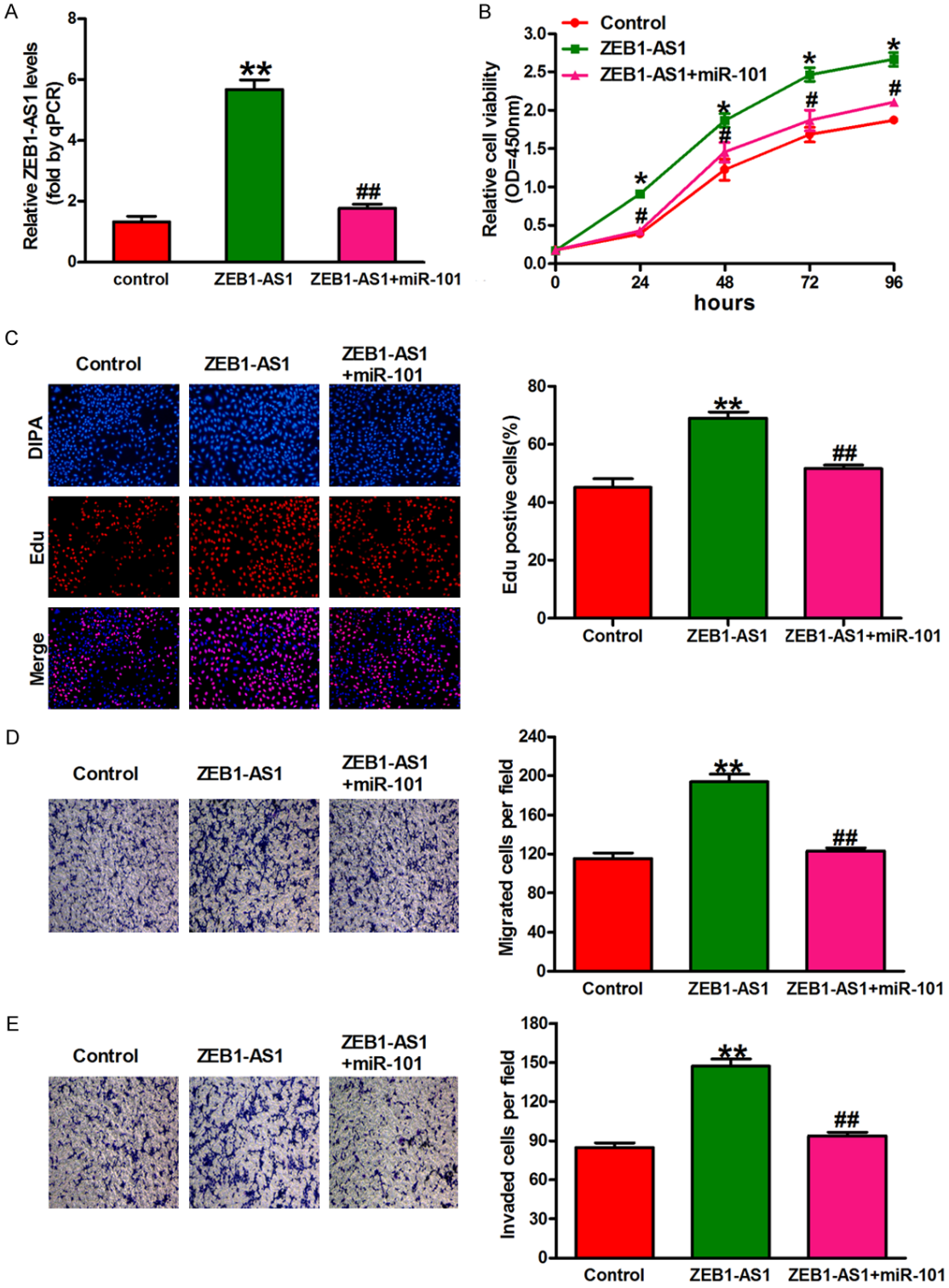

Figure 5. MiR-101 overexpression partially reversed the proliferation- and migration-promoting effects of ZEB1-AS1 in CRC cells. A: ZEB1-AS1 expression levels in ZEB1-AS1 stably overexpressed HCT116 cells or cotransfected with miR-101 mimics were measured by qRT-PCR. ZEB1-AS1 expression levels were increased significantly in HCT116 cells after transfection with ZEB1-AS1. MiR-101 suppressed the ZEB1-AS1 expression levels in ZEB1-AS1 stably overexpressed HCT116 cells. GAPDH was used as internal controls. B: The proliferation of ZEB1-AS1 stably overexpressed HCT116 cells or cotransfected with miR-101 mimics were assessed by CCK-8 assays. The proliferation ability of ZEB1-AS1 stably overexpressed HCT116 cells were improved compared with that of the control group. Meanwhile, miR-101 partially reversed the proliferation effects of ZEB1-AS1. C: The effects of miR-101 overexpression on ZEB1-AS1-induced HCT116 cell proliferation were determined by EdU incorporation assays. The red color indicates EdU-+ nuclei. The EdU-+ nuclei were increased significantly in ZEB1-AS1 stably overexpressed HCT116 cells. Conversely, miR-101 partially neutralized the proliferation effect of ZEB1-AS1. D: Cell migration was determined by Transwell assays. The migration ability of HCT116 cells was improved after ZEB1-AS1 was stably overexpressed. Introduction of miR-101 partially reversed the promigration effects of ZEB1-AS1. E: The invasion ability of HCT116 cells were improved after ZEB1-AS1 was stably overexpressed, while introduction of miR-101 partially reversed the pro-invasion effects of ZEB1-AS1, as determined by Transwell assays. Total magnification of all images is 100×. Data are shown as mean ± SD based on at least three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, ZEB1-AS1 versus control, $^{\#}P<0.05$, $^{\#\#}P<0.01$, ZEB1-AS1+miR-101 versus ZEB1-AS1.

