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Original Article

miR-34b-5p suppresses the epithelial-mesenchymal transition and metastasis in endometrial cancer AN3CA cells by targeting *ZEB1*

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Abstract: Objectives: Tumor metastasis is a primary cause of recurrence and mortality in endometrial cancer. miR-34b-5p is abnormally expressed in various cancers and participates in tumor cell progression and metastasis. The objective of this study was to elucidate the biological functions and molecular mechanisms of miR-34b-5p in regulating the epithelial-mesenchymal transition (EMT) and metastasis in AN3CA endometrial cancer cells. Methods: The expression levels of miR-34b-5p and zinc finger E-box-binding homeobox 1 (ZEB1) in endometrial cancer cells were analyzed by qRT-PCR, and ZEB1 expression in endometrial cancer tissues was examined by immunohistochemistry. Proliferation, migration, and invasion of endometrial cancer AN3CA cells were evaluated using CCK8, scratch, and transwell assays, respectively. Bioinformatic analysis and dual-luciferase reporter gene assays were used to validate the targeting relationship between miR-34b-5p and *ZEB1*. Western blotting was performed to analyze the expression levels of ZEB1 and EMT-related proteins. Results: miR-34b-5p was significantly downregulated in endometrial cancer AN3CA cells. Overexpression of miR-34b-5p significantly inhibited proliferation, invasion, migration, and the EMT of endometrial cancer AN3CA cells. *ZEB1,* which was identified as a direct target gene of miR-34b-5p, exhibited high expression in endometrial cancer cells and tissues. Additionally, *ZEB1* upregulation partially reversed the inhibitory effects of miR-34b-5p on proliferation, migration, invasion, and the EMT of endometrial cancer AN3CA cells. Conclusions: miR-34b-5p suppresses the EMT and metastasis in endometrial cancer AN3CA cells by targeting *ZEB1*, indicating that the miR-34b-5p-*ZEB1*-EMT axis may be a therapeutic target for endometrial cancer.

Keywords: miR-34b-5p, *ZEB1*, endometrial cancer, EMT, metastasis

Introduction

Endometrial cancer, owing to its increasing global incidence and mortality rate, is the most prevalent malignant neoplasm in the female reproductive tract [1]. There has been significant progress in preventive measures, diagnostic methods, and treatment strategies for endometrial cancer, yet the lack of specific clinical manifestations and effective early screening methods causes a large number of patients to succumb to postoperative recurrence and systemic metastasis [2, 3]. Hence, it is imperative to elucidate the molecular mechanisms underlying the invasion and metastasis of endometrial cancer in order to identify effective therapeutic targets and specific biomarkers for improving prognosis.

MicroRNAs (miRNAs), which are a class of endogenous noncoding RNAs comprising 19-25 nucleotides, regulate gene expression by binding to the 3'-untranslated region (3'UTR) of target mRNAs, thus leading to degradation or translational suppression of the mRNA [4]. miRNAs function as oncogenes or tumor suppressor genes, participating in such biological processes as cell proliferation, metastasis, cell cycle regulation, apoptosis, and chemotherapy resistance [5]. It has been confirmed that miR-34b-5p, directly regulated by the p53 transcription factor, exerts tumor-suppressive effects [6]. According to previous research, miR-34b-5p is downregulated in various cancers, including breast cancer, colorectal cancer, and bladder cancer, and plays a crucial role in regulating tumor cell proliferation, invasion, and metasta-

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sis [7-9]. However, the role of miR-34b-5p and its molecular mechanisms in endometrial cancer remain uncertain.

The epithelial-mesenchymal transition (EMT) is a critical mechanism by which tumor cells undergo invasion and metastasis. During the EMT, epithelial cells lose their polarity and transform into a mesenchymal phenotype characterized by decreased expression of the epithelial marker E-cadherin and increased expression of mesenchymal markers, such as N-cadherin and vimentin [10]. The occurrence of the EMT depends on the regulation of transcription factors, such as ZEB1, Twist, and Snail, which serve as transcriptional repressors of E-cadherin. They activate the EMT and promote tumor cell invasion and metastasis by binding to E-box elements in the promoter region of E-cadherin [11]. Inhibiting EMT has been demonstrated to effectively suppress or prevent the dissemination and metastasis of tumor cells.

Located on human chromosome 10p11.22, *ZEB1* is an important transcription factor regulating the EMT. Its abnormal expression is closely associated with an adverse prognosis in cancer patients, and it serves as a major driver of cancer progression and metastasis [12]. It has been reported that various cancers, including lung cancer, liver cancer, gastric cancer, and breast cancer, exhibit heightened levels of *ZEB1*, correlating with advanced tumor stage, recurrence, and metastasis [13-16]. Nevertheless, the mechanisms underlying *ZEB1* overexpression in endometrial cancer remain unclear. Our research team identified *ZEB1* as a target gene of miR-34b-5p. Afterward, we functionally validated the miR-34b-5p-*ZEB1*-EMT axis, confirming that miR-34b-5p inhibits the EMT and metastasis of endometrial cancer by directly targeting *ZEB1*. These findings provide new insights and treatment strategies for targeted therapy in endometrial cancer.

Materials and methods

Bioinformatic analysis

The expression levels of miR-34b-5p and corresponding clinical information on survival were obtained from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.com) for endometrial cancer patients. There were 538 endometrial cancer samples and 33 normal tissue samples. The TCGA database was updated on May 3, 2022.

TargetScan (http://www.targetscan.org/), StarBase (http://starbase.sysu.edu.cn/), and miRWalk (http://www.mirwalk.umm.uni-heidelberg.de) websites were used to predict the binding site of miR-34b-5p to *ZEB1*.

Isolation of human endometrial glandular epithelial cells

Endometrial tissue was collected from patients undergoing diagnostic curettage who attended the Department of Gynecology at the General Hospital of Ningxia Medical University from January to March 2023. Based on the inclusion criteria, patients aged 28-45 years, with regular menstrual cycles, and no prior exposure to estrogen-progestin therapy within the preceding 6 months were included in the study. Ethical approval was granted by the Ethics Committee of the General Hospital of Ningxia Medical University (Approval No: 2019-032). Informed consent was obtained from all patients, who willingly participated in the study and signed informed consent forms. The obtained uterine endometria were pathologically confirmed to be normal tissue.

In accordance with the experimental approach outlined by Satu Kuokkanen et al [17], we isolated glandular epithelial cells from the uterine endometrium. After sampling, blood and mucus on the surface of the endometrium were removed, and the tissue was cut into 1 mm^3 fragments. Tissue digestion was performed at a constant temperature of 37°C using 1 mg/mL type I collagenase (Biotopped, China) for 1 h, with agitation every 10 min. The digested tissue and cell suspension were homogenized by oscillation, filtered through a 100-μm cell strainer to remove excess tissue and mucus, and further filtered through a 40-μm cell strainer to obtain glandular epithelial cells. The collected glandular epithelial cells were centrifuged, and the cell pellets were cultured in a DMEM-F12 medium (Gibco, USA) containing 10% fetal bovine serum (Biological Industries, Israel) and a 1% penicillin-streptomycin mixture (Solarbio, China). Cells were cultured in a humidified incubator at 37°C with 5% CO_2.

