OncoTargets and Therapy

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ORIGINAL RESEARCH

miRNA-641 inhibits the proliferation, migration, and invasion and induces apoptosis of cervical cancer cells by directly targeting *ZEB1*

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Background: miRNAs have been found to be dysregulated in cervical cancer. The dysregulation of miRNA has been implicated in cervical carcinogenesis and progression. Therefore, further studies of the specific roles of deregulated miRNAs in cervical cancer and underlying molecular mechanisms may facilitate the identification of novel therapeutic techniques for patients with this disease. miRNA-641 (miR-641) was previously reported to serve an important role in lung cancer. However, the expression pattern and roles of miR-641 in cervical cancer remain unclear.

Method: In this study, the expression level of miR-641 in cervical cancer tissues and cell lines was detected using RT-qPCR. The influence of miR-641 upregulation in cervical cancer cell proliferation, apoptosis, migration and invasion was evaluated using CCK-8 assay, flow cytometry assay, migration and invasion assays, respectively. In vivo tumor growth assay was utilized to determine the effect of miR-641 overexpression in the tumor growth of cervical cancer cells in vivo. The molecular mechanisms underlying the action of miR-641 in cervical cancer cells were also explored.

Results: We found that miR-641 expression was obviously decreased in cervical cancer tissues and cell lines, which was strongly correlated with the International Federation of Gynecology and Obstetrics stage and lymph node metastasis. Upregulation of miR-641 inhibited cell proliferation, induced apoptosis, and reduced metastasis in cervical cancer. Additionally, bioinformatics analysis predicted *ZEB1* as a novel target gene of miR-641. Notably, luciferase reporter assay, RT-qPCR, and Western blot analysis revealed that miR-641 decreased *ZEB1* expression in cervical cancer cells by directly targeting its 3′-untranslated region. Furthermore, *ZEB1* was upregulated in cervical cancer tissues, which was negatively correlated with miR-641 expression. Moreover, recovered ZEB1 expression attenuated the tumor suppressive action of miR-641 overexpression in the malignant phenotypes of cervical cancer cells. Besides, miR-641 could hinder cervical cancer tumor growth in vivo by inhibiting *ZEB1*.

Conclusion: These results indicate that miR-641 has tumor suppressive roles in the development of cervical cancer by directly targeting *ZEB1*, suggesting that miR-641 is a novel, effective therapeutic target for treating patients with this disease.

Keywords: microRNA-641, target therapy, zinc finger E-Box binding homeobox 1, aggressive behaviors

Introduction

Cervical cancer, one of the most common gynecological malignancies, is the fourth leading cause of cancer-related death worldwide.[1] In the People's Republic of China, the morbidity and mortality rates of cervical cancer in recent years have gradually increased each year.[2] Approximately 98,900 novel cervical cancer cases were diagnosed and 30,500 deaths were caused by cervical cancer in 2015.[3] Infection with high-risk human papillomavirus plays a crucial role in the pathogenesis of cervical cancer;[4] however,

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the detailed molecular mechanisms remain unclear. Despite remarkable improvements in diagnosis and therapy, the prognosis of patients with cervical cancer remains unsatisfactory, with a 5-year survival rate of less than 40%.[5,6] Recurrence and metastasis are the main factors affecting the poor therapeutic outcomes of patients with cervical cancer.[7] Therefore, studies are needed to determine the mechanism responsible for cervical carcinogenesis and progression, which may be particularly useful for identifying attractive therapeutic targets for treating patients with this malignancy.

miRNAs are a subset of conserved, single-strand, noncoding, and short RNA molecules 18–24 nucleotides in length.[8] miRNAs have been demonstrated to negatively regulate gene expression by directly interacting with the 3′-untranslated regions (3′-UTRs) of their target genes to cause mRNA degradation and/or translational suppression.[9] More than 2,500 miRNAs have been validated in the human genome.[10] Recently, miRNAs were found to be dysregulated in most human cancer types, such as lung cancer,[11] colorectal cancer,[12] breast cancer,[13] and bladder cancer.[14] Particularly, a variety of miRNAs are aberrantly expressed in cervical cancer.[15] Aberrant expression of miRNAs is important in the malignant development of cervical cancer by dysregulation of multiple cellular biological processes, including cell proliferation, cell cyc apoptosis, metastasis, motility, and angiogenesis.[16,17] miRNA may play oncogenic or tumor suppressive roles in th genesis and progression of cervical cancer, which are m inly at but able to the biological roles of their target ge s.[18] Th fore miRNAs may be effective diagnostic a ther ic targets for treating patients with cervical ca r.

miRNA-641 (miR-641) was found t play an important role in lung cancer.[19,20] How er, the expre on pattern and roles of miR-641 in cer al canc r remain unclear. In this study, we detected mi 641 e pression in cervical cancer, examined its role i malig phenot pes, and investigated its underlying echan ms. Ou ts indicate that miR-641 can be dev ped as lecular target to treat patients with cervical cance

Material and methods

Clinical specimens and cell lines

Paired cervical cancer and adjacent non-tumor tissues were collected from 51 patients with cervical cancer who had undergone surgical resection at Wenzhou Hospital of Integrated Traditional Chinese and Western Medicine between June 2015 and April 2017. No patients had been treated with preoperative radiotherapy or chemotherapy. Fresh tissues were quickly snap-frozen in liquid nitrogen after surgical removal and then stored at −80°C until use. This study was granted approval by Ethics Committee of Wenzhou Hospital of Integrated Traditional Chinese and Western Medicine, and was performed in accordance with the Declaration of Helsinki and the guidelines of the Ethics Committee of Wenzhou Hospital of Integrated Traditional Chinese and Western Medicine. Written informed consent was provided by all individuals enrolled in this study.

A normal human cervix epithelial cell line (Ect1/E6E7) and four cervical cancer cell lines (C-33A, HeLa, SiHa, and CaSki) were purchased from American Type Culture Collection (Manassas, VA, USA). DME ontaining 10% FBS, 100 U/mL penicillin, and 10 µg/mL eptomycin (all from Invitrogen, Thermo Fishe ientific, Wa ham, MA, USA) was used for cell cultu . Cultu were m intained in a humidified incubator at 7°C su plied th % CO_2.

Oligonucleotide an plasmid transfection

miR-641 mi and negati ntrol miRNA mimics (miR-NC) were c nically synthesized by Shanghai GenePh Co., Ltd. Shanghai, People's Republic of Chi a). siRNA against the expression of *ZEB1* (*ZEB1* siR A) and neg ive control siRNA (NC siRNA) were obtai d from G angzhou RiboBio Co., Ltd. (Guangzhou, People's R blic of China). The *ZEB1* siRNA sequence was 5′- GCCAAUAAGCAAACGA-3′ and the NC siRNA equence was 5′-UUCUCCGAACGUGUCACGUTT-3′. The *ZEB1* overexpression vector pcDNA3.1-ZEB1 (pc-ZEB1) and pcDNA3.1 blank vector were produced by the Chinese Academy of Sciences (Changchun, People's Republic of China). For cell transfection, the cells were inoculated into 6-well plates at an initial density of 6×10^5 cells/well. Lipofectamine 2000 reagent (Invitrogen, Thermo Fisher Scientific) was used for all transfections in accordance with the manufacturer's instructions.