the first exon of ZEB1, and extending further upstream (**Figure 2A**). We first measured the mRNA and protein levels of ZEB1 in ZEB1-AS1 stably overexpressed HCT116 cells and ZEB1-AS1 stably depleted SW480 to identify whether and how ZEB1-AS1 regulate ZEB1 expression. The results showed that knockdown of ZEB1-AS1 markedly decreased the mRNA and protein levels of ZEB1 in SW480 cells (**Figure 2B** and **2C**). Conversely, ZEB1-AS1 over-expression significantly increased the mRNA and protein levels of ZEB1 in HCT116 cells (**Figure 2D** and **2E**).

ZEB1-AS1 expression was associated with miR-101/ZEB1 axis in CRC patients

A complementary sequence of miR-101 to the 3'-UTR of ZEB1 mRNA is illustrated in **Figure 3A**. The dual-luciferase reporter assay showed that miR-101 reduced the activity of the luci= ferase reporter fused to the 3'-UTR-WT of ZEB1 but did not suppress that of the reporter fused to the MUT version (**Figure 3B**). Introduction of miR-101 reduced ZEB1 expression at the mRNA and protein levels in the SW480 cells (**Figure 3C** and **3D**). Considering that ZEB1 is a well-known miR-101 target, we next investigated whether ZEB1-AS1 expression level and miR-101 level in CRC tissues were correlated. We first measured the miR-101 level in 50 pairs of CRC tissues and adjacent normal tissues. The results showed that ZEB1-AS1 and ZEB1 expression was inversely correlated with miR-101 level in CRC tissues (**Figure 3F** and **3G**). Considering that ZEB1-AS1 transcript level was positively correlated with ZEB1 mRNA level, these data support the possible interplay between ZEB1-AS1 and miR-101/ZEB1 axis.

