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MiR-873-5p inhibits cell migration, invasion and epithelial-mesenchymal transition in colorectal cancer via targeting ZEB1

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ABSTRACT

Recent studies have demonstrated that dysregulation of mircoRNAs (miRNAs) greatly affected biological processes of human cancers, including colorectal cancer. As a member of miRNAs family, miR-873-5p has been proved to be a tumor suppressor in some human cancers. Here, we aim to investigate the effects of miR-873-5p on the migration, invasion and epithelial-mesenchymal transition (EMT) of colorectal cancer cells. The low expression of miR-873-5p in colorectal cancer cells was identified by conducting qRT-PCR analysis. Gain of function assays were designed and conducted to demonstrate the specific function of miR-873-5p overexpression in colorectal cancer progression. Transwell assay and western blot assay were conducted and revealed that miR-873-5p inhibited cell migration, invasion and EMT formation. To find the downstream molecular mechanism of miR-873-5p, mechanism assays were designed and performed to find the downstream target of miR-873-5p. ZEB1 (Zinc finger E-box-binding homeobox 1) was certified to be the target of miR-873-5p through bioinformatics analysis, luciferase activity assay and pull-down assay. Finally, rescue assays were carried out to demonstrate the effects of miR-873-5p-ZEB1 axis on the migration, invasion and EMT process of colorectal cancer cells. In conclusion, we confirmed that miR-873-5p suppressed cell migration, invasion and EMT in colorectal cancer via targeting ZEB1.

1. Introduction

Colorectal cancer (CRC) belongs to the globally commonest malignancies, with more than one million new-diagnosed cases every year [9]. Colorectal cancer is caused by various reasons, such as colorectal adenoma, intestinal inflammation and intestinal microflora disorders. The progression of CRC is generally accompanied with the mutations and dysregulations of anti-oncogenic genes and oncogenes [41]. As we all know, migration and epithelial-mesenchymal transition (EMT) are two important factors for the formation of malignant phenotype [30]. This study aims to investigate the molecular mechanism contributed to cell migration, invasion and EMT process in colorectal cancer.

MicroRNAs (miRNAs), are a class of small non-coding RNAs which are comprised of 19–25 nucleotides [1]. MiRNAs are a class of cellular regulators that regulate various gene expression, thereby affect cell function [3,10,27,28]. Generally, miRNAs exert function by down-regulating their target mRNAs through sequence complementarity [24,31]. So far, a large quantity of miRNAs has been studied in human cancers. Based on the functions, miRNAs are divided into tumor suppressors and oncogenes. MiRNAs with either oncogenic function or tumor-suppressive function have been widely reported in human cancers [13,14,32,37], including CRC [2,11,29,42]. MiR-873-5p has been reported in gastric cancer [4]. The specific function of miR-873-5p in other cancers is still unknown.

In this study, we studied the biological function of muR-873-5p in human colorectal cancers. The expression condition of miR-873-5p in CRC cells and the normal cell lines was examined by qRT-PCR. Gain or loss-of functional assays were conducted in HCT116 and SW480 cells. The effects of miR-873-5p on migration, invasion and EMT process were separately tested by transwell assay and western blot analysis. Some

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potential targets of miR-873-5p were predicted by using bioinformatics analysis. Among these target mRNAs, Zinc finger E-box-binding homeobox 1 (ZEB1) is a famous master inducer of EMT program. Numerous studies have demonstrated that ZEB1 promoted cell migration, invasion and EMT progress in various human malignant tumors [17,26,33,40,43], including colorectal cancer [8,39]. Therefore, we chose ZEB1 to do further study. Luciferase reporter assay and pull down assay further demonstrate the combination between ZEB1 and miR-873-5p. Finally, rescue assays demonstrated the effects of miR-873-5p-ZEB1 axis on cell migration, invasion and EMT process in CRC.