RT-qPCR

RT-qPCR was conducted to detect miR-641 and ZEB1 mRNA levels. Total RNA was extracted from tissue specimens or cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocol. To evaluate miR-641 expression, cDNA was produced from total RNA using a miScript Reverse Transcription kit (Qiagen NV, Venlo, the Netherlands). Subsequently, quantitative PCR was conducted using a miScript SYBR-Green PCR kit (Qiagen NV). To measure ZEB1 mRNA expression, total RNA was reverse-transcribed into cDNA using a PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Shiga, Japan). Next, SYBR Premix Ex Taq™ (Takara Biotechnology Co., Ltd.) was used to perform qPCR according to the manufacturer's instructions. Relative miR-641

and *ZEB1* mRNA expression were normalized to that of U6 snRNA and GAPDH, respectively. The primers were designed as follows: miR-641, 5′-TTATACTCTCACCATTTGGATC-3′ (forward) and 5′-TGACAAGATTTTACATCAAGAA-3′ (reverse); U6, 5′-CTTCGGCAGCACATATACT-3′ (forward) and 5′-AAAATATGGAACGCTTCACG-3′ (reverse); ZEB1, 5′-TTGTAGCGACTGGATTTT-3′ (forward) and 5′-AGACGATAGTTGGGTCCCGGC-3′ (reverse); and GAPDH, 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ (forward) and 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′ (reverse). Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method.[21]

Cell counting kit-8 (CCK-8) assay

Transfected cells were collected after 24 hours of incubation at 37°C with 5% CO_2. Cells were resuspended and plated into 96-well plates at a density of 3×10^3 cells/well. Cellular proliferation was assessed by conducting a CCK-8 assay (Dojindo, Kumamoto, Japan) at four time points: 0, 1, 2, and 3 days after incubation. A total of 10 µL CCK-8 reagent was added to each well and incubated at 37°C with 5% CO_2 for another 2 hours. Finally, the optical density of each well was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, U[illegible]

Flow cytometry assay

Transfected cells were harvested at 48 hou[illegible] post-tr[illegible]sfecti[illegible] and washed twice with ice-cold P[illegible] An[illegible]xin V fluorescein isothiocyanate (FITC) apopto[illegible] detection kit (Biolegend, San Diego, CA, U[illegible]) was utiliz[illegible] to evaluate apoptosic rate. Briefly, tran[illegible]fected [illegible]lls were stained in the dark with 5 µL of Anne[illegible]n V FITC an[illegible] 5 µL of propidium iodide diluted in [illegible]0 µL of binding buffer. Following incubation at roo[illegible] temp[illegible]ature for 20 minutes, the percentage of ap[illegible]totic c[illegible]s was d[illegible]ected by flow cytometry (FACSca[illegible] BD B[illegible]scienc[illegible] San Jose, CA, USA).

Migrati[illegible] and invasion assays

For migration[illegible]ssay, cells were collected after 48 hours of transfection, suspended in FBS-free DMEM, and inoculated into the upper compartment of Transwell chambers (24-well insert; pore size, 8 µm; Corning Incorporated, Corning, NY, USA). The lower compartments were covered with 500 µL DMEM containing 20% FBS to serve as the chemoattractant. Following incubation for 24 hours, non-invading cells were carefully removed with a cotton swab, whereas invasive cells were fixed with 95% methanol and stained with 0.5% crystal violet. Stained cells were observed and counted under an inverted microscope (200× magnification; IX83; Olympus Corporation, Tokyo, Japan) in five randomly chosen fields in each chamber. The experimental procedure of the invasion assay was similar to that of the migration assay except that the Transwell chambers were precoated with Matrigel (BD Biosciences) before examination.

In vivo tumor growth assay

All procedures involving animals were approved by the Experimental Animal Ethics Committee of Wenzhou Hospital of Integrated Traditional Chinese and Western Medicine, and were carried out in accordanc[illegible]h the Declaration of Helsinki and the guidelines of th[illegible] Experim[illegible]tal Animal Ethics Committee of Wenzhou Ho[illegible]ital of Integr[illegible]ed Traditional Chinese and Western M[illegible]icine. I[illegible]total, $2\times$[illegible]^6 miR-NC- or miR-641 mimic-tr[illegible]sfected cells [illegible] subcutaneously seeded into the fla[illegible] of [illegible]de mice (Shanghai Laboratory Animal Cent[illegible], Chine[illegible] Acaden[illegible] of Sciences, Shanghai, People's R[illegible]blic of Chin[illegible] The mice were checked daily, and the tumor v[illegible]me was measured for 4 days using the fol[illegible]g formula: [illegible]mor volume =1/2 × tumor length × [illegible]mor width.[2] Four weeks later, all nude mice were sacrificed [illegible]nder deep a[illegible]esthesia. The formed tumor xenografts were e[illegible]ised and [illegible]eighed.

[illegible]ciferase reporter assay

The 3′-UTR fragments of *ZEB1* containing putative wild-type (wt) and mutant (mut) miR-641-binding sites were constructed by Shanghai GenePharma Co., Ltd., inserted into the pMIR-REPORT miRNA Expression Reporter vector (Ambion; Thermo Fisher Scientific), and named pMIR-ZEB1-3′-UTR wt and pMIR-ZEB1-3′-UTR mut, respectively. Cells were plated into 24-well plates 1 night before transfection. miR-641 mimics or miR-NC were co-transfected with pMIR-ZEB1-3′-UTR wt or pMIR-ZEB1-3′-UTR mut into cells using Lipofectamine 2000 reagent according to the manufacturer's recommendations. Transfected cells were collected after 48 hours of incubation at 37°C and then luciferase activity was measured using a dual-luciferase reporter assay system (Promega Corporation, Fitchburg, WI, USA) in accordance with the manufacturer's instructions. Firefly luciferase activity was normalized to that of Renilla luciferase.

Western blot analysis

Total protein was extracted from cultured cells or tissue samples using RIPA Lysis Buffer (Beyotime Institute of Biotechnology, Haimen, People's Republic of China) in the presence of a protease inhibitor cocktail (Pierce Biotechnology, Inc., Rockford, IL, USA). A bicinchoninic acid protein assay (Aidlab Biotechnologies Co., Ltd., Beijing, People's Republic

of China) was conducted to determine the concentration of total protein. Equal amounts of protein were separated by 10% SDS-PAGE, transferred to polyvinylidene fluoride membranes (Sigma-Aldrich Co., St Louis MO, USA), and then blocked at room temperature for 1 hour in 5% fat-free milk diluted in Tris-buffered saline containing 0.1% Tween-20 (TBST). The membranes were incubated overnight at 4°C with the following primary antibodies: rabbit anti-human ZEB1 primary antibody (1:1,000 dilution; cat no: ab203829) or rabbit anti-human GAPDH primary antibody (1:1,000 dilution; cat no: ab181603; both from Abcam, Cambridge, MA, USA). After washing three times with TBST, horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:5,000 dilution; cat no: ab205718; Abcam) were incubated with the membranes at room temperature for 2 hours. Finally, an ECL Protein Detection kit (Pierce Biotechnology, Inc.) was applied to develop the protein bands.