Overexpression of miR-101 or downregulation of ZEB1 inhibited proliferation and migration of CRC cells

We overexpressed miR-101 or inhibited ZEB1 in SW480 cells to investigate the effects of ZEB1-AS1-miR-101-ZEB1 regulating axis on the proliferation and migration of CRC cells. The results supported the inhibition of ZEB1 by miR-101 in SW480 cells (**Figure 4A**). CCK-8 assays and EdU incorporation assays showed that miR-101 overexpression and ZEB1 depletion inhibited SW480 cell proliferation (**Figure 4B** and **4C**). This result was consistent with the proliferation-inhibiting effect of ZEB1-AS1 depletion shown by a previous report [19]. Transwell assays showed that miR-101 overexpression and ZEB1 depletion significantly inhibited SW480 cell migration and invasion (**Figure 4D** and **4E**), which was also consistent with the migration-inhibiting effect of ZEB1-AS1 depletion revealed in our previous report [19]. These data suggested that depletion of ZEB1-AS1, overexpression of miR-101, and depletion of ZEB1 have inhibitory effects on the proliferation and migration of CRC cells.

Overexpression of miR-101 partially reversed the effects of ZEB1-AS1 on the cell proliferation and migration of CRC cells

Considering the association of ZEB1-AS1 with miR-101/ZEB1 axis in CRC, we next explored whether miR-101 mediated the effects of ZEB1-AS1 on the cell proliferation and migration of CRC cells. We overexpressed miR-101 in ZEB1-AS1 stably overexpressed HCT116 cells (**Figure 5A**). CCK-8 assays and EdU incorporation assays showed that overexpression of miR-