Tissue microarray and IHC assay

A human endometrial cancer tissue microarray (HUteA045PG01) was purchased from Outdo BioTech (Shanghai, China) and comprised of 34 endometrial cancer tissues and nine paracancerous tissues. IHC was conducted on the tissue microarray samples using a standardized procedure. In the beginning, the tissue microarray chips were deparaffinized in xylene, which was followed by antigen retrieval using an EDTA buffer (pH 9.0) after graded ethanol hydration. Subsequently, the tissue microarray was blocked with goat serum before incubating overnight at 4°C with a ZEB1 antibody (1:3000, ABclonal, China). Then, a secondary antibody was applied to the tissue microarray. Following washing with PBS, chromogenic staining was conducted using 3,3'-diaminobenzidine (DAB), and counterstaining was performed with hematoxylin. The dehydrated samples were then scanned using an Aperio digital pathology slide scanner. Two experienced pathologists evaluated the IHC results using the modified IHC score (H-score). The scoring system included assessing staining intensity as follows: negative = 0; weak = 0.5; moderate = 1; strong = 2; and very strong = 3. The percentage of positively stained cells ranging from 0-100% was also determined. The total score for the quantitative analysis of ZEB1 expression was calculated by multiplying the staining intensity score by the percentage of positively stained cells, resulting in an H-score range of 0-300.

Cell culture

The AN3CA cell line derived from human endometrial adenocarcinoma was procured from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (Biological Industries, Israel) and a 1% penicillin-streptomycin mixture (Solarbio, China). Cell cultivation was performed in a humidified incubator at 37°C with 5% CO_2, and passaging was performed every 2-3 days.

Cell transfection

miR-34b-5p overexpression lentivirus, miR-NC lentivirus, *ZEB1* overexpression adenovirus, and NC adenovirus were purchased from HANBIO (Shanghai, China). AN3CA cells were seeded at a density of 5×10^5 cells per well in a six-well plate, according to the manufacturer's instructions. Once the cells adhered completely the following day, each well was treated with 1 mL of culture medium mixed with the respective viral vectors. After a 4-h incubation at 37°C, the medium was replenished to a total volume of 2 mL, and the culture medium was replaced after 24 h. Fluorescence expression was observed 48-72 h post-culturing. Stable cell lines expressing miR-34b-5p or miR-NC were selected from cells transfected with a lentivirus using 4 μg/μL puromycin (Solarbio, China). Subsequently, the efficiency of infection was assessed using qRT-PCR or western blot experiments.

RNA extraction and qRT-PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, USA), and the concentration and purity of RNA were measured using a Colibri spectrophotometer (Berthold, Germany). According to the instructions of the PrimeScript RT kit (Takara, Japan), RNA was reverse-transcribed into cDNA, with the reverse transcription of miR-34b-5p using the stem-loop method. Real-time fluorescent quantitative PCR (qRT-PCR) reactions were performed using a TB Green Premix Ex Taq™ II kit (Takara, Japan). U6 served as the internal reference for miR-34b-5p, and *GAPDH* was the internal reference for *ZEB1*. The $2^{-\Delta\Delta CT}$ method was employed to calculate relative expression levels of miR-34b-5p and *ZEB1*. The primers used in the experiment were designed and synthesized by Sangon Biotech (Shanghai, China). The specific primer sequences were as follows: miR-34b-5p: stem-loop: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCAACCAGC-3'; forward 5'-ACACTCCAGCTGGCTAGGCAGTGTCATTAGC-3' and reverse 5'-TGGTGTCGTGGAGTCG-3'; U6: forward 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'-AACGCTTCACGAATTTGCGT-3'; *ZEB1*: forward 5'-CAATGATCAGCCTCAATCTGCA-3' and reverse 5'-CCATTGGTGGTTGATCCCA-3'; and *GAPDH*: forward 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse 5'-TGGTGAAGACGCCAGTGGA-3'.

Dual-luciferase reporter gene assay

293T cells were seeded in a 96-well plate and co-transfected with miR-34b-5p mimics or miR-NC, along with ZEB1-3'UTR-wt or ZEB1-3'UTR-mut constructs (all synthesized compounds

and plasmids were procured from HANBIO, China). After 48 h of transfection, cells were collected and lysed, and the fluorescent enzyme activity was assessed using the dual-luciferase system kit (Promega, China).

Cell proliferation assay (CCK8)

Cells (2×10^3 cells/well) were seeded in a 96-well plate and cultured for 0, 24, 48, 72, and 96 h. At the same time point each day, 10 μL of CCK8 solution (APExBIO, China) was added to each well, followed by a 1-h incubation at 37°C. Subsequently, the absorbance of each well was measured at 450 nm using a microplate reader.

Scratch assay

Cells (5×10^5 cells/well) were inoculated in six-well plates. After the growth density reached 100%, a linear scratch wound was made on the confluent surface of the cells with a 200-μL sterile pipette tip. The floating cells that were scratched off were removed with PBS. The remaining adhered cells were then cultured in serum-free medium. At 0 and 24 h, the scratch area (magnification, ×100) was recorded using an inverted microscope. The migration rate was calculated as: cell migration rate = [(scratched area at 0 h - scratched area at 24 h)/scratched area at 0 h] ×100%.

Transwell assay

In the upper chamber of a transwell chamber (8-μm pore size, LABSELECT, China), 50 μL of diluted VitroGel hydrogel matrix (TheWell, USA) was added, and cells were incubated at 37°C for 3 h. Subsequently, cells (1×10^5 cells/well) were suspended in 100 μL of serum-free medium and seeded onto the upper chamber of the transwell plate, while 700 μL of complete medium containing 10% FBS was added to the lower chamber. After 24 h of incubation, noninvasive cells in the upper chamber were gently wiped off with a cotton swab. The cells that invaded the lower chamber surface were fixed with 4% paraformaldehyde (Biosharp, China) for 30 min and stained with 0.2% crystal violet (Solarbio, China) for 10 min. Finally, five random fields were captured and counted under an inverted microscope (magnification, ×200) to determine the number of invading cells.

Protein extraction and western blot

Extraction of total protein from cells was performed using a total protein extraction kit (KeyGEN BioTECH, China), and the protein concentration was determined using a BCA assay kit (KeyGEN BioTECH, China). Thirty micrograms of protein was separated by SDS-PAGE gel electrophoresis and transferred onto a PVDF membrane. The membrane was then blocked with 5% skim milk at room temperature for 2 h. Afterward, the membrane was incubated overnight at 4°C with specific primary antibodies, followed by washing with TBST and incubation with secondary antibodies for 1.5 h. Finally, protein bands were visualized using an enhanced chemiluminescence detection kit (PerkinElmer, USA). The grayscale values of the protein bands were analyzed and quantified using ImageJ software with GAPDH as an internal reference. Primary antibodies used in the experiment included E-cadherin (1:2000, Proteintech, China), N-cadherin (1:5000, Proteintech, China), Vimentin (1:12000, Proteintech, China), α-SMA (1:2000, Proteintech, China), SNAI1 (1:1000, Proteintech, China), GAPDH (1:50000, Proteintech, China), and ZEB1 (1:1000, ABclonal, China). The secondary antibodies used included HRP-conjugated goat anti-rabbit IgG (1:5000, Proteintech, China) and goat anti-mouse IgG (1:5000, ABclonal, China).

Statistical analysis

All data were, presented as the mean ± SD from at least three independent experiments. Data were statistically analyzed using SPSS 23.0 and GraphPad Prism 9.0. Bioinformatic data were statistically analyzed using R software. Student's *t*-tests were used for comparisons between two groups, while one-way analysis of variance (ANOVA), followed by Tukey's post hoc test, was used for comparisons among multiple groups. Kaplan-Meier curves were generated for survival analysis. A *P* value less than 0.05 was considered significant.