2. Materials and methods

2.1. Cell culture

All cell lines used in this study (the normal human intestinal epithelial cell line: HIEC; five human CRC cell lines: DLD-1, HCT-116, SW-480, HT-29, SW-620) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL; Invitrogen, Carlsbad, CA, USA) in which 10% fetal bovine serum (HyClone, Logan, UT, USA), 100U/ml penicillin and 100 mg/ml streptomycin were added. The condition for cell culture is as follow: 37 °C, humidified air atmosphere containing 5% CO_2.

2.2. Transfection

To prepare for functional assays, miR-873-5p mimic (miR-873-5p), miR-873-5p inhibitor (anti-miR-873-5p) as well as NC for miR-873-5p mimic (miR-NC) and NC for miR-873-5p inhibitor (anti-miR-NC) were synthesized and purified by Molbase Co Ltd (Shanghai, China). Additionally, the pcDNA-ZEB1 expression vector and the control empty vector (pcDNA-NC) were synthesized and purchased from GenePharma (Shanghai, China). According to the instructions, transfections were performed and finished by using Lipofectamine2000 (Invitrogen).

2.3. RNA extraction and qRT-PCR

Total messenger RNAs (mRNAs) were extracted from CRC cells by using TRIZOL (Invitrogen). Reverse transcription was performed and finished using PrimeScript RT reagent kit (TaKaRa, Dalian, China). Accomplish quantitative real-time polymerase chain reaction (qRT-PCR) was carried out through using miSYBRGreenPCR kit (TransGen Biotech, China). U6 snRNA was taken as an endogenous control for miR-873-5p, while GAPDH was used as the internal control for ZEB1 expression. The relative expression of miR-873-5p and ZEB1 was quantified with $2^{-\Delta\Delta Ct}$ method.

2.4. Transwell assay

After necessary transfections, HCT-116 and SW-480 cells (1×10^4) were suspended in 200 µl of serum-free medium. To measure invasion and migration abilities of indicated CRC cells, cells were then seeded into the upper chambers of transwell chambers (8 µm pore size, Costar) which were coated or uncoated with Matrigel (BD Biosciences, USA). Medium containing 10% FBS was used as a chemoattractant and added into the bottom chamber. Cell incubation was proceeded at 37 °C and 5% CO_2 for 48 h (invasion) and for 24 h (migration). Cells stayed in the top chamber were wiped out by cotton swabs, while cells on the lower surface were fixed with methanol and stained with 0.1% crystal violet. At length, the visual fields were observed under a ×200 microscope (Olympus, Japan).

2.5. Luciferase reporter assay

HCT-116 cell was co-transfected with wild-type and mutant 3′-UTR sequence of ZEB1 as well as miR-NC and miR-873-5p. Whereas, SW-480 cell was co-transfected with wild-type and mutant 3′-UTR sequence of ZEB1 as well as anti-miR-NC and anti-miR-873-5p. Transfections were finished using Lipofectamine 2000 (Invitrogen). The luciferase activity was measured with Luc-PairmiR Luciferase Assay (GeneCopoeia, Rockville, Maryland).

2.6. Pull-down assay

MiR-873-5p, miR-873-5p-MUT, and miR-873-5p-NC were biotinylated to be bio-miR-873-5p-WT, bio-miR-873-5p-MUT, and bio-NC by GenePharma Company (Shanghai, China). Next, they were transfected into HCT-116 and SW-480 cells. 48 h later, cells were collected and incubated with Dynabeads M-280 Streptavidin (Invitrogen) for about 10 min. After cells was washed with buffer, the bound RNAs were quantified and analyzed by qRT-PCR.