Statistical analysis

Data were expressed as mean ± SD from at least three independent experiments. The chi-squared test was used to assess the correlation among miR-641 expression and clinicopathological indices of cervical cancer patients. Student's *t*-test was utilized to evaluate the differences between two groups, while the significance of multiple groups was determined by one-way ANOVA followed by a Tukey's post hoc test. SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA) was applied for all statistical analysis. The association between miR-641 and ZEB1 mRNA levels was examined by Spearman's correlation analysis. A *P*-value less than 0.05 was considered to indicate statistical significance.

Results

miR-641 expression is decreased in cervical cancer tissues and cell lines

To determine the expression status of miR-641 in cervical cancer, RT-qPCR was conducted to detect miR-641 expression in 51 pairs of cervical cancer and adjacent non-tumor tissues. Compared to that in adjacent non-tumor tissues, miR-641 showed low expression in cervical cancer tissues (Figure 1A, $P<0.05$). Additionally, the expression level of miR-641 in four cervical cancer cell lines (C-33A, HeLa, SiHa, and CaSki) and a normal human cervix epithelial cell line (Ect1/E6E7) was determined by RT-qPCR. miR-641 was downregulated in all four cervical cancer cell lines relative to that in Ect1/E6E7 (Figure 1B, $P<0.05$). These results suggest that downregulation of miR-641 may be closely related with the malignant progression of cervical cancer.

Association of miR-641 expression with clinicopathological factors in cervical cancer patients

To determine the clinical significance of miR-641 in cervical cancer, all patients with cervical cancer enrolled in this study were divided into two groups: miR-641 low expression group and miR-641 high expression group. The median value of miR-641 was used as a cut-off point. Statistical analysis revealed that decreased miR-641 expression was clearly correlated with the Federation of Gynecology and Obstetrics (FIGO) stage ($P=0.016$) and lymph node metastasis ($P=0.036$), while no significant correlation with any other clinicopathological indices was observed (Table 1, all $P>0.05$).

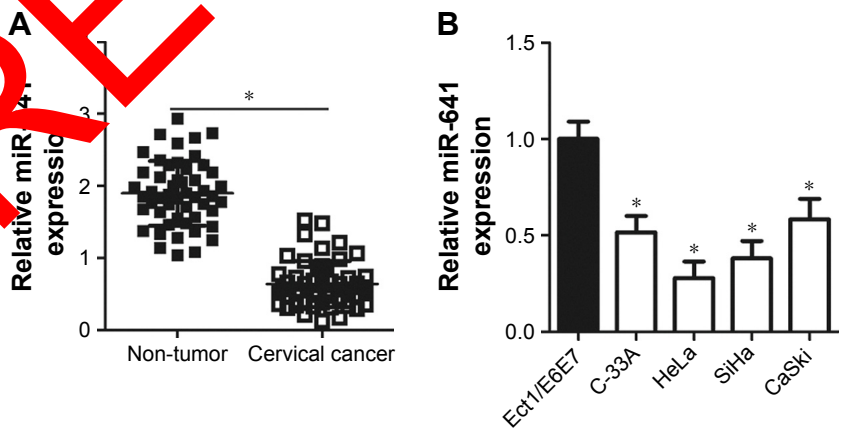

Figure 1 Expression status of miR-641 in cervical cancer tissues and cell lines.
Notes: (**A**) RT-qPCR analysis was employed to determine miR-641 expression in 51 pairs of cervical cancer and adjacent non-tumor tissues. *$P<0.05$ vs non-tumor tissues. (**B**) The expression level of miR-641 in four cervical cancer cell lines (C-33A, HeLa, SiHa, and CaSki) and a normal human cervix epithelial cell line (Ect1/E6E7) was detected by using RT-qPCR. *$P<0.05$ vs Ect1/E6E7.
Abbreviation: miR-641, miRNA-641.

Table 1 The association between miR-641 and clinicopathological indices in cervical cancer patients

Indices	miR-641 expression		*P*-value
	Low	High	
Age (years)			0.242
<60	9	5	
≥60	17	20	
Tumor size (cm)			0.488
<4	15	12	
≥4	11	13	
Family history of cancer			0.343
Yes	7	4	
No	19	21	
FIGO stage			0.016
I–II	6	14	
III–IV	20	11	
Lymph node metastasis			0.036
No	8	15	
Yes	18	10	

Abbreviations: FIGO, Federation of Gynecology and Obstetrics; miR-641, miRNA-641.

miR-641 inhibits cervical cancer cell proliferation, migration, and invasion, and promotes cell apoptosis in vitro

HeLa and SiHa cell lines showed relatively lower miR-641 expression among the four cervical cancer cell lines, and therefore the two cell lines were used in subsequent functional experiments. To explore the biological function of miR-641 in cervical cancer, HeLa and SiHa cells were transfected with miR-641 mimics to increase endogenous miR-641 expression (Figure 2A, $P<0.05$). Subsequently, the regulatory influence of miR-641 on the proliferation of cervical cancer cells was examined. HeLa and SiHa cells transfected with miR-641 mimics exhibited obvious growth suppression compared to cells transfected with miR-NC (Figure 2B, $P<0.05$). The role of miR-641 in regulating cervical cancer cell apoptosis was then investigated. Flow cytometry analysis revealed that miR-641 upregulation significantly promoted the apoptosis of HeLa and SiHa cells (Figure 2C, $P<0.05$). Furthermore, migration and invasion assays were performed to determine the effect of miR-641 on the metastasis of cervical cancer cells. Ectopic miR-641 expression attenuated the migratory (Figure 2D, $P<0.05$) and invasive (Figure 2E, $P<0.05$) abilities of HeLa and SiHa cells. These results suggest that miR-641 plays a tumor suppressive role in the growth and metastasis of cervical cancer cells.

ZEB1 is a direct target gene of miR-641 in cervical cancer cells

To clarify the molecular mechanism responsible for the tumor suppressor activity of miR-641 in cervical cancer, bioinformatics analysis was carried out to search for the putative target of miR-641. Two highly conserved putative binding sites were observed in the 3′-UTR of *ZEB1* (Figure 3A). *ZEB1* was selected for further verification because it is a well-known oncogene and has been implicated in the initiation and progression of cervical cancer.[22–27] A luciferase reporter assay was then conducted to determine whether miR-641 directly binds to the 3′-UTR of *ZEB1* in cervical cancer cells. As shown in Figure 3B, miR-641 overexpression noticeably reduced the luciferase activity of the plasmid harboring the wt (1 and 2) miR-641 binding site ($P<0.05$) and failed to affect that of the plasmid carrying mutated (1 and 2) *ZEB1* 3′-UTR in HeLa and SiHa cells (Figure 3B). Additionally, RT-qPCR and Western blot analysis revealed that restoration of miR-641 expression suppressed the mRNA (Figure 3C, $P<0.05$) and protein (Figure 3D, $P<0.05$) expression of *ZEB1* in HeLa and SiHa cells. Collectively, these results demonstrate that *ZEB1* is a direct target of miR-641 in cervical cancer cells.