Results

miR-34b-5p is downregulated in endometrial cancer

To elucidate the expression pattern of miR-34b-5p in endometrial cancer, we extracted RNA from endometrial glandular epithelial cells

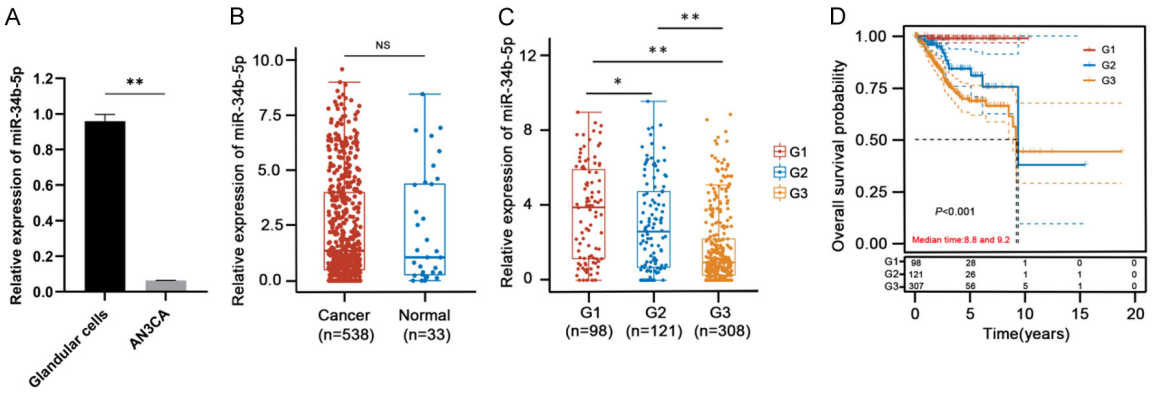

Figure 1. miR-34b-5p is downregulated in endometrial cancer. A. Expression of miR-34b-5p in endometrial glandular epithelial cells and AN3CA cells by qRT-PCR. B. Comparative analysis of miR-34b-5p expression between endometrial cancer tissue and normal tissue. C. miR-34b-5p expression across different histologic grades of endometrial cancer, as obtained from the TCGA database. D. Kaplan-Meier survival curve illustrating prognostic outcomes in endometrial cancer patients with different histologic tumor grades. *P<0.05, **P<0.01.

and AN3CA cells, and analyzed it through qRT-PCR. The results showed a significant downregulation of miR-34b-5p in AN3CA cells compared to normal endometrial glandular epithelial cells (**Figure 1A**). In addition, an analysis of miR-34b-5p expression in endometrial cancer tissues from the TCGA database indicated no significant difference between tumor and normal tissue (**Figure 1B**). Based on this observation, we conducted a stratified analysis across different pathological tumor grades of endometrial cancer patients. The findings revealed a progressive decrease in miR-34b-5p expression levels with increasing malignancy. Kaplan-Meier survival analysis indicated a markedly reduced overall survival period in patients with low miR-34b-5p expression (G2 and G3 stages) compared to those with high miR-34b-5p expression (G1 stage) (**Figure 1C**, **1D**). This suggested a close association between low miR-34b-5p levels and adverse patient prognosis.

Overexpression of miR-34b-5p suppresses proliferation, migration, invasion, and the EMT in endometrial cancer AN3CA cells

To study the biological functions of miR-34b-5p in endometrial cancer, we transduced AN3CA cells with lentiviruses overexpressing miR-34b-5p or miR-NC. The results revealed a significant increase in miR-34b-5p expression in cells transduced with the overexpression lentivirus, confirming the establishment of stably transduced cell lines (**Figure 2A**). CCK8 assays showed that miR-34b-5p overexpression effectively inhibited the proliferation of AN3CA cells (**Figure 2B**). Scratch assays indicated a significant reduction in the migration capacity of AN3CA cells upon miR-34b-5p overexpression (**Figure 2C**). Moreover, transwell experiments revealed a noticeable decrease in the invasive potential of AN3CA cells overexpressing miR-34b-5p (**Figure 2D**). To further elucidate the impact of miR-34b-5p on the progression of EMT, the expression of EMT-related proteins were assessed using western blot analysis. The results revealed that miR-34b-5p overexpression increased the expression of the epithelial marker E-cadherin, while the mesenchymal markers N-cadherin, vimentin, α-SMA, and SNAI1 were significantly downregulated (**Figure 2E**). These findings indicated that, by inhibiting tumor cell proliferation, migration, invasion, and EMT processes, miR-34b-5p overexpression exerts anticancer effects in endometrial cancer.

ZEB1 is a direct target of miR-34b-5p

The crucial role of miRNAs in tumor progression is intricately linked to their target genes. Employing bioinformatic analyses through TargetScan, miRDB, and miRwalk algorithms, we identified putative targets of miR-34b-5p (**Figure 3A**). The results revealed a complementary sequence between miR-34b-5p and the 3'UTR of *ZEB1* (**Figure 3B**). To ascertain whether miR-34b-5p directly targeted the 3'UTR of *ZEB1*, we conducted dual-luciferase reporter gene assays. The findings demonstrated a sig-