2.7. Western blot analysis

When transfection was terminated, HCT-116 and SW-480 cells were lysed in an ice-cold RIPA buffer (Solaibo, China). Total protein concentration was determined with BCA Protein Assay kit (Vigorous Biotechnology Beijing Co Ltd, Beijing, China). Next, proteins were isolated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% non-fat milk for one hour at room temperature. And then incubated with primary antibodies (E-cadherin, β-catenin, ZO-1, N-cadherin, Vimentin, and ZEB1) at 4 °C overnight. Goat Anti-Rabbit IgG H&L was used as the secondary antibody. All these antibodies were purchased from Abcam (Cambridge, UK). The protein bands were quatified with the ImageJ software (National Institutes of Health, Bethesda, Maryland).

2.8. Immunofluorescence

Cells were cultured on glass slices and fixed with 4% formaldehyde for about ten minutes. Next, 0.3% Triton X-100 was used for cell permeation. The slices were blocked by the goat serum for about 15 min at 37 °C. Subsequently, samples were incubated with anti-E-cadherin (1:80, Abcam) and anti-*N*-cadherin (1:80, Abcam) at 4 °C overnight and with goat TRITC labeled secondary antibody (1:70, Abcam) at 37 °C for one hour. Meanwhile, DAPI (Genview Inc, Shanghai, China) was utilized for staining. At last, the fluorescence was visualized under a microscope (×400).

2.9. Statistical analysis

SPSS Statistics 18.0 (IBM, Armonk, NY, USA) was used for all statistical analyses in this study. Data are displayed as mean ± SD of more than two independent experiments. Two group comparison was made and analyzed with Student's t-test, while multiple group comparisons were analyzed using one-way ANOVA. P-value < 0.05 indicated differences are statistically significant.

3. Results

3.1. MiR-873a-5p was downregulated in CRC cells and suppressed cell migration and invasion

To investigate the specific biological role of miR-873-5p in colorectal cancer, the expression pattern of miR-873-5p both in five CRC cell lines in several human CRC cell lines (DLD-1, HCT-116, SW-480, HT-29, SW-620) was detected with qRT-PCR. We uncovered that the level of miR-873-5p was higher in CRC cells compared with that in HIEC normal human intestinal epithelial cell line (Fig. 1A). According to the data in Fig. 1A, the expression of miR-873-5p was highest in SW480 cell and lowest in HCT-116 cell. Therefore, we decided to conduct gain-of