Upregulation of ZEB1 is negatively correlated with miR-641 expression in cervical cancer tissues

To further evaluate the relationship between miR-641 and *ZEB1* in cervical cancer, the expression of *ZEB1* in 60 pairs of cervical cancer and adjacent non-tumor tissues was determined by RT-qPCR. The expression level of *ZEB1* mRNA was higher in cervical cancer tissues than in adjacent non-tumor tissues (Figure 4A, $P<0.05$). Additionally, Western blot analysis revealed that ZEB1 protein level was upregulated in cervical cancer tissues compared to that in adjacent non-tumor tissues (Figure 4B, $P<0.05$). Furthermore, an inverse correlation was found between miR-641 and *ZEB1* mRNA levels in cervical cancer tissues (Figure 4C; $r=-0.5231$, $P<0.0001$). These results suggest that upregulation of ZEB1 expression in cervical cancer tissues is, at least partly, caused by low miR-641 expression.

Knockdown of *ZEB1* simulates the tumor suppressing effects of miR-641 overexpression in cervical cancer cells

Because *ZEB1* was identified as a direct target gene of miR-641 in cervical cancer, we then investigated the functional roles of *ZEB1* in cervical cancer.

HeLa and SiHa cells were transfected with *ZEB1* siRNA to knock down endogenous ZEB1 levels. *ZEB1* siRNA efficiently decreased *ZEB1* protein expression in HeLa and SiHa cells compared to cells transfected with NC siRNA (Figure 5A, $P<0.05$). The CCK-8 assay revealed that downregulation of ZEB1 led to a decreased

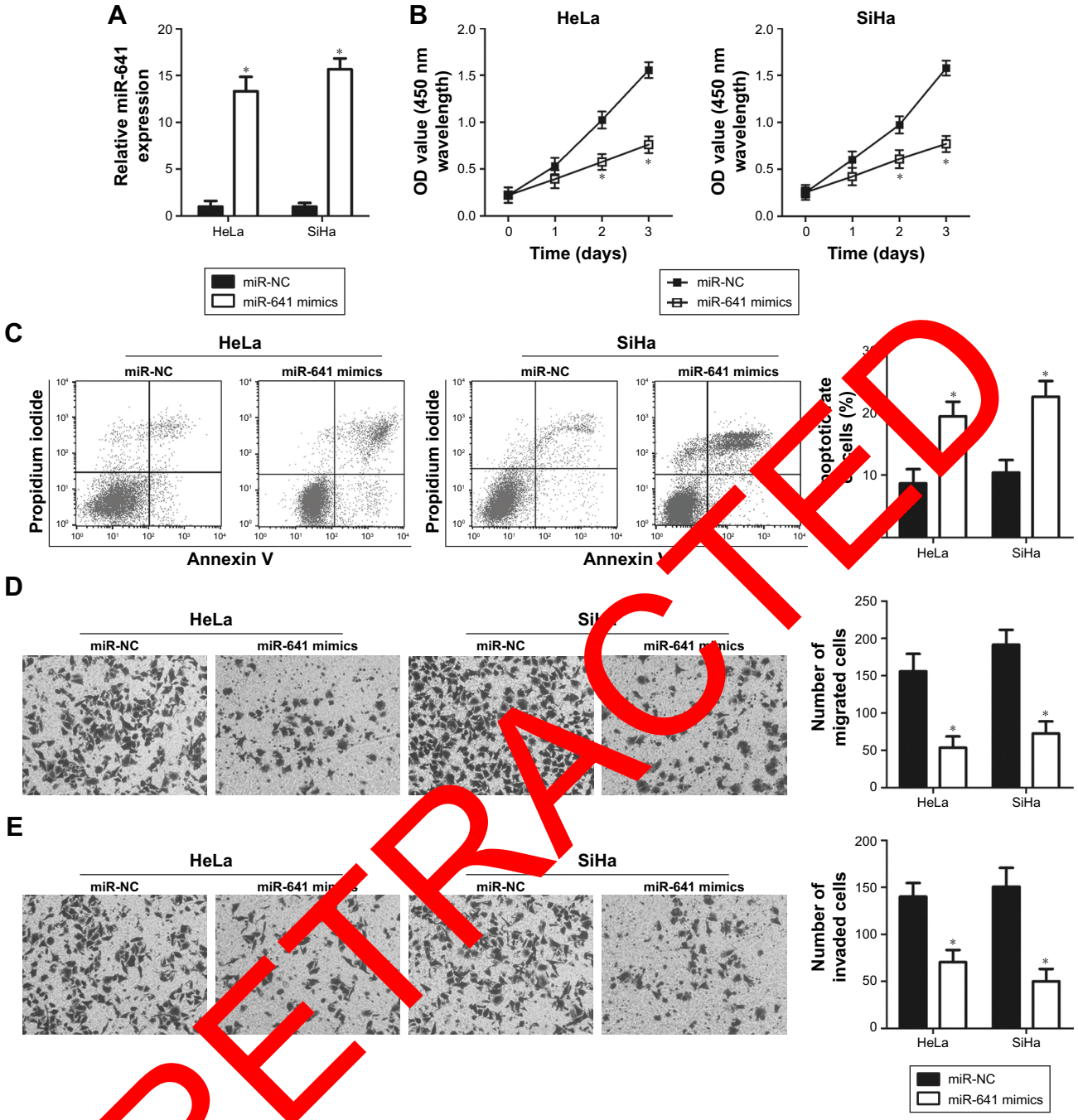

Figure 2 miR-641 regulates the proliferation, apoptosis, migration, and invasion of HeLa and SiHa cells.

Notes: miR-641 mimics or miR-NC was introduced into HeLa and SiHa cells, and the transfected cells were used in the functional analyses. (**A**) RT-qPCR analysis was performed to examine the expression level of miR-641 in previously mentioned cells. miR-641 was notably upregulated in HeLa and SiHa cells after transfection with miR-641 mimics. *$P<0.05$ vs miR-NC. (**B**, **C**) CCK-8 and flow cytometry assays were used to analyze the proliferation and apoptosis of HeLa and SiHa cells following transfection with miR-641 mimics or miR-NC. Upregulation of miR-641 significantly inhibited the proliferation and induced the apoptosis of HeLa and SiHa cells. *$P<0.05$ vs miR-NC. (**D**, **E**) The effects of miR-641 overexpression on the migration and invasion of HeLa and SiHa cells were evaluated using migration and invasion assays. miR-641 overexpression decreased the migratory and invasive abilities of HeLa and SiHa cells. *$P<0.05$ vs miR-NC.

Abbreviations: CCK-8, Cell counting kit-8; miR-641, miRNA-641; miR-NC, negative control miRNA mimics.

proliferative ability of HeLa and SiHa cells (Figure 5B, $P<0.05$). Flow cytometry analysis indicated that inhibition of *ZEB1* increased the apoptosis rate of HeLa and SiHa cells compared to the NC siRNA groups (Figure 5C, $P<0.05$). Furthermore, the number of migrated (Figure 5D, $P<0.05$) and invaded (Figure 5E, $P<0.05$) cells was significantly reduced in *ZEB1* siRNA transfectants compared to that in NC siRNA-transfected HeLa and SiHa cells. These results demonstrate that the effects of ZEB1 knockdown on cervical cancer cells were similar to that of miR-641 overexpression, further suggesting that *ZEB1* is a functional downstream target of miR-641 in cervical cancer cells.