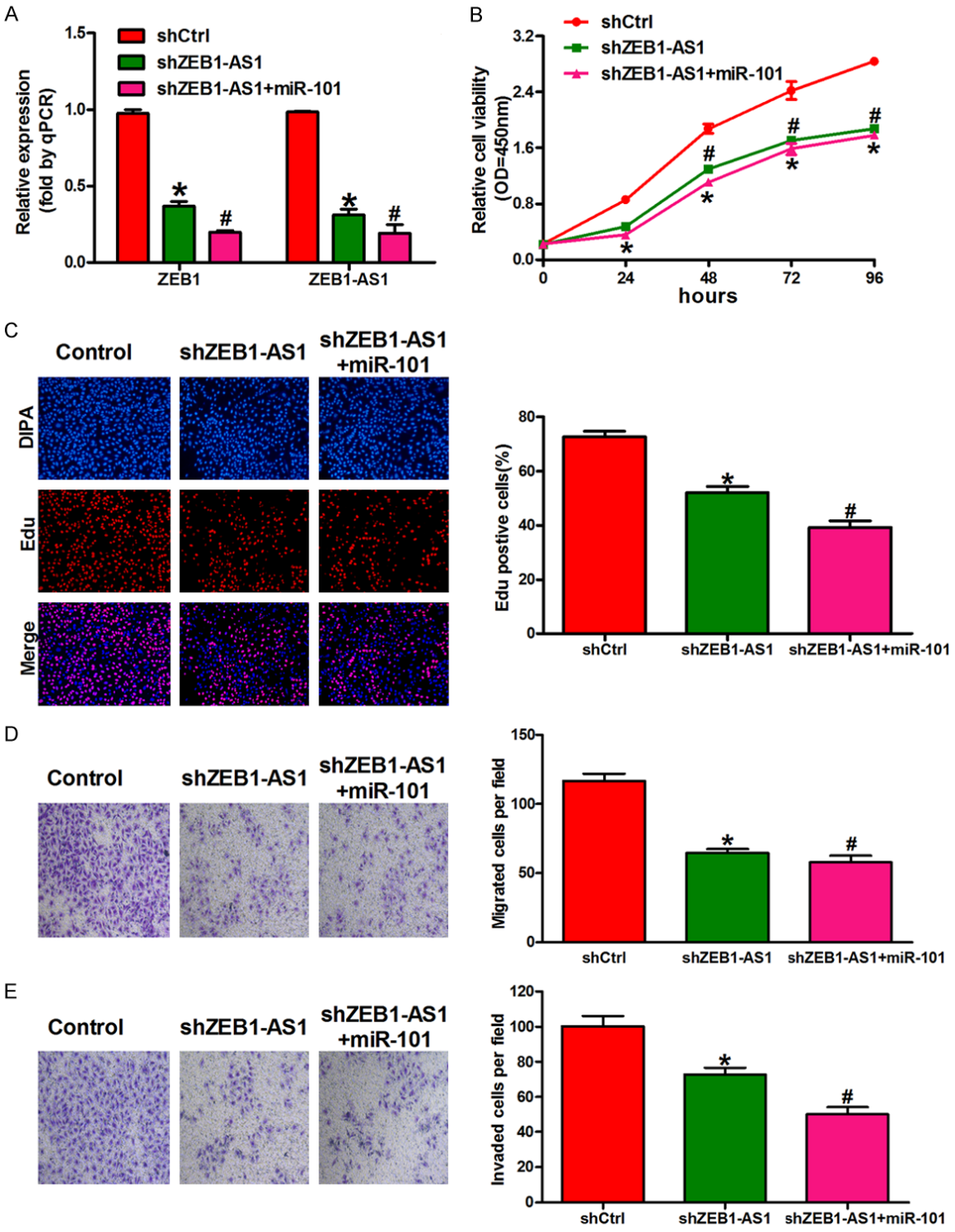

Figure 6. Combined ZEB1-AS1 depletion and miR-101 overexpression significantly inhibited the proliferation and migration of CRC cells. A: ZEB1-AS1 and ZEB1 expression levels in ZEB1-AS1 stably depleted SW480 cells or cotransfected with miR-101 mimics. ZEB1-AS1 and ZEB1 expression was significantly inhibited by combining ZEB1-AS1 depletion and miR-101 overexpression. B: The effects of ZEB1-AS1 depletion or ZEB1-AS1 depletion combined with miR-101 overexpression on SW480 cell proliferation were assessed using CCK-8 assays. The proliferation ability of SW480 cell ZEB1-AS1 depletion or co-transfected with miR-101 was reduced compared with that of the control group. MiR-101 partially enhanced the inhibitory effect of ZEB1-AS1 depletion on cell proliferation. C: The effects of ZEB1-AS1 depletion or cotransfection with miR-101 overexpression on SW480 cell proliferation were determined by EdU incorporation assays. The red color indicates EdU-+ nuclei. EdU-+ nuclei were decreased significantly after ZEB1-AS1 depletion in SW480 cells. MiR-101 partially reinforced the suppression effect of ZEB1-AS1 depletion. D:

The migration ability of SW480 cells was suppressed after ZEB1-AS1 depletion, which when combined with miR-101 overexpression enhanced the suppression effect of ZEB1-AS1 depletion, as determined by Transwell assays. E: The invasion ability of SW480 cells was inhibited after ZEB1-AS1 depletion while cotransfected miR-101 partially enhanced the inhibitory effect of shZEB1-AS1, as determined by Transwell assays. Total magnification of all images is 100×. Data are shown as mean ± SD based on at least three independent experiments. *P<0.05, **P<0.01, shZEB1-AS1 versus control, $^{\#}P$<0.05, $^{\#\#}P$<0.01, shZEB1-AS1+miR-101 versus shZEB1-AS1.

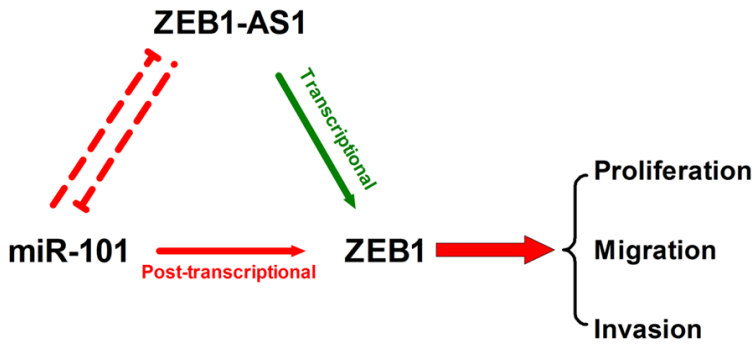

Figure 7. The interplay between ZEB1-AS1 and miR-101 in the proliferation and migration of CRC cells. In addition to the direct transcriptional activation of ZEB1 by ZEB1-AS1, ZEB1-AS1 also post-transcriptionally upregulated ZEB1 via binding and inhibiting miR-101. ZEB1-AS1 promoted proliferation and migration of the CRC cells by regulating miR-101 and ZEB1.