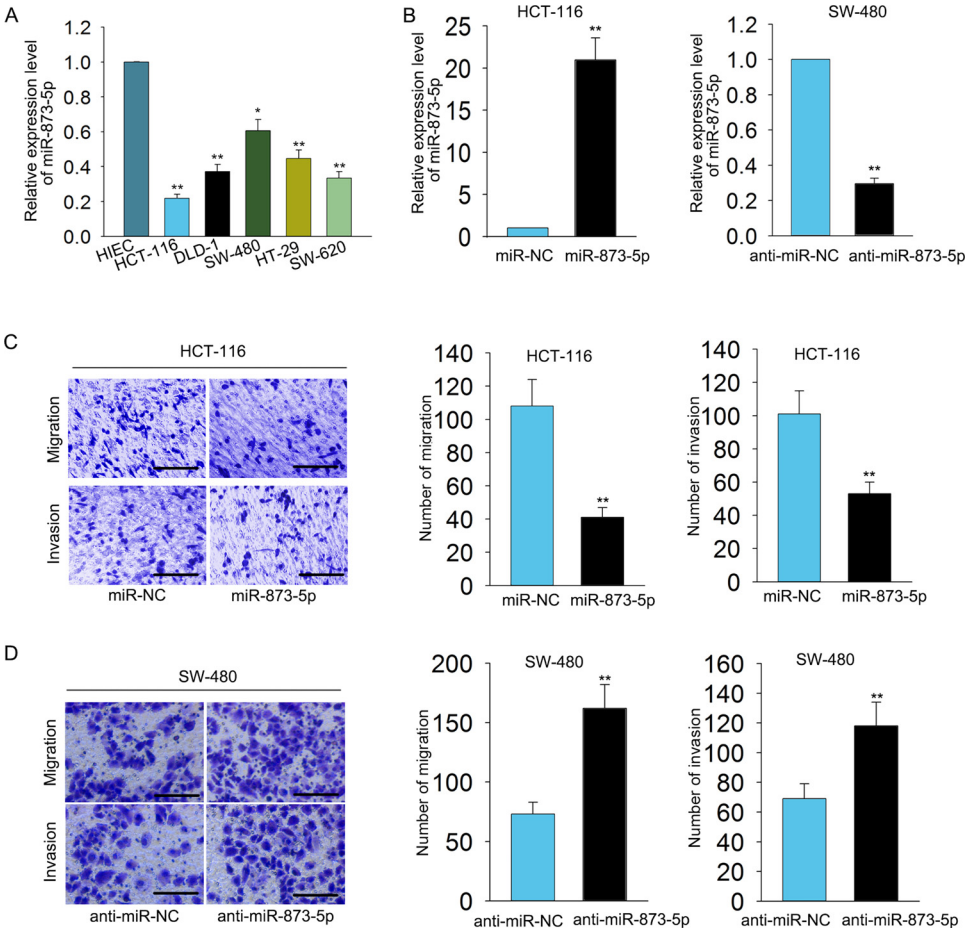

Fig. 1. MiR-873a-5p was downregulated in CRC cells and suppressed cell migration and invasion. A. The expression pattern of miR-873-5p both in five CRC cell lines in several human CRC cell lines (DLD-1, HCT-116, SW-480, HT-29, SW-620) was detected with qRT-PCR. **B.** HCT-116 cell was transfected with miR-873-5p mimics (miR-873-5p), while SW-480 cell was transfected with miR-873-5p inhibitors (anti-miR-873-5p). After 48 h, qRT-PCR was used to harvest the transfection result. **C-D**. Transwell assay revealed the migratory and invasive capacity of HCT-116 (**C**) and SW-480 (**D**) cells after transfections. Images for migration and invasion were obtained under a ×200 microscope. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group.

function assay in HCT-116 cell and perform loss-of function assay in SW-480 cell. Accordingly, HCT-116 cell was transfected with miR-873-5p mimics (miR-873-5p), while SW-480 cell was transfected with miR-873-5p inhibitors (anti-miR-873-5p). After 48 h, qRT-PCR was used to harvest the transfection result. The expression level of miR-873-5p was efficiently increased or decreased in transfected cells (Fig. 1B). Transwell assay indicated that miR-873-5p suppressed the migratory and invasive capacity of HCT-116 cell (Fig. 1C). Whereas, anti-miR-873-5p enhanced the migratory and invasive capacity of SW-480 cell (Fig. 1D). These results suggested that miR-873-5p acted as a tumor suppressor in CRC through regulating cell migration and invasion.

3.2. MiR-873a-5p reversed epithelial-mesenchymal transition in CRC cells

Here, we continued to explore whether miR-873-5p can reverse the phenotype from EMT to MET by examining the protein levels of EMT markers (E-cadherin, β-catenin, ZO-1, N-cadherin, Vimentin). Upregulated miR-873-5p greatly enhanced the levels of E-cadherin, β-catenin and ZO-1, while reduced the levels of N-cadherin, Vimentin (Fig. 2A). Whereas, the completely opposite results were observed in SW-480 cell transfected with anti-miR-873-5p. Consistent with western blot analysis, immunofluorescence showed the same effects of miR-873-5p on EMT progress in CRC cells (Fig. 2B). These data indicated that miR-873-5p reversed EMT phenotype into EMT.