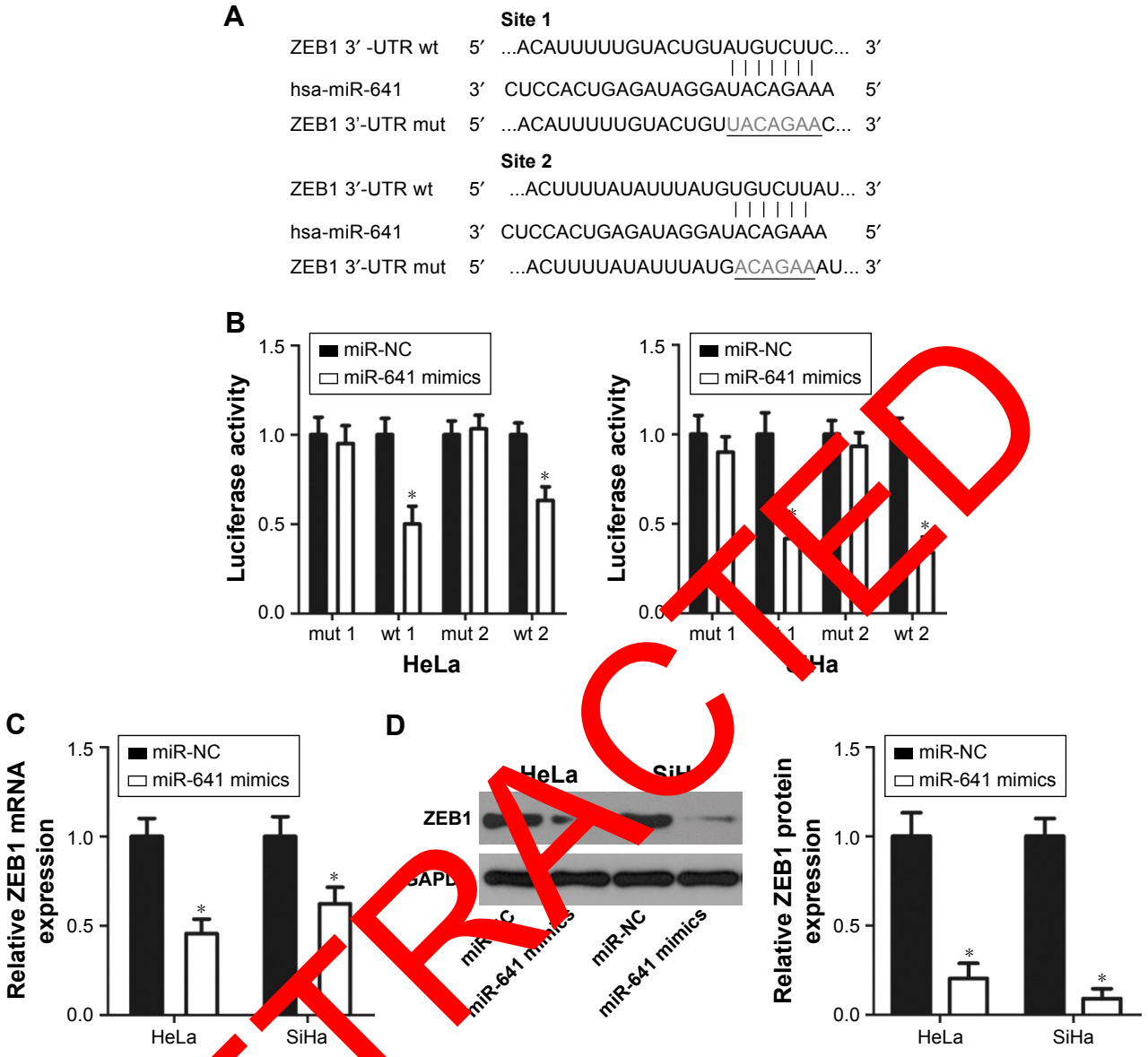

Figure 3 *ZEB1* gene is a direct target of miR-641 in cervical cancer cells.

Notes: (**A**) Predicted wild-type (wt) and mutant (mut) binding sites of miR-641 in the 3′-UTR of *ZEB1*. (**B**) Luciferase activity was determined in HeLa and SiHa cells that were co-transfected with miR-641 mimics or miR-NC and pMIR-ZEB1-3′-UTR (wt 1 and 2) or pMIR-ZEB1-3′-UTR (mut 1 and 2). Firefly luciferase activity was normalized to that of Renilla luciferase activity. $^{*}P<0.05$ vs miR-NC. (**C**, **D**) The mRNA and protein expression of *ZEB1* in HeLa and SiHa cells after transfection with miR-641 mimics or miR-NC was detected by RT-qPCR and Western blot analysis, respectively. $^{*}P<0.05$ vs miR-NC.

Abbreviations: 3′-UTR, 3′-untranslated region; miR-641, miRNA-641; miR-NC, negative control miRNA mimics.

Restored *ZEB1* expression attenuates the suppressive effects of miR-641 overexpression in malignant phenotypes of cervical cancer cells

A series of rescue experiments were performed to confirm that *ZEB1* mediates the tumor suppressive action of miR-641 in cervical cancer cells. Thus, miR-641-overexpressing HeLa and SiHa cells were transfected with *ZEB1* overexpression plasmid (pc-ZEB1) or pcDNA3.1 blank vector. After transfection, miR-641 overexpression significantly reduced the protein level of *ZEB1* in HeLa and SiHa cells; however, the *ZEB1* protein expression was recovered by co-transfection with pc-ZEB1 (Figure 6A, $P<0.05$). Functional analyses revealed that restoration of ZEB1 expression rescued the effects of miR-641 overexpression on the proliferation (Figure 6B, $P<0.05$), apoptosis (Figure 6C, $P<0.05$), migration (Figure 6D, $P<0.05$), and invasion (Figure 6E, $P<0.05$) of HeLa and SiHa cells. These data confirm that the tumor suppressor activity of miR-641 in the malignant phenotypes of cervical cancer cells is, at least in part, attributable to downregulation of *ZEB1*.