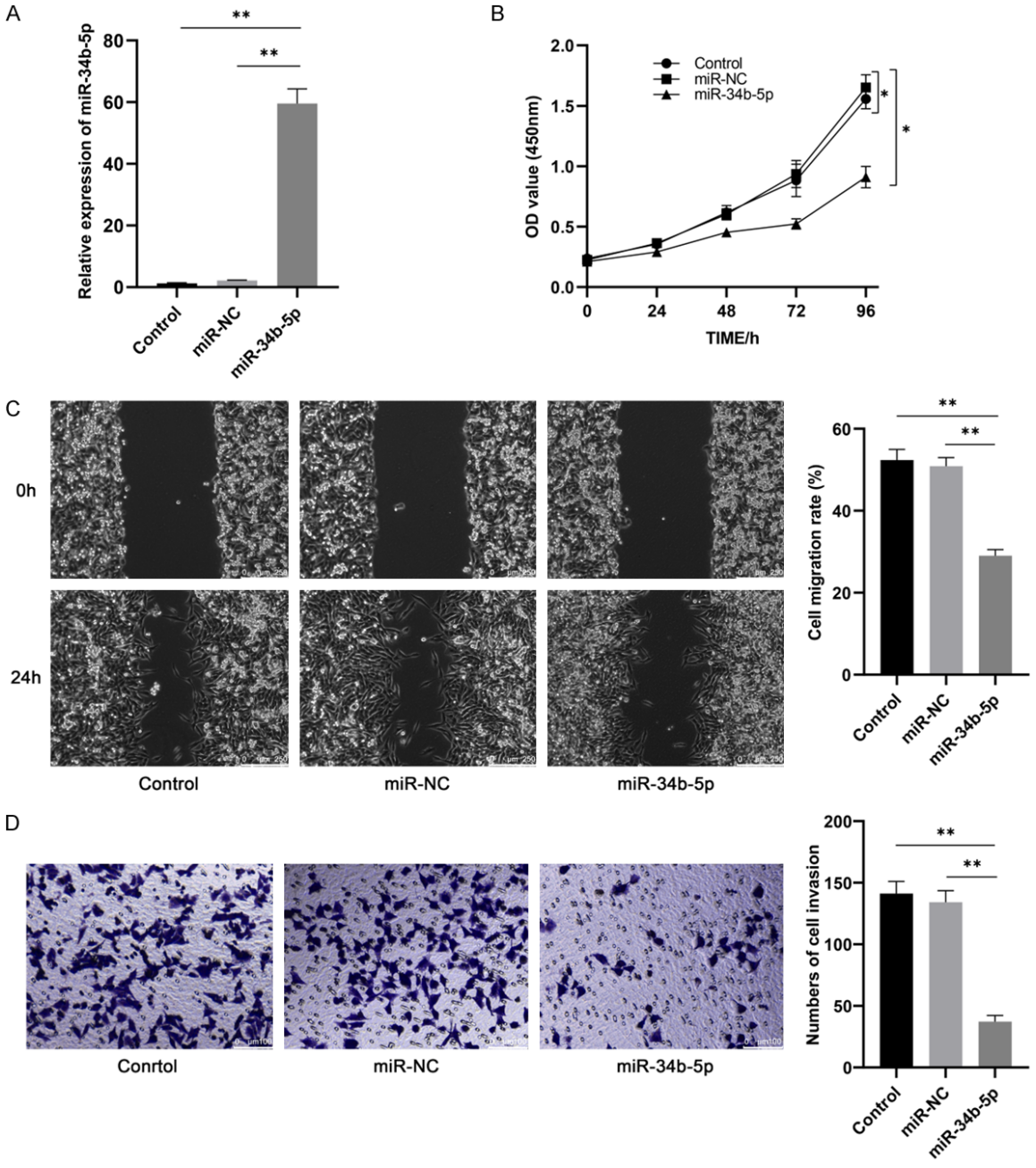
A
Relative expression of miR-34b-5p
Control
miR-NC
miR-34b-5p
B
OD value (450nm)
TIME/h
Control
miR-NC
miR-34b-5p
C
0h
24h
Control
miR-NC
miR-34b-5p
Cell migration rate (%)
D
Conrtol
miR-NC
miR-34b-5p
Numbers of cell invasion
E
E-cadherin
N-cadherin
Vimentin
α-SMA
SNAI1
GAPDH
Relative expression of protein

Figure 2. Overexpression of miR-34b-5p suppresses proliferation, migration, invasion, and the EMT in endometrial cancer AN3CA cells. A. Transfection efficiency of miR-NC lentivirus and miR-34b-5p overexpression lentivirus in AN3CA cells assessed by qRT-PCR. B. Impact of miR-34b-5p overexpression on the proliferation of AN3CA cells measured using CCK8 assays. C. Effects of miR-34b-5p overexpression on the migration of AN3CA cells examined through scratch assays (magnification, ×100). D. Influence of miR-34b-5p overexpression on the invasion of AN3CA cells evaluated by transwell assays (magnification, ×200). E. Western blot analysis illustrating the impact of miR-34b-5p overexpression on EMT-related proteins in AN3CA cells. *P<0.05, **P<0.01.

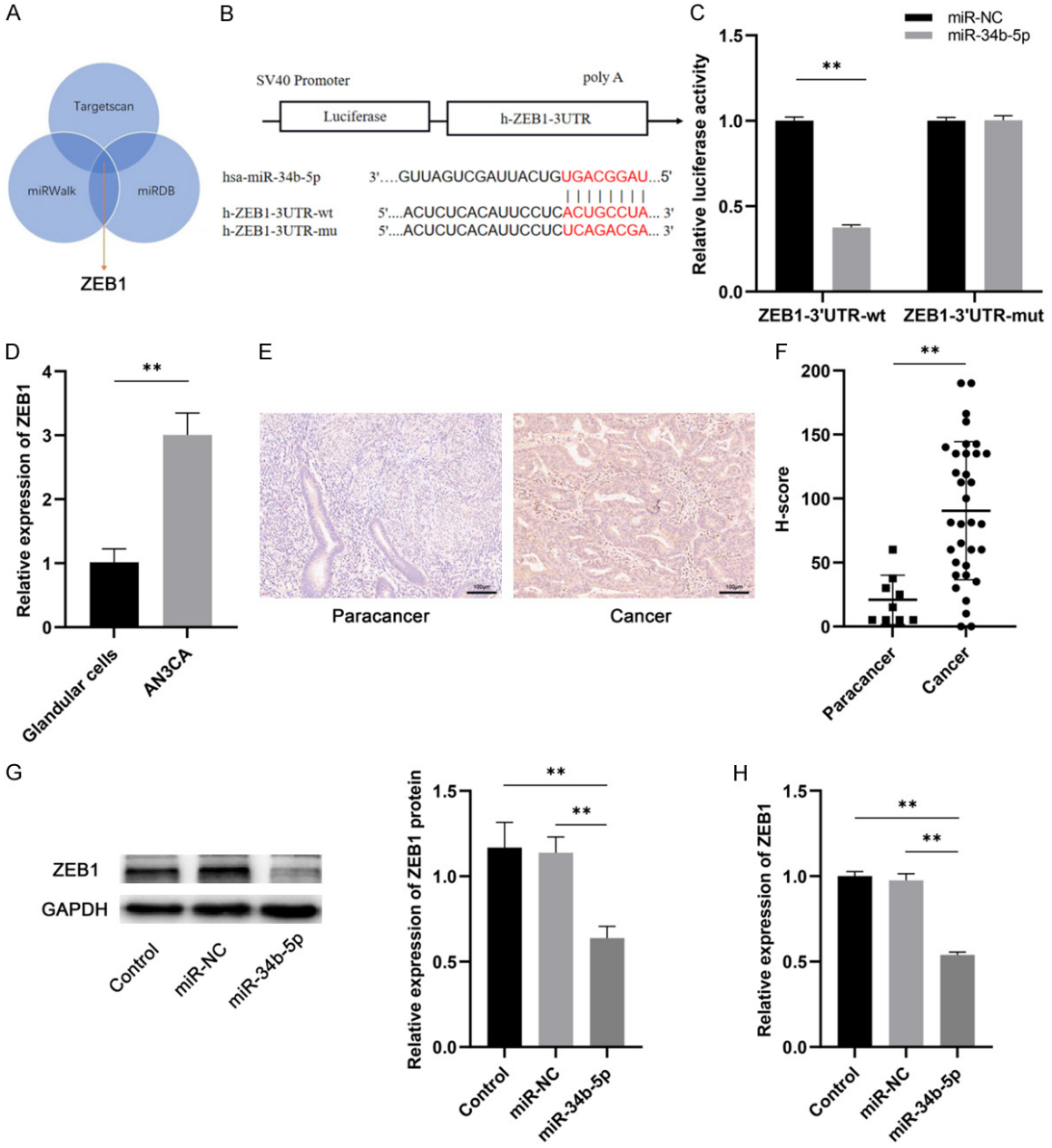

Figure 3. *ZEB1* is a direct target of miR-34b-5p. A. Using the bioinformatic tools TargetScan, miRDB, and miRwalk, prediction algorithms were employed to identify *ZEB1* as a candidate target of miR-34b-5p. B. The binding sequence between miR-34b-5p and *ZEB1* was obtained through the bioinformatic tool TargetScan. C. Dual-luciferase reporter gene assays were conducted to confirm the direct targeting interaction between miR-34b-5p and *ZEB1*. D. qRT-PCR was employed to assess *ZEB1* expression in endometrial glandular epithelial cells and AN3CA cells. E, F. IHC analysis was conducted to evaluate ZEB1 expression in endometrial cancer tissues and paracancerous tissues (magnification, ×100). G. The impact of miR-34b-5p overexpression on ZEB1 protein was assessed through western blot. H. qRT-PCR was employed to examine the influence of miR-34b-5p overexpression on *ZEB1*. *P<0.05, **P<0.01.