3.3. ZEB1 is a target of miR-873-5p

77 potential target mRNAs of miR-873-5p were predicted by using two bioinformatics prediction tools (targetScan and miRanda) (Fig. 3A). Next, we examined the expression level of all these 77 mRNAs in response to the overexpression or knockdown of miR-873-5p. As shown in Fig. 3B, 13 of 77 mRNAs were negatively regulated by miR-873-5p. Among these 13 mRNAs, Zinc finger E-box-binding homeobox 1 (ZEB1) is closely correlated with migration and epithelial-mesenchymal transition [36]. Numerous reports have validated that ZEB1 can act as a target mRNA of miRNAs to regulate cell migration and epithelial-mesenchymal transition in human malignant tumors [5,7,20,36]. Therefore, we continued to explore whether ZEB1 participated in miR-873-5p-mediated cell migration and EMT process in colorectal cancer. The putative binding sequence between miR-873-5p and ZEB1 was obtained by using the public bioinformatics tool targetScan (Fig. 3C). To make further confirmation, dual luciferase reporter assay was conducted in indicated CRC cells. MiR-873-5p overexpression significantly decreased the luciferase activity of wild type 3′ UTR of ZEB1 (ZEB1-WT) but not that of mutant type 3′ UTR of ZEB1 (ZEB1-MUT) (Fig. 3D). In SW-480 cell, inhibition of miR-873-5p expression efficiently enhanced the luciferase activity of wild type 3′ UTR of ZEB1-WT but not that of mutant type 3′ UTR of ZEB1-MUT (Fig. 3D). Furthermore, pull-down assay revealed that ZEB1 could be pulled down by Bio-miR-873-5p but not Bio-miR-873-5p-MUT or Bio-NC (Fig. 3E). Moreover, both mRNA level and protein level of ZEB1 were examined in response to miR-873-5p upregulation or downregulation. Both levels were found to be negatively regulated by miR-873-5p (Fig. 3F-G). All findings above reflected a fact that ZEB1 was a target of miR-873-5p in CRC.

3.4. ZEB1 reversed the miR-873-5p-mediated cell migration, invasion and EMT progress

Rescue assays were conducted to further validate the influences of ZEB1 on miR-873-5p-mediated cell migration, invasion and EMT process in CRC. As expected, overexpression of ZEB1 partially reversed the inhibitory effects of miR-873-5p on the migratory and invasive capacity