101 partially reversed the proproliferation effects of ZEB1-AS1 (**Figure 5B** and **5C**). Transwell assays showed that overexpression of miR-101 partially reversed the promigration effects of ZEB1-AS1 (**Figure 5D** and **5E**). These results suggested that ZEB1-AS1 promoted the cell proliferation and migration of CRC cells partially through binding and inhibiting miR-101.

Inhibition of ZEB1-AS1 combined with miR-101 overexpression significantly inhibited CRC cell proliferation and migration

We overexpressed miR-101 in ZEB1-AS1 stably depleted SW480 cells to further explore the biological significance of the interplay between ZEB1-AS1 and miR-101. Combined ZEB1-AS1 depletion and miR-101 overexpression significantly inhibited ZEB1-AS1 and ZEB1 expression (**Figure 6A**). CCK-8 assays and EdU incorporation assays showed that this combination significantly inhibited SW480 cell proliferation (**Figure 6B** and **6C**). Moreover, Transwell assays showed that migration and invasion of SW480 cell were also significantly inhibited (**Figure 6D** and **6E**). These data further confirm the important roles of the interplay between ZEB1-AS1 and miR-101/ZEB1 axis in the proliferation and migration of CRC cell.

Discussion

In this study, we identified a new regulation mechanism of ZEB1-AS1 in promoting the proliferation and migration of CRC cells. This mechanism is involved in regulating the miR-101/ZEB1 axis. First, ZEB1-AS1 was confirmed to be upregulated and positively correlated with higher histological grade and advanced tumor stage T. These characteristics predicted poor prognosis in CRC cancer. Second, a correlation between ZEB1 levels and ZEB1-AS1 transcript level in CRC cells further supported the regulation of ZEB1 by ZEB1-AS1. Third, ZEB1 was confirmed as a direct target of miR-101, and miR-101 expression levels were negatively correlated with ZEB1-AS1 and ZEB1 expression levels in CRC. Fourth, miR-101 ectopic expression, depletion of ZEB1, or depletion of ZEB1-AS1 repressed the proliferation and migration of CRC cells. The effects of ZEB1-AS1 on CRC progression were partially dependent on miR-101. Moreover, the combination of ZEB1-AS1 depletion and miR-101 overexpression showed more intensive inhibiting effects on the proliferation and migration of CRC cells. These findings suggested that the interplay between ZEB1-AS1 and miR-101/ZEB1 is functionally significant in CRC.

LncRNAs are ncRNAs and frequently observed to be involved in carcinogenesis and cancer progression [8]. Recent studies showed that aberrant expression of lncRNAs has been identified in types of human cancer, including CRC [22-25]. These molecules may provide new biomarkers for the diagnosis and prognosis of cancers [26, 27]. Upregulation of lncRNA ZEB1-AS1 promotes tumor metastasis in hepatocellular carcinoma [16]. LncRNA ZEB1-AS1 epigenetically regulates the expression of ZEB1 and downstream molecules in prostate cancer

[19]. Although previous studies showed that overexpression of ZEB1-AS1 increased ZEB1 expression and ZEB1-AS1 acted as an oncogene in HCC and osteosarcoma cells [16, 20]. the relation between ZEB1-AS1 and ZEB1 in CRC cancer has not been investigated yet. We measured the mRNA and protein levels of ZEB1 in ZEB1-AS1 stably overexpressed HCT116 and ZEB1-AS1 stably depleted SW480 cells to identify whether and how ZEB1-AS1 regulate ZEB1 expression. The results showed that ZEB1-AS1 upregulated ZEB1 expression in CRC cells. Thus, ZEB1-AS1 upregulated ZEB1 expression transcriptionally and posttranscriptionally in CRC.