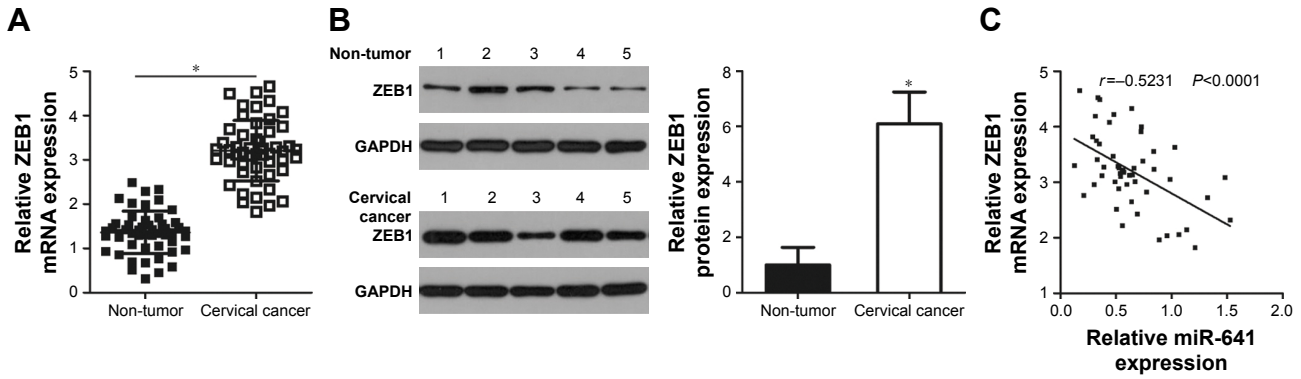

Figure 4 *ZEB1* is upregulated in cervical cancer tissues, and inversely correlated with miR-641 level.
Notes: (**A**) The mRNA level of *ZEB1* in 51 pairs of cervical cancer and adjacent non-tumor tissues was determined using RT-qPCR. **P*<0.05 vs non-tumor tissues. (**B**) Western blot analysis was carried out to measure the protein expression of *ZEB1* in several pairs of cervical cancer and adjacent non-tumor tissues. **P*<0.05 vs non-tumor tissues. (**C**) Spearman's correlation analysis was applied to explore the relationship between miR-641 and *ZEB1* mRNA levels in cervical cancer tissues. *r*=–0.5231, *P*<0.0001.
Abbreviation: miR-641, miRNA-641.

miR-641 inhibits cervical cancer tumor growth in vivo

To determine the effect of miR-641 on tumor growth in vivo, in vivo tumor growth assay was carried out by subcutaneous implantation of miR-641 mimics- or miR-NC-transfected HeLa cells in nude mice. The miR-641-overexpressing tumor xenografts had an obvious reduction of tumor volume relative to that in miR-NC groups (Figure 7A and B, $P<0.05$). On day 29, all nude mice were sacrificed, and the tumor xenografts were excised and weighed. It was observed that the weight of tumor xenografts in miR-641 group was significantly lower than that of miR-NC group (Figure 7C, $P<0.05$). Additionally, RT-qPCR analysis revealed the expression level of miR-641 in tumor xenografts from miR-641 mimic-transfected HeLa cells was markedly overexpressed (Figure 7D, $P<0.05$). Furthermore, Western blot analysis was applied to detect *ZEB1* protein expression, and confirmed that *ZEB1* protein expression was noticeably reduced in the tumor xenografts derived from the HeLa cells transfected with miR-641 mimics (Figure 7E). Together, these results suggest that miR-641 can hinder cervical cancer tumor growth in vivo by inhibiting *ZEB1*.

Discussion

miRNAs were found to be dysregulated in cervical cancer, and their dysregulation has been implicated in cervical carcinogenesis and progression by regulating major tumor-related biological behaviors.[28,29] Notably, exploring the underlying mechanisms of cervical cancer formation and progression may be helpful for early diagnosis and effective treatment.[30] Therefore, further studies on the specific roles of deregulated miRNAs in cervical cancer and underlying molecular mechanisms may facilitate the identification of novel therapeutic techniques for patients with this disease. In this study, we detected miR-641 expression in cervical cancer tissues and determined its clinical significance in this disease. Additionally, the functional roles and underlying mechanisms of miR-641 in cervical cancer were investigated. Our results indicate that miR-641 can be used as a diagnostic biomarker and therapeutic agent in patients with cervical cancer.

miR-641 has been widely studied in lung cancer. For example, miR-641 expression is decreased in lung cancer tissues and cell lines. Ectopic miR-641 expression suppressed the proliferation and induced the apoptosis of lung cancer cells by directly targeting *MDM2*.[19] Additionally, the expression level of miR-641 was increased in the serum of patients with non-small-cell lung cancer showing acquired resistance to erlotinib treatment. miR-641 induces erlotinib resistance by directly targeting *NF1* and regulating *ERK* signaling in non-small-cell lung cancer.[20] However, the expression status and specific roles of miR-641 in cervical cancer remain largely unknown. Here, we found that miR-641 was downregulated in cervical cancer, and the decreased miR-641 expression was significantly correlated with FIGO stage and lymph node metastasis. Functional analyses demonstrated that miR-641 overexpression restricted cervical cancer cell proliferation, promoted apoptosis, and attenuated migration and invasion in vitro as well as hindered tumor growth in vivo. Hence, this miRNA may be developed as a diagnostic biomarker and valuable therapeutic agent for patients with lung and cervical cancers.

Validation of the direct targets of miR-641 is important for understanding its roles in the progression of cervical cancer and may be helpful for identifying promising therapeutic