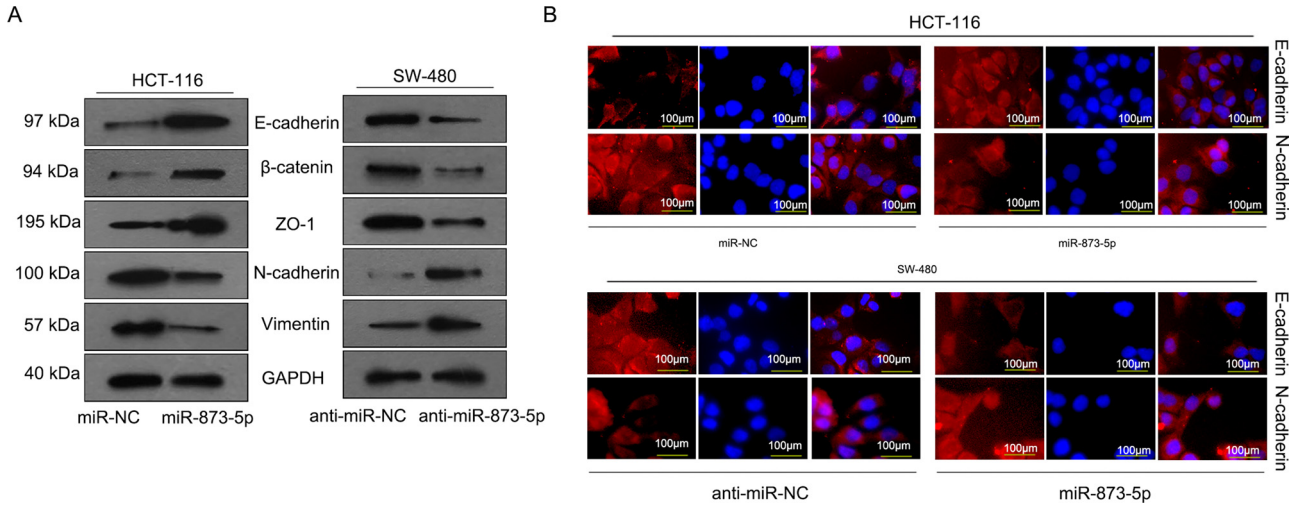

Fig. 2. MiR-873a-5p reversed epithelial-mesenchymal transition in CRC cells. A. The levels of EMT markers (E-cadherin, β-catenin, ZO-1, N-cadherin, Vimentin) in HCT-116 and SW-480 cells were examined by western blotting. B. The expression of EMT markers was analyzed with immunofluorescence experiment. Scale bar = 100 μm. $^{**}P < 0.01$ vs. control group.

of HCT-116 cells (Fig. 4A-B). In addition, overexpression of ZEB1 attenuated the reversal effect of miR-873-5p on the EMT progress of HCT-116 cell (Fig. 4C). Whereas, knockdown of ZEB1 reversed anti-miR-873-5p-mediated migration, invasion and EMT progress of SW-480 cells (Fig. 4D–F). In summary, miR-873-5p suppresses cell migration, invasion and EMT progress in CRC via targeting ZEB1.

4. Discussion

According to the report of the American Cancer Society in 2016, the new cases of CRC and new deaths caused by CRC is unceasingly increased, which making CRC become the third leading cause of cancer-related death [25]. Although many therapeutic methods for treating CRC have been developed, CRC remains a severe public health problem. The clinical behavior of CRC is formed due to interactions at multiple levels [22]. Among these interactions, the expression changes of anti-oncogenic genes and oncogenes are the major driving force for tumorigenesis and tumor development. ZEB1 is a famous oncogene which can promote migration and epithelial-mesenchymal transition in various malignant tumors [12,18,19,34], including colorectal cancer [38]. In this study, tried to detect the molecular mechanism by which ZEB1 was silenced, thereby the migration and EMT progress were inhibited.