Table 1. Clinical correlation of ZEB1 expression in endometrial cancer

Data	Cases (n)	ZEB1 expression		*P* value
		Low	High	
Age				
≤60 years	20	10	10	0.738
>60 years	14	6	8	
Tumor Size				
≤4 cm	21	6	15	0.014
>4 cm	12	9	3	
Grade				
G1+G2	16	13	13	0.693
G3	8	3	5	

nificant reduction in luciferase activity of ZEB1-3'UTR-wt upon miR-34b-5p transfection compared to the control group, while ZEB1-3'UTR-mut showed no apparent change (**Figure 3C**). Afterward, we evaluated *ZEB1* expression in AN3CA cells. qRT-PCR results exhibited a significant upregulation of *ZEB1* in AN3CA cells compared to normal endometrial glandular epithelial cells (**Figure 3D**). IHC analysis revealed elevated ZEB1 expression in endometrial cancer compared to paracancerous tissue (**Figure 3E**, **3F**). In these endometrial cancer samples, ZEB1 expression was unrelated to patient age and clinical grade but correlated with tumor size (**Table 1**). Furthermore, western blot analysis and qRT-PCR results showed that miR-34b-5p overexpression decreased ZEB1 protein and mRNA expression in AN3CA cells (**Figure 3G**, **3H**). In summary, these findings strongly indicate that *ZEB1* is a direct target gene of miR-34b-5p.

Overexpression of ZEB1 promotes proliferation, migration, invasion, and the EMT in endometrial cancer AN3CA cells

To explain in detail the biological functions of ZEB1 in endometrial cancer, we transiently transfected AN3CA cells with *ZEB1* overexpression adenovirus or NC adenovirus. The infection efficiency of cells was validated through western blotting and qRT-PCR (**Figure 4A**, **4B**). CCK8 results demonstrated that *ZEB1* overexpression significantly enhanced the proliferation of AN3CA cells (**Figure 4C**). Scratch and transwell assays revealed that *ZEB1* overexpression promoted the migration (**Figure 4D**) and invasion (**Figure 4E**) of AN3CA cells. Additionally, western blot results showed that ZEB1 overexpression markedly reduced the expression of the epithelial marker E-cadherin and increased the expression of mesenchymal markers N-cadherin, vimentin, α-SMA, and SNAI1 (**Figure 4F**). In summary, *ZEB1* overexpression promoted proliferation, migration, invasion, and EMT processes in AN3CA cells, thereby facilitating the malignant progression of endometrial cancer.

miR-34b-5p suppresses proliferation, migration, invasion, and the EMT in endometrial cancer AN3CA cells by targeting ZEB1

We conducted a series of rescue experiments to explain the molecular mechanisms underlying the regulatory role of miR-34b-5p and ZEB1 in endometrial cancer. Initially, AN3CA cells were co-transfected with miR-34b-5p overexpression lentivirus and *ZEB1* overexpression adenovirus. Western blot and qRT-PCR experiments revealed that the overexpression of miR-34b-5p led to a reduction in ZEB1 protein and mRNA levels, which were restored by transfection with *ZEB1* overexpression adenovirus (**Figure 5A**, **5B**). CCK8 assays demonstrated that *ZEB1* overexpression partially rescued the inhibitory effect of miR-34b-5p on proliferation of AN3CA cells (**Figure 5C**). Furthermore, scratch and transwell invasion assays indicated that *ZEB1* overexpression reversed the inhibitory effects of miR-34b-5p overexpression on the migration (**Figure 5D**) and invasion (**Figure 5E**) of AN3CA cells. Western blot experiments revealed that the overexpression of miR-34b-5p decreased the expression of N-cadherin, vimentin, α-SMA, and SNAI1 while increasing the expression of E-cadherin. This inhibitory effect on EMT was reversed by elevating *ZEB1* expression (**Figure 5F**). In summary, *ZEB1* overexpression partially restored or reversed the inhibitory effects of miR-34b-5p on the proliferation, migration, invasion, and EMT of AN3CA cells.

Discussion

Endometrial cancer, a carcinoma of the endometrial lining, predominantly affects perimenopausal and postmenopausal women, with endometrioid adenocarcinoma the most prevalent histologic subtype [18, 19]. Patients diagnosed in the early stages who receive effective treatment generally exhibit favorable outcomes.