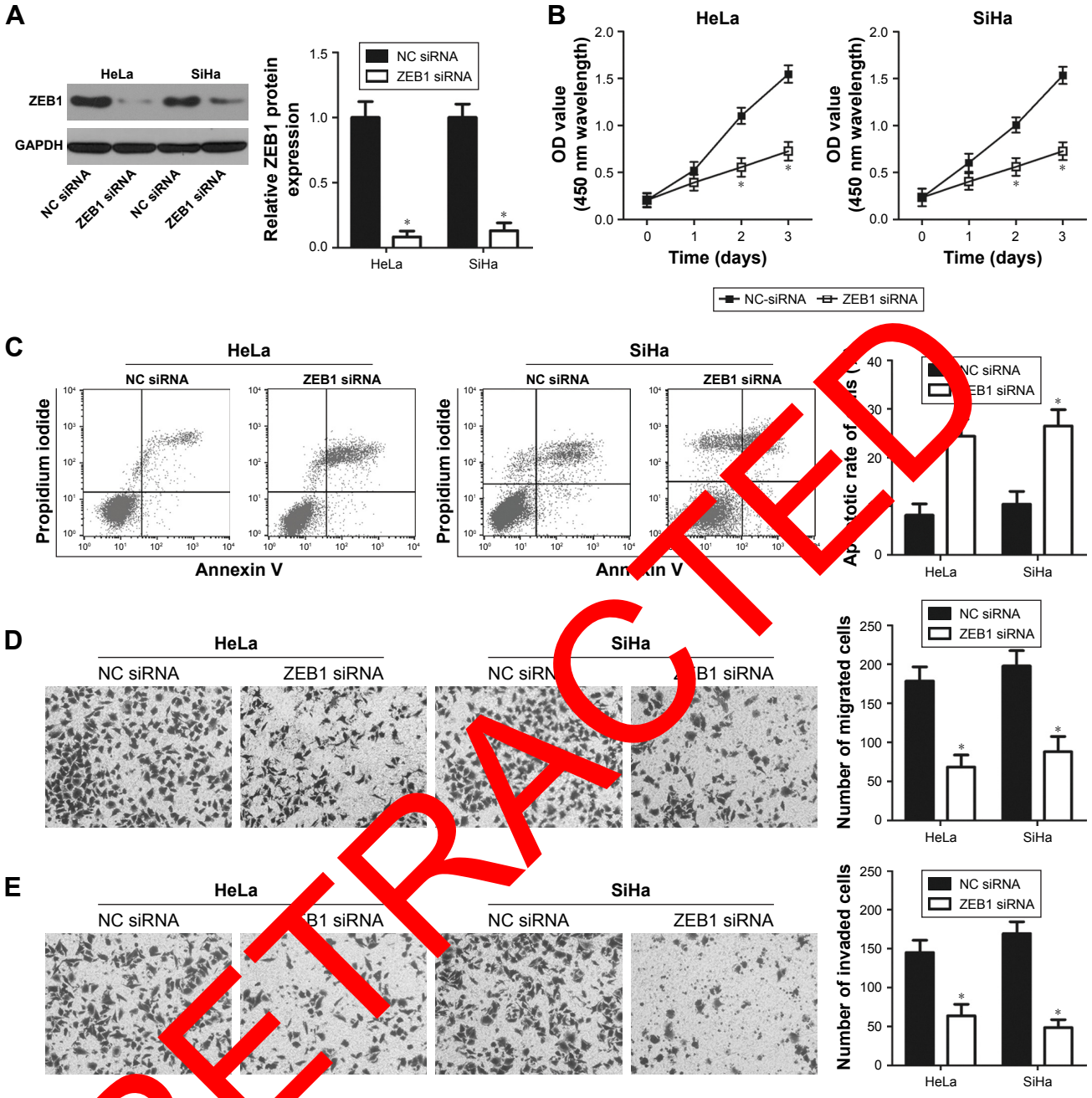

Figure 5 ZEB1 knockdown inhibits the proliferation, promotes apoptosis, and attenuates metastasis of cervical cancer cells.
Notes: (**A**) Western blot analysis was utilized to measure *ZEB1* protein expression in HeLa and SiHa cells that were treated with *ZEB1* siRNA or NC siRNA. *ZEB1* was significantly downregulated in *ZEB1* siRNA-transfected HeLa and SiHa cells. *$P<0.05$ vs NC siRNA. (**B**–**E**) CCK-8, flow cytometry, migration, and invasion assays were performed to evaluate the proliferation, apoptosis, migration, and invasion of HeLa and SiHa cells following *ZEB1* siRNA or NC siRNA transfection. *ZEB1* silencing obviously suppressed the proliferation, promoted apoptosis, and attenuated migration and invasion of HeLa and SiHa cells. *$P<0.05$ vs NC siRNA.
Abbreviations: CCK-8, Cell counting kit-8; NC siRNA, negative control siRNA.

approaches. Therefore, we investigated the molecular mechanisms responsible for the tumor suppressive actions of miR-641 in cervical cancer. First, bioinformatics analysis was conducted to search for potential targets of miR-641. One highly conserved putative binding site was observed at the 3′-UTR of *ZEB1*. Second, a luciferase reporter assay, RT-qPCR, and Western blot analysis revealed that miR-641 directly binds to the 3′-UTR of *ZEB1* and inhibits endogenous *ZEB1* expression in cervical cancer cell lines. Third, ZEB1 was upregulated in cervical cancer tissues, which is negatively correlated with miR-641 expression. Fourth, inhibition of *ZEB1* mimicked the tumor suppressing roles of

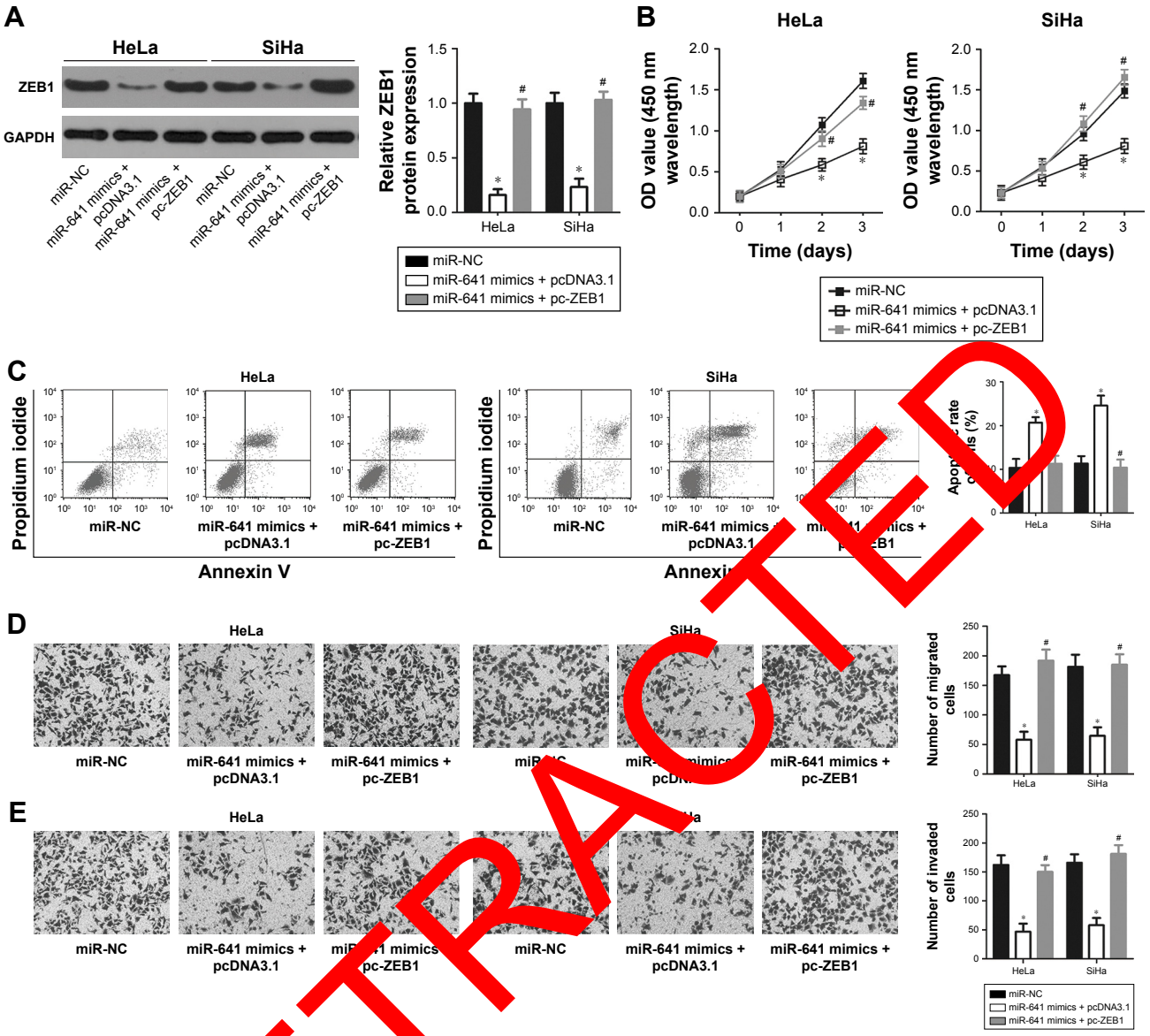

Figure 6 *ZEB1* expression restored the effects of miR-641 overexpression in the malignant phenotypes of HeLa and SiHa cells.