The relevance of ZEB1 to tumor progression has been studied in several human cancers [28-31]. Accumulating evidence has suggested that ZEB1 is a direct target of miR-101 in various cancers, such as breast, oral squamous cell, ovarian, and hepatocellular carcinomas [32-35]. Similarly, in this study, ZEB1 was directly targeted by tumor suppressor miR-101 in CRC. MiR-101 was downregulated in CRC tissues than in adjacent normal tissues. ZEB1-AS1 and ZEB1 were upregulated in the same CRC tissues. Furthermore, miR-101 expression levels were negatively correlated with ZEB1-AS1 and ZEB1 expression levels in CRC. These results supported the inter-regulation between ZEB1-AS1 and miR-101/ZEB1 axis. Moreover, the loss of balance between ZEB1-AS1, ZEB1, and miR-101 in CRC may contribute to CRC progression.

Functional experiments showed that ZEB1 depletion, miR-101 ectopic expression, and ZEB1-AS1 depletion suppress the proliferation and migration of CRC cells. Furthermore, the silencing of ZEB1 mimicked the inhibitory effects of miR-101 on the proliferation, migration, and invasion of the CRC cells. The effects of ZEB1-AS1 on proliferation and migration of the CRC cells were partially neutralized by the introduction of miR-101. Moreover, miR-101 overexpression enhanced the inhibitory effects on proliferation and migration in CRC cells induced by ZEB1-AS1 depletion. These data suggested that the interplay between ZEB1-AS1 and miR-101/ZEB1 axis had important functional significance on CRC and supported the roles of ZEB1-AS1, ZEB1, and miR-101 dysregulation in CRC progression. In addition to ZEB1, miR-101 also targets and inhibits EZH2 [36], COX2 [37], and EP4R [38] in CRC cells. However, further investigation is needed to determine whether ZEB1-AS1 also regulates the expression of these targets. Overall, this study reveals a new regulation mechanism of ZEB1-AS1 on ZEB1.

In summary, this study revealed the reciprocally negative regulation between ZEB1-AS1 and miR-101 and the post-transcriptional upregulation of ZEB1 by ZEB1-AS1 by binding miR-101. Furthermore, the interplay between ZEB1-AS1 and miR-101/ZEB1 axis played critical roles in the proliferation and migration of CRC cells (**Figure 7**). The results implied that targeting the interplay between ZEB1-AS1 and miR-101/ZEB1 axis would be a novel therapeutic strategy for CRC.

Acknowledgements

This work was supported by the Funding Scheme for Key Scientific Research Projects in Universities of Henan Province (No. 15A-320060) and the Departmental Funding of General Surgery Department in the First Affiliated Hospital of Xinxiang Medical University. The funders had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Peng He and Wan-Cheng Xiong, Department of General Surgery, First Affiliated Hospital of Xinxiang Medical University, Weihui 453100, Henan, China. Tel: 86 0373-4404713; Fax: 86 0373-4404713; E-mail: hepeng163001@163.com (PH); wanchengxiong2011@163.com (WCX)

References

[1] Siegel RL, Miller KD, Fedewa SA, Ahnen DJ, Meester RGS, Barzi A and Jemal A. Colorectal cancer statistics, 2017. CA Cancer J Clin 2017; 67: 177-193.

[2] Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J. Cancer statistics in China, 2015. CA Cancer J Clin 2016; 66: 115-132.

[3] Carvalho C and Glynne-Jones R. Challenges behind proving efficacy of adjuvant chemotherapy after preoperative chemoradiation for rectal cancer. Lancet Oncol 2017; 18: e354-e363.