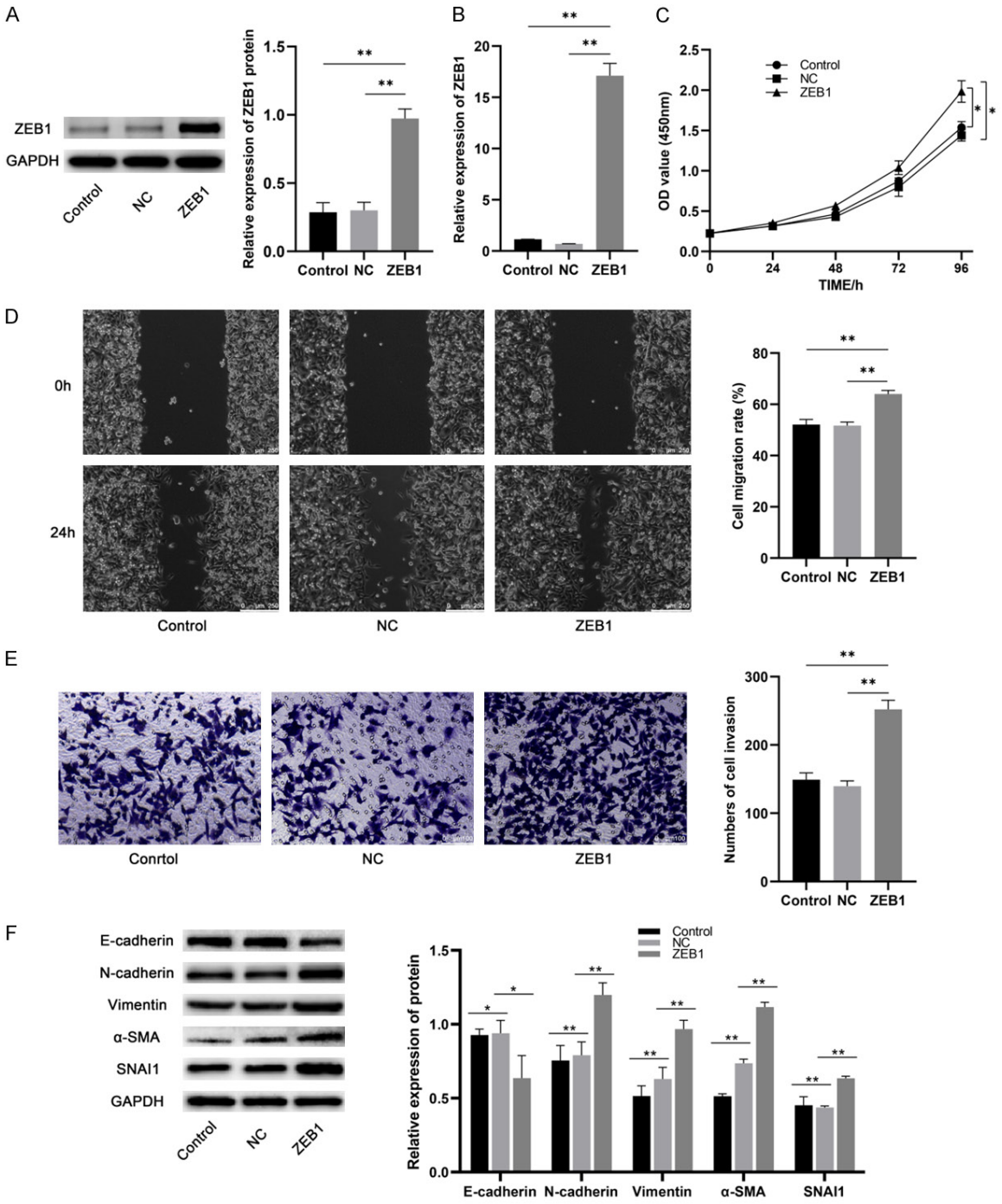

Figure 4. Overexpression of *ZEB1* promotes proliferation, migration, invasion, and the EMT in endometrial cancer AN3CA cells. A, B. Transfection efficiency of NC adenovirus and ZEB1 overexpression adenovirus in AN3CA cells was assessed by western blot and qRT-PCR. C. The impact of *ZEB*1 on the proliferation of AN3CA cells was evaluated using CCK8 assays. D. The influence of *ZEB1* overexpression on the migration of AN3CA cells was examined through scratch assays (magnification, ×100). E. Transwell assays were conducted to assess the effect of *ZEB1* overexpression on the invasion of AN3CA cells (magnification, ×200). F. Western blot analysis was performed to investigate the impact of *ZEB1* overexpression on EMT-related proteins in AN3CA cells. **P*<0.05, ***P*<0.01.

However, therapeutic options for those in advanced stages, experiencing recurrence, or manifesting metastasis are significantly limited, leading to a dismal prognosis with a five-

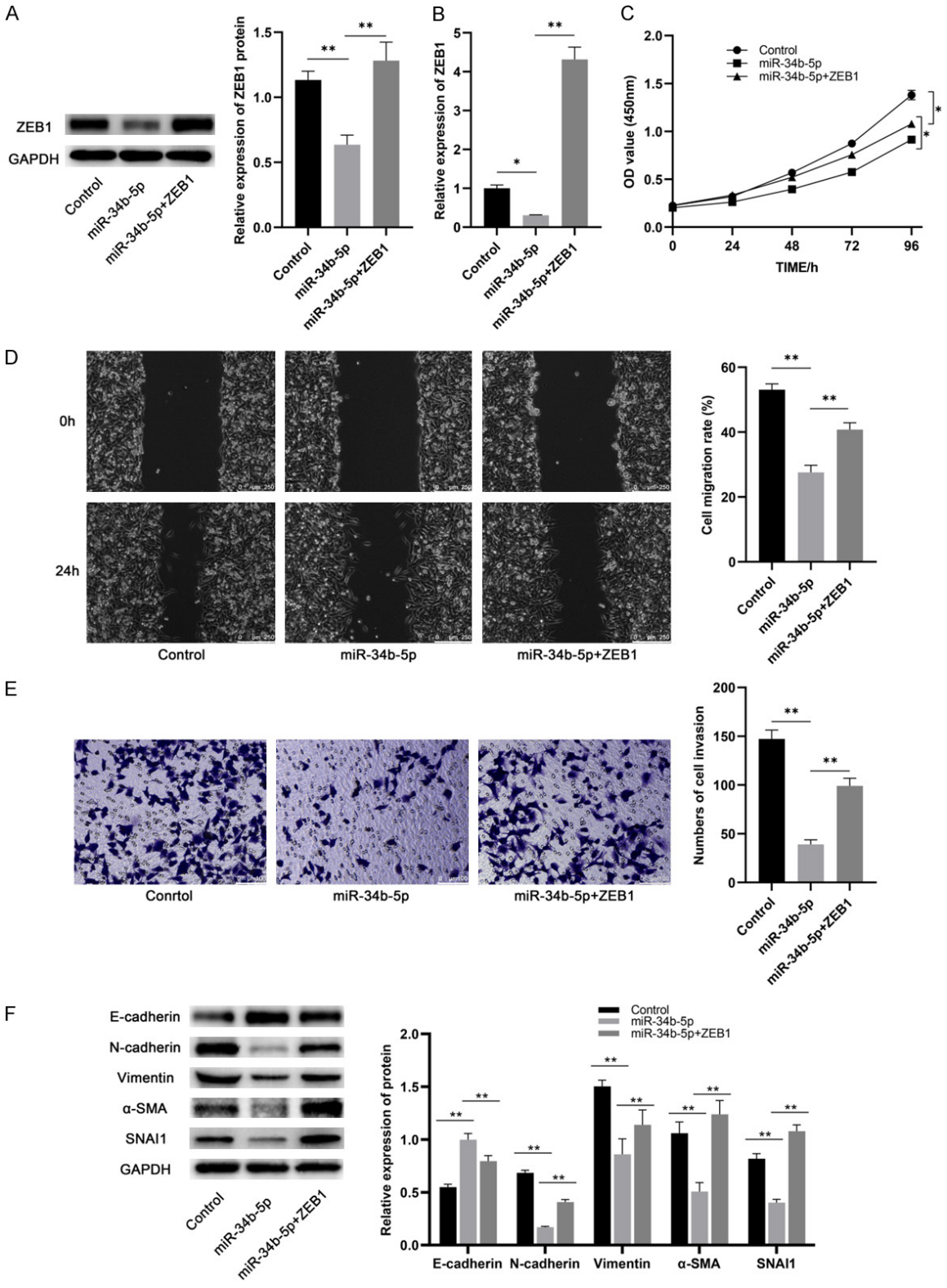

Figure 5. miR-34b-5p suppresses proliferation, migration, invasion, and the EMT in endometrial cancer AN3CA cells by targeting *ZEB1*. A, B. AN3CA cells were co-transfected with miR-34b-5p overexpression lentivirus or miR-34b-5p overexpression lentivirus combined with ZEB1 adenovirus. The expression levels of ZEB1 were examined by western blot and qRT-PCR. C. The proliferative capacity of AN3CA cells in each group was assessed using the CCK8 method. D. The migration ability of AN3CA cells in each group was evaluated through scratch assays (magnification, ×100). E. The invasive potential of AN3CA cells in each group was determined by transwell assays (magnification, ×200). F. Changes in EMT-related proteins in AN3CA cells of each group were examined by western blot. **P*<0.05, ***P*<0.01.

year survival rate of less than 17% [20, 21]. Metastasis and recurrence are the primary contributors to the elevated mortality rate among endometrial cancer patients [22]. Numerous studies underscore the pivotal role of the EMT in the invasion and metastasis of endometrial cancer [23]. Nevertheless, the precise mechanisms underlying the progression and metastasis of endometrial cancer remain largely unexplained. Hence, it is imperative to explore novel strategies for precision medicine in endometrial cancer management.