Notes: The miR-641 mimics-transfected HeLa and SiHa cells were further co-transfected with pc-ZEB1 or pcDNA3.1 blank vector. (**A**) Western blot analysis was conducted to detect the protein level of *ZEB1* in HeLa and SiHa cells treated as previously mentioned. The downregulation of *ZEB1* protein expression in HeLa and SiHa cells caused by miR-641 overexpression was restored after transfection with pc-ZEB1. $^{*}P<0.05$ vs miR-NC. $^{\#}P<0.05$ vs miR-641 mimics + pcDNA3.1. (**B**–**E**) The proliferation, apoptosis, migration, and invasion of previously mentioned cells were examined using CCK-8, flow cytometry, migration, and invasion assays, respectively. *ZEB1* reintroduction abolished the inhibitory effects of miR-641 overexpression on HeLa and SiHa cell proliferation, apoptosis, migration, and invasion. $^{*}P<0.05$ vs miR-NC. $^{\#}P<0.05$ vs miR-641 mimics + pcDNA3.1.

Abbreviations: CCK-8, Cell Counting Kit-8; miR-641, miRNA-641; miR-NC, negative control miRNA mimics.

miR-641 overexpression in cervical cancer cell lines. Finally, rescue experiments confirmed that the downregulation of *ZEB1* was essential for miR-641 to be effective in the malignant phenotypes of cervical cancer cells. These results clearly demonstrate that *ZEB1* is a direct and functional downstream target of miR-641 in cervical cancer.

ZEB1 is a member of the deltaEF1 family of two-handed zinc-finger factors.[31] *ZEB1* was reported to be overexpressed in a variety of human malignant tumors, such as thyroid cancer,[32] lung cancer,[33] colorectal cancer,[34] and endometrial cancer.[35] Its expression is high in cervical cancer tissues and cell lines. Increased expression of *ZEB1* is strongly correlated with the differentiation status, FIGO stages, lymph node metastasis, and occurrence of vascular invasion in cervical cancer patients.[22,23] Emerging studies have shown that *ZEB1* is closely related to the carcinogenesis and progression of cervical cancer by affecting cell proliferation, migration, invasion, epithelial–mesenchymal

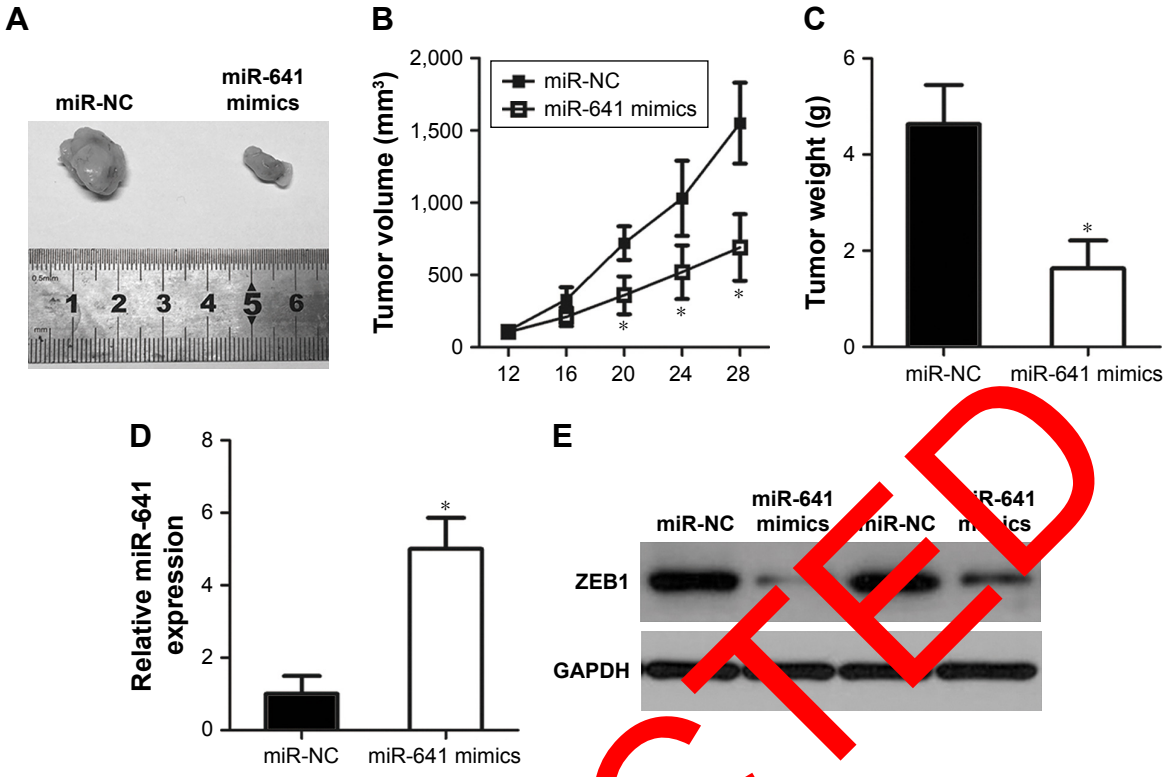

Figure 7 miR-641 hindered cervical cancer in vivo tumor growth.

Notes: miR-641 mimics- or miR-NC-transfected HeLa cells were harvested after 24 hours incubation and subcutaneously inoculated into the nude mice. (**A**) Photographs of tumor xenografts derived from miR-641 mimics- or miR-NC-transfected HeLa cells. (**B**) Tumor volume of tumor xenografts was measured every 4 days. The tumor xenograft volumes derived from miR-641 mimics were obviously lower than in miR-NC group. *P<0.05 vs miR-NC. (**C**) Tumor xenografts derived from miR-641 mimics or miR-NC-transfected cells were excised and weighed 4 weeks after injection. *P<0.05 vs miR-NC. (**D**) RT-qPCR analysis was performed to measure miR-641 expression in tumor xenografts. *P<0.05 vs miR-NC. (**E**) *ZEB1* protein expression in tumor xenografts was determined through Western blot analysis.

Abbreviations: miR-641, miRNA-641; miR-NC, negative control miRNA mimics.

transition, and motility.[24–27] Here, we revealed that *ZEB1* is upregulated in cervical cancer and serves oncogenic roles in the genesis and development of cervical cancer. miR-641 directly targeted *ZEB1* to inhibit the malignant phenotypes of cervical cancer in vitro and in vivo. Thus, *ZEB1* knockdown using miR-641-based targeted therapy may be a suitable therapeutic strategy for the prevention and treatment of patients with cervical cancer.

In conclusion, we showed that miR-641 expression was decreased in cervical cancer and significantly associated with FIGO stage and lymph node metastasis. Recovery of miR-641 expression suppressed the development and progression of cervical cancer both in vitro and in vivo. Mechanistically, ZEB1 was validated as a direct target of miR-641 in cervical cancer cells. These results highlight that *ZEB1* is a potential therapeutic target in cervical cancer. However, in this study, we did not explore whether miR-641 has an impact on cervical cancer epithelial–mesenchymal transition, cancer stem cell properties in vitro, and metastasis in vivo. These limitations of the present study may be resolved in future investigations.

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Disclosure

The authors report no conflicts of interest in this work.

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