[4] Petrelli F, Coinu A, Lonati V and Barni S. A systematic review and meta-analysis of adjuvant chemotherapy after neoadjuvant treatment and surgery for rectal cancer. Int J Colorectal Dis 2015; 30: 447-457.
[5] Shaikh I, Askari A, Ouru S, Warusavitarne J, Athanasiou T and Faiz O. Oncological outcomes of local excision compared with radical surgery after neoadjuvant chemoradiotherapy for rectal cancer: a systematic review and meta-analysis. Int J Colorectal Dis 2015; 30: 19-29.
[6] Berindan-Neagoe I, Monroig Pdel C, Pasculli B and Calin GA. MicroRNAome genome: a treasure for cancer diagnosis and therapy. CA Cancer J Clin 2014; 64: 311-336.
[7] Wahlestedt C. Targeting long non-coding RNA to therapeutically upregulate gene expression. Nat Rev Drug Discov 2013; 12: 433-446.
[8] Beermann J, Piccoli MT, Viereck J and Thum T. Non-coding RNAs in development and disease: background, mechanisms, and therapeutic approaches. Physiol Rev 2016; 96: 1297-1325.
[9] Huarte M. The emerging role of lncRNAs in cancer. Nat Med 2015; 21: 1253-1261.
[10] Schmitt AM and Chang HY. Long noncoding RNAs in cancer pathways. Cancer Cell 2016; 29: 452-463.
[11] Martens-Uzunova ES, Bottcher R, Croce CM, Jenster G, Visakorpi T and Calin GA. Long noncoding RNA in prostate, bladder, and kidney cancer. Eur Urol 2014; 65: 1140-1151.
[12] Mehler MF and Mattick JS. Noncoding RNAs and RNA editing in brain development, functional diversification, and neurological disease. Physiol Rev 2007; 87: 799-823.
[13] Okugawa Y, Grady WM and Goel A. Epigenetic alterations in colorectal cancer: emerging biomarkers. Gastroenterology 2015; 149: 1204-1225, e1212.
[14] Parasramka MA, Maji S, Matsuda A, Yan IK and Patel T. Long non-coding RNAs as novel targets for therapy in hepatocellular carcinoma. Pharmacol Ther 2016; 161: 67-78.
[15] Hayes EL and Lewis-Wambi JS. Mechanisms of endocrine resistance in breast cancer: an overview of the proposed roles of noncoding RNA. Breast Cancer Res 2015; 17: 40.
[16] Li T, Xie J, Shen C, Cheng D, Shi Y, Wu Z, Deng X, Chen H, Shen B, Peng C, Li H, Zhan Q and Zhu Z. Upregulation of long noncoding RNA ZEB1-AS1 promotes tumor metastasis and predicts poor prognosis in hepatocellular carcinoma. Oncogene 2016; 35: 1575-1584.
[17] Su W, Xu M, Chen X, Chen N, Gong J, Nie L, Li L, Li X, Zhang M and Zhou Q. Long noncoding RNA ZEB1-AS1 epigenetically regulates the expressions of ZEB1 and downstream molecules in prostate cancer. Mol Cancer 2017; 16: 142.
[18] Lin J, Zhan Y, Liu Y, Chen Z, Liang J, Li W, He A, Zhou L, Mei H, Wang F and Huang W. Increased expression of ZEB1-AS1 correlates with higher histopathological grade and promotes tumorigenesis in bladder cancer. Oncotarget 2017; 8: 24202-24212.
[19] Gong H, Wen H, Zhu X, Lian Y, Yang X, Qian Z and Zhu J. High expression of long non-coding RNA ZEB1-AS1 promotes colorectal cancer cell proliferation partially by suppressing p15 expression. Tumour Biol 2017; 39: 1010428-317705336.
[20] Liu C and Lin J. Long noncoding RNA ZEB1-AS1 acts as an oncogene in osteosarcoma by epigenetically activating ZEB1. Am J Transl Res 2016; 8: 4095-4105.
[21] Li JT, Jia LT, Liu NN, Zhu XS, Liu QQ, Wang XL, Yu F, Liu YL, Yang AG and Gao CF. MiRNA-101 inhibits breast cancer growth and metastasis by targeting CX chemokine receptor 7. Oncotarget 2015; 6: 30818-30830.
[22] Batista PJ and Chang HY. Long noncoding RNAs: cellular address codes in development and disease. Cell 2013; 152: 1298-1307.
[23] Prensner JR and Chinnaiyan AM. The emergence of lncRNAs in cancer biology. Cancer Discov 2011; 1: 391-407.
[24] Gutschner T and Diederichs S. The hallmarks of cancer: a long non-coding RNA point of view. RNA Biol 2012; 9: 703-719.
[25] Fatima R, Akhade VS, Pal D and Rao SM. Long noncoding RNAs in development and cancer: potential biomarkers and therapeutic targets. Mol Cell Ther 2015; 3: 5.
[26] Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, Tsai MC, Hung T, Argani P, Rinn JL, Wang Y, Brzoska P, Kong B, Li R, West RB, van de Vijver MJ, Sukumar S and Chang HY. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature 2010; 464: 1071-1076.
[27] Yang Z, Zhou L, Wu LM, Lai MC, Xie HY, Zhang F and Zheng SS. Overexpression of long noncoding RNA HOTAIR predicts tumor recurrence in hepatocellular carcinoma patients following liver transplantation. Ann Surg Oncol 2011; 18: 1243-1250.
[28] Spaderna S, Schmalhofer O, Hlubek F, Berx G, Eger A, Merkel S, Jung A, Kirchner T and Brabletz T. A transient, EMT-linked loss of basement membranes indicates metastasis and poor survival in colorectal cancer. Gastroenterology 2006; 131: 830-840.
[29] Matsubara D, Kishaba Y, Yoshimoto T, Sakuma Y, Sakatani T, Tamura T, Endo S, Sugiyama Y, Murakami Y and Niki T. Immunohistochemical analysis of the expression of E-cadherin and ZEB1 in non-small cell lung cancer. Pathol Int 2014; 64: 560-568.