In recent years, the rapid advancement of high-throughput sequencing and bioinformatic technologies has enabled the observation of abnormal expression and dysregulation of miRNAs at various stages of cancer progression [24, 25]. Therefore, a better understanding of the biological mechanisms of miRNAs holds the potential to facilitate the development of specific therapeutic strategies against various malignancies. Substantial evidence indicates that miR-34b-5p is downregulated in various malignant tumors, acting as a tumor suppressor. For example, miR-34b-5p inhibited the proliferation, migration, and invasion of breast cancer cells by targeting ARHGAP1 [7]. miR-34b-5p exerted tumor-suppressive effects on retinoblastoma by modulating the Notch signaling pathway [26]. Downregulation of miR-34b-5p promoted the growth and proliferation of colorectal cancer cells while inhibiting apoptosis [8, 27]. Moreover, miR-34b-5p exhibited low expression in pancreatic ductal adenocarcinoma, correlating negatively with distant metastasis and TNM staging [28]. A similar trend characterized by a significant downregulation of miR-34b-5p in endometrial cancer cells was observed in this study. By analyzing the TCGA data, we found no significant difference in the expression levels of miR-34b-5p between endometrial cancer tissue and normal tissue. However, there was a significant difference in miR-34b-5p expression among endometrial cancer samples with different histologic grades. Specifically, as the malignancy of the tumor increased, the expression of miR-34b-5p progressively decreased. Furthermore, patients with high miR-34b-5p expression exhibited a better prognosis than those with low miR-34b-5p expression. Subsequently, functional experiments were conducted to assess the impact of miR-34b-5p on the endometrial cancer cell line AN3CA. The results showed that miR-34b-5p overexpression inhibited the proliferation, migration, invasion, and EMT of endometrial cancer AN3CA cells. These findings emphasize the tumor-suppressive role of miR-34b-5p in endometrial cancer, suggesting its use as a therapeutic target in the management of this malignancy.

ZEB1, a zinc finger transcription factor, has been identified as a key regulator of tumor progression, metastasis, and dissemination through the induction of EMT [29]. Elevated expression of *ZEB1* has been reported in various cancers, correlating with more invasive cancer types and poorer prognosis [30]. Notably, *ZEB1* not only promoted the movement and spread of tumor cells, but also facilitated cellular dedifferentiation, treatment resistance, tumor metastasis, and immune escape [31]. In hepatocellular carcinoma, *ZEB1* was shown to play a carcinogenic role by accelerating cell proliferation and inhibiting apoptosis [15]. In squamous cell lung carcinoma, *ZEB1* promoted the EMT and metastasis of tumors by activating the expression of Notch1 [32]. Furthermore, *ZEB1* overexpression in melanoma enhanced tumor growth and invasive capabilities both in vitro and in vivo [33]. These studies substantiate our research findings. In our current investigation, we observed an upregulation of *ZEB1* expression in both endometrial cancer tissues and cells. We also validated these observations by establishing a *ZEB1*-overexpressing endometrial cancer cell model and conducting functional experiments. The results showed that *ZEB1* overexpression promoted the proliferation, migration, invasion, and EMT processes of AN3CA cells, in stark contrast to the outcomes observed with miR-34b-5p overexpression. This suggested that *ZEB1* might function as an oncogene, driving endometrial cancer initiation, progression, and metastasis by enhancing cell proliferation, migration, invasion, and EMT. These findings shed light on the potential role of ZEB1 as a therapeutic target in the context of endometrial cancer.

It is well established that miRNAs exert their biological functions at the post-transcriptional level by binding to target genes [34]. Numerous miRNAs function as negative regulators of *ZEB1*, influencing the progression and metasta-

sis of tumor cells. For example, miR-128b-3p inhibited the EMT and metastasis of pancreatic cancer cells by targeting *ZEB1* [35]. In osteosarcoma, miR-144-3p regulated cell proliferation, migration, and invasion by inhibiting *ZEB1* [36]. Additionally, miR-572 modulated the invasive phenotype and cisplatin resistance of gastric cancer cells through *ZEB1* regulation [16]. In this study, we confirmed the targeting relationship between miR-34b-5p and *ZEB1*. Furthermore, overexpression of miR-34b-5p significantly downregulated the expression levels of *ZEB1* mRNA and protein. Conversely, *ZEB1* overexpression partially restored or reversed the inhibitory effects of miR-34b-5p on the proliferation, migration, invasion, and EMT of endometrial cancer AN3CA cells. This indicated that miR-34b-5p may suppress the progression and metastasis of endometrial cancer by targeting ZEB1.

While these findings provide vital theoretical guidance and experimental foundations for personalized therapeutic strategies in endometrial cancer, our study is not without limitations. First, our investigation primarily elucidated the regulatory mechanisms of miR-34b-5p on endometrial cancer at the cellular level, but the applicability to clinical samples is uncertain. Second, our focus on the AN3CA cell line may overlook possible variations among different cell lines, influencing the robustness of our results. Moreover, the absence of in vivo experiments warrants further exploration in subsequent studies. Therefore, future research demands an expansion of the experimental scope to comprehensively uncover the functions and mechanisms of miR-34b-5p and *ZEB1* in endometrial cancer EMT and metastasis. Addressing these limitations will fortify the foundation of our understanding and may support new clinical therapy for endometrial cancer.

To summarize, this study elucidated the downregulation of miR-34b-5p in endometrial cancer, highlighting its role as a suppressor in cell proliferation, migration, invasion, and EMT. Furthermore, *ZEB1* emerged as a direct target of miR-34b-5p, and the upregulation of *ZEB1* expression counteracted the inhibitory effects of miR-34b-5p on endometrial cancer cell proliferation, migration, invasion, and EMT. miR-34b-5p likely inhibits EMT and metastasis in endometrial cancer cells by directly targeting *ZEB1*. This suggests that miR-34b-5p and ZEB1 could serve as therapeutic targets for molecularly targeted treatment in endometrial cancer.

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Disclosure of conflict of interest

None.

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References

[1] Jamieson A and McAlpine JN. Molecular profiling of endometrial cancer from TCGA to clinical practice. J Natl Compr Canc Netw 2023; 21: 210-216.

[2] Del Carmen MG, Boruta DM 2nd and Schorge JO. Recurrent endometrial cancer. Clin Obstet Gynecol 2011; 54: 266-277.

[3] Mao W, Wei S, Yang H, Yu Q, Xu M, Guo J and Gao L. Clinicopathological study of organ metastasis in endometrial cancer. Future Oncol 2020; 16: 525-540.

[4] Shi Y, Liu Z, Lin Q, Luo Q, Cen Y, Li J, Fang X and Gong C. MiRNAs and cancer: key link in diagnosis and therapy. Genes (Basel) 2021; 12: 1289.

[5] Mishra S, Yadav T and Rani V. Exploring miRNA based approaches in cancer diagnostics and therapeutics. Crit Rev Oncol Hematol 2016; 98: 12-23.

[6] Jang H, Park S, Kim J, Kim JH, Kim SY, Cho S, Park SG, Park BC, Kim S and Kim JH. The tumor suppressor, p53, negatively regulates non-canonical NF-κB signaling through miRNAInduced Silencing of NF-κB-inducing kinase. Mol Cells 2020; 43: 23-33.

[7] Dong L, Chen F, Fan Y and Long J. MiR-34b-5p inhibits cell proliferation, migration and invasion through targeting ARHGAP1 in breast cancer. Am J Transl Res 2020; 12: 269-280.