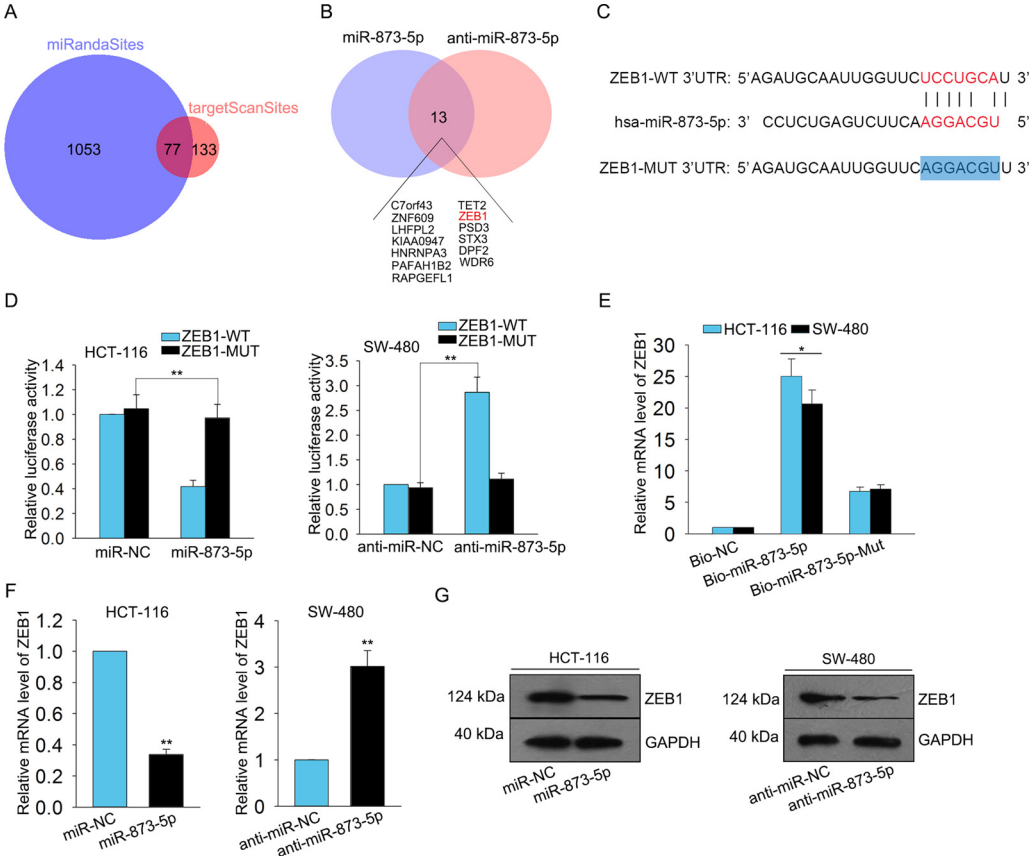

Fig. 3. ZEB1 is a target of miR-873-5p. A. 77 potential target mRNAs of miR-873-5p were predicted by using two bioinformatics tools (targetScan and miRanda). **B.** The expression level of all these 77 mRNAs in response to the overexpression or knockdown of miR-873-5p. **C.** The putative binding sequence between miR-873-5p and ZEB1 was obtained from public bioinformatics tool targetScan. **D.** Dual luciferase reporter assay was conducted in indicated HCT-116 and SW-480 cells to demonstrate the interaction between miR-873-5p and ZEB1. **E.** pull-down assay revealed that ZEB1 could be pulled down by Bio-miR-873-5p-wt but not Bio-miR-873-5p-MUT or Bio-NC. **F–G.** Both mRNA level and protein level of ZEB1 were examined in response to miR-873-5p upregulation or downregulation. Results were obtained by using qRT-PCR and western blotting. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group.