[30] Bronsert P, Kohler I, Timme S, Kiefer S, Werner M, Schilling O, Vashist Y, Makowiec F, Brabletz T, Hopt UT, Bausch D, Kulemann B, Keck T and Wellner UF. Prognostic significance of Zinc finger E-box binding homeobox 1 (ZEB1) expression in cancer cells and cancer-associated fibroblasts in pancreatic head cancer. Surgery 2014; 156: 97-108.

[31] Liu Y, Zhang N, Wang Y, Xu M, Liu N, Pang X, Cao J, Ma N, Pang H, Liu L and Zhang H. Zinc finger E-box binding homeobox 1 promotes invasion and bone metastasis of small cell lung cancer in vitro and in vivo. Cancer Sci 2012; 103: 1420-1428.

[32] Chandra Mangalhara K, Manvati S, Saini SK, Ponnusamy K, Agarwal G, Abraham SK and Bamezai RN. ERK2-ZEB1-miR-101-1 axis contributes to epithelial-mesenchymal transition and cell migration in cancer. Cancer Lett 2017; 391: 59-73.

[33] Wu B, Lei D, Wang L, Yang X, Jia S, Yang Z, Shan C, Yang X, Zhang C and Lu B. MiRNA-101 inhibits oral squamous-cell carcinoma growth and metastasis by targeting zinc finger E-box binding homeobox 1. Am J Cancer Res 2016; 6: 1396-1407.

[34] Guo F, Cogdell D, Hu L, Yang D, Sood AK, Xue F and Zhang W. MiR-101 suppresses the epithelial-to-mesenchymal transition by targeting ZEB1 and ZEB2 in ovarian carcinoma. Oncol Rep 2014; 31: 2021-2028.

[35] Zhao S, Zhang Y, Zheng X, Tu X, Li H, Chen J, Zang Y and Zhang J. Loss of MicroRNA-101 promotes epithelial to mesenchymal transition in hepatocytes. J Cell Physiol 2015; 230: 2706-2717.

[36] Peng Z and Zhang Y. Methyl jasmonate induces the apoptosis of human colorectal cancer cells via downregulation of EZH2 expression by microRNA101. Mol Med Rep 2017; 15: 957-962.

[37] Strillacci A, Griffoni C, Sansone P, Paterini P, Piazzi G, Lazzarini G, Spisni E, Pantaleo MA, Biasco G and Tomasi V. MiR-101 downregulation is involved in cyclooxygenase-2 overexpression in human colon cancer cells. Exp Cell Res 2009; 315: 1439-1447.

[38] Chandramouli A, Onyeagucha BC, Mercado-Pimentel ME, Stankova L, Shahin NA, LaFleur BJ, Heimark RL, Bhattacharyya AK and Nelson MA. MicroRNA-101 (miR-101) post-transcriptionally regulates the expression of EP4 receptor in colon cancers. Cancer Biol Ther 2012; 13: 175-183.