[8] Tian J, Cui P, Li Y, Yao X, Wu X, Wang Z and Li C. LINC02418 promotes colon cancer progression by suppressing apoptosis via interaction with miR-34b-5p/BCL2 axis. Cancer Cell Int 2020; 20: 460.
[9] Luo G, Zhang Y, Wu Z, Zhang L, Liang C and Chen X. Exosomal LINC00355 derived from cancer-associated fibroblasts promotes bladder cancer cell resistance to cisplatin by regulating miR-34b-5p/ABCB1 axis. Acta Biochim Biophys Sin (Shanghai) 2021; 53: 558-566.
[10] Aiello NM, Maddipati R, Norgard RJ, Balli D, Li J, Yuan S, Yamazoe T, Black T, Sahmoud A, Furth EE, Bar-Sagi D and Stanger BZ. EMT subtype influences epithelial plasticity and mode of cell migration. Dev Cell 2018; 45: 681-695, e4.
[11] Yeung KT and Yang J. Epithelial-mesenchymal transition in tumor metastasis. Mol Oncol 2017; 11: 28-39.
[12] Park MK, Lee H and Lee CH. Post-translational modification of ZEB family members in cancer progression. Int J Mol Sci 2022; 23: 15127.
[13] Wu HT, Zhong HT, Li GW, Shen JX, Ye QQ, Zhang ML and Liu J. Oncogenic functions of the EMT-related transcription factor ZEB1 in breast cancer. J Transl Med 2020; 18: 51.
[14] Larsen JE, Nathan V, Osborne JK, Farrow RK, Deb D, Sullivan JP, Dospoy PD, Augustyn A, Hight SK, Sato M, Girard L, Behrens C, Wistuba II, Gazdar AF, Hayward NK and Minna JD. ZEB1 drives epithelial-to-mesenchymal transition in lung cancer. J Clin Invest 2016; 126: 3219-3235.
[15] Li LY, Yang JF, Rong F, Luo ZP, Hu S, Fang H, Wu Y, Yao R, Kong WH, Feng XW, Chen BJ, Li J and Xu T. ZEB1 serves an oncogenic role in the tumourigenesis of HCC by promoting cell proliferation, migration, and inhibiting apoptosis via Wnt/β-catenin signaling pathway. Acta Pharmacol Sin 2021; 42: 1676-1689.
[16] Wang M, Zhang R, Zhang S, Xu R and Yang Q. MicroRNA-574-3p regulates epithelial mesenchymal transition and cisplatin resistance via targeting ZEB1 in human gastric carcinoma cells. Gene 2019; 700: 110-119.
[17] Kuokkanen S, Chen B, Ojalvo L, Benard L, Santoro N and Pollard JW. Genomic profiling of microRNAs and messenger RNAs reveals hormonal regulation in microRNA expression in human endometrium. Biol Reprod 2010; 82: 791-801.
[18] Liao Y, Li R, Pei J, Zhang J, Chen B, Dong H, Feng X, Zhang H, Shang Y, Sui L and Kong Y. Melatonin suppresses tumor proliferation and metastasis by targeting GATA2 in endometrial cancer. J Pineal Res 2024; 76: e12918.
[19] Akar S, Çelik ZE, Fındık S, İlhan TT, Ercan F and Çelik Ç. Prognostic significance of solid growth in endometrioid endometrial adenocarcinoma. Int J Clin Oncol 2020; 25: 195-202.
[20] Tronconi F, Nero C, Giudice E, Salutari V, Musacchio L, Ricci C, Carbone MV, Ghizzoni V, Perri MT, Camarda F, Gentile M, Berardi R, Scambia G and Lorusso D. Advanced and recurrent endometrial cancer: state of the art and future perspectives. Crit Rev Oncol Hematol 2022; 180: 103851.
[21] Banz-Jansen C, Helweg LP and Kaltschmidt B. Endometrial cancer stem cells: where do we stand and where should we go? Int J Mol Sci 2022; 23: 3412.
[22] Yu J, Chen L, Bao Z, Liu Y, Liu G, Li F and Li L. BMI-1 promotes invasion and metastasis in endometrial adenocarcinoma and is a poor prognostic factor. Oncol Rep 2020; 43: 1630-1640.
[23] Liu J, Cui G, Shen S, Gao F, Zhu H and Xu Y. Establishing a prognostic signature based on epithelial-mesenchymal transition-related genes for endometrial cancer patients. Front Immunol 2022; 12: 805883.
[24] Saliminejad K, Khorram Khorshid HR, Soleymani Fard S and Ghaffari SH. An overview of microRNAs: biology, functions, therapeutics, and analysis methods. J Cell Physiol 2019; 234: 5451-5465.
[25] Pinto R, De Summa S, Petriella D, Tudoran O, Danza K and Tommasi S. The value of new high-throughput technologies for diagnosis and prognosis in solid tumors. Cancer Biomark 2014; 14: 103-117.
[26] Zhang S and Cui Z. MicroRNA-34b-5p inhibits proliferation, stemness, migration and invasion of retinoblastoma cells via Notch signaling. Exp Ther Med 2021; 21: 255.
[27] Zhao J, Lin H, Huang K and Li S. Cancer-associated fibroblasts-derived extracellular vesicles carrying lncRNA SNHG3 facilitate colorectal cancer cell proliferation via the miR-34b-5p/HuR/HOXC6 axis. Cell Death Discov 2022; 8: 346.
[28] Guo X, Zhou Q, Su D, Luo Y, Fu Z, Huang L, Li Z, Jiang D, Kong Y, Li Z, Chen R and Chen C. Circular RNA circBFAR promotes the progression of pancreatic ductal adenocarcinoma via the miR-34b-5p/MET/Akt axis. Mol Cancer 2020; 19: 83.
[29] Perez-Oquendo M and Gibbons DL. Regulation of ZEB1 function and molecular associations in tumor progression and metastasis. Cancers (Basel) 2022; 14: 1864.
[30] Caramel J, Ligier M and Puisieux A. Pleiotropic roles for ZEB1 in cancer. Cancer Res 2018; 78: 30-35.
[31] Lu J, Fei F, Wu C, Mei J, Xu J and Lu P. ZEB1: catalyst of immune escape during tumor me-

tastasis. Biomed Pharmacother 2022; 153: 113490.
[32] Gao Y, Cheng X and Han M. ZEB1-activated Notch1 promotes circulating tumor cell migration and invasion in lung squamous cell carcinoma. Clin Transl Oncol 2023; 25: 817-829.
[33] Xu Y, Guo B, Liu X and Tao K. miR-34a inhibits melanoma growth by targeting ZEB1. Aging (Albany NY) 2021; 13: 15538-15547.
[34] Chen L, Heikkinen L, Wang C, Yang Y, Sun H and Wong G. Trends in the development of miRNA bioinformatics tools. Brief Bioinform 2019; 20: 1836-1852.
[35] Zheng T, Han W, Wang A and Wang Y. Functional mechanism of hsa-miR-128-3p in epithelial-mesenchymal transition of pancreatic cancer cells via ZEB1 regulation. PeerJ 2022; 10: e12802.
[36] Jiang M, Jike Y, Liu K, Gan F, Zhang K, Xie M, Zhang J, Chen C, Zou X, Jiang X, Dai Y, Chen W, Qiu Y and Bo Z. Exosome-mediated miR-144-3p promotes ferroptosis to inhibit osteosarcoma proliferation, migration, and invasion through regulating ZEB1. Mol Cancer 2023; 22: 113.