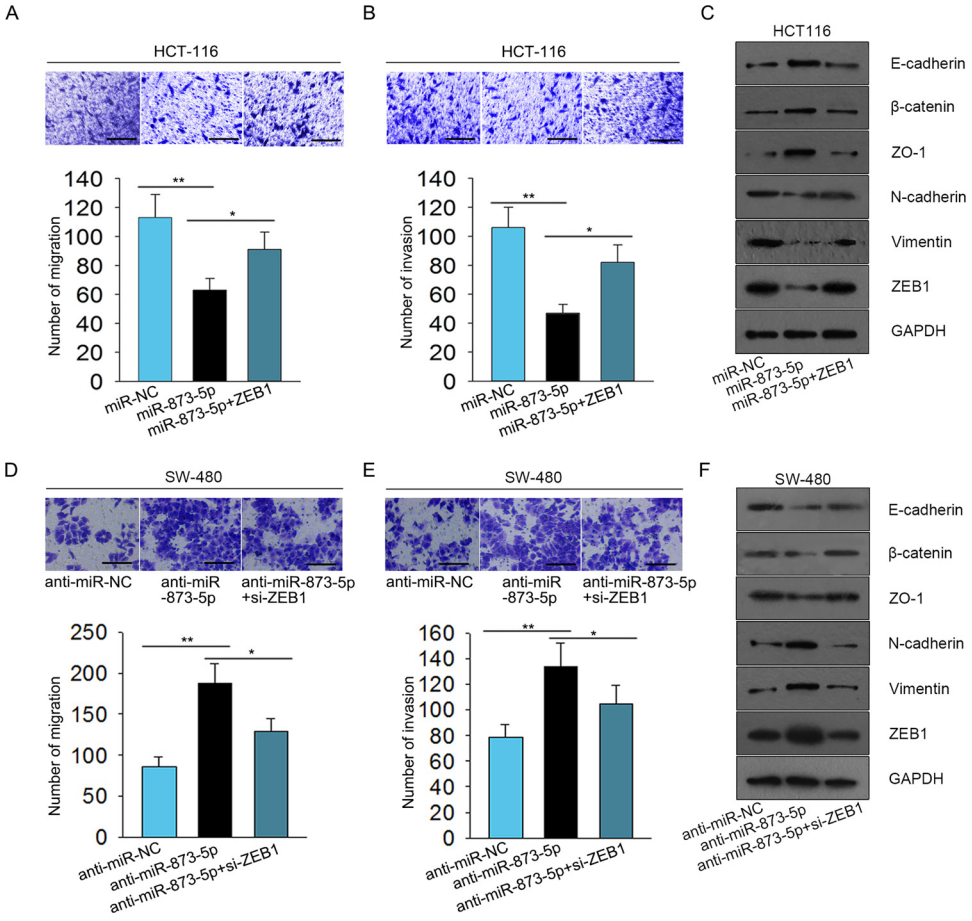

Fig. 4. ZEB1 reversed the miR-873-5p-mediated cell migration, invasion and EMT progress. A–B. Tranwell assay revealed the reversal effects of ZEB1 overexpression on miR-873-5p-mediated migratory and invasive capacity of HCT-116 cells. Images for migration and invasion were obtained under a ×200 microscope. **C.** The effect of ZEB1 on miR-873-5p-mediated EMT progress was detected with western blotting. **D–E.** The reversal effect of si-ZEB1 on anti-miR-873-5p-mediated cell invasion and migration was identified with transwell assay. Images for migration and invasion were obtained under a ×200 microscope. **F.** The expression of EMT markers was determined by western blot in SW-480 cell which was transfected with anti-miR-873-5p and si-ZEB1. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group.

miRNA profiles are generally altered during the initiation and development of various types of malignant tumors, including CRC [6,23]. More importantly, more and more studies have revealed that miRNAs can be taken as therapeutic molecules against cancer [15,16,21,35]. MiR-873-5p has been reported in gastric cancer as a tumor suppressor. In this study, we explored the biological effects of miR-873-5p on migration, invasion and EMT phenotype in CRC cells. At first, the expression pattern of miR-873-5p was tested in CRC cells. Compared with that in HIEC, the expression of miR-873-5p was obviously decreased in CRC cell lines. The highest level of miR-873-5p was observed in SW-480 cell, while the lowest level of miR-873-5p was observed in HCT-116 cell. Accordingly, we conducted gain-of function in HCT-116 cells but performed loss-of function assays in SW-480 cells. To determine the influences of miR-873-5p on cell migration and invasion, transwell assay was carried out in two CRC cells. Upregulated miR-873-5p was found to be able to inhibited migration and invasion of HCT-116 cells, whereas downregulated miR-873-5p enhanced migration and invasion of SW-480 cells. According to the result of western blotting, the expression of EMT markers were greatly affected by miR-873-5p. Upregulated miR-873-5p reversed the phenotype of EMT to MET, while downregulated miR-873-5p promoted the formation of EMT phenotype. Furthermore, the results of immunofluorescence experiments further identified the effects of miR-873-5p on EMT progress in CRC cells. All these findings suggested that miR-873-5p suppressed cell migration, invasion and EMT progress in CRC. Considering ZEB1 is closely correlated with migration and EMT progress in human cancers, we hypothesized miR-873-5p suppressed migration and EMT progress in CRC via targeting ZEB1. The putative binding sites between miR-873-5p and ZEB1 were obtained by using public bioinformatics prediction tools targetScan. Dual luciferase reporter assay and pull down assay further validated the interaction between miR-873-5p and ZEB1. Moreover, ZEB1 was negatively modulated by miR-873-5p in CRC cells. Finally, rescue assays demonstrated that ZEB1 could reverse the effects of miR-873-5p on migration, invasion and EMT progress of CRC cells. All findings in this study may help to find novel therapeutic target for CRC.

Conflict of interest

None.

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