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MicroRNA-200c Inhibits Epithelial-Mesenchymal Transition by Targeting the *BMI-1* Gene Through the Phospho-AKT Pathway in Endometrial Carcinoma Cells *In Vitro*

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: MicroRNA-200c (miR-200c) is a short non-coding RNA that has a role in tumorigenesis and cancer progression. The aims of this study were to investigate the role of miR-200c in cell migration and epithelial-mesenchymal transition (EMT) in endometrial carcinoma cells *in vitro*.

Material/Methods: Potential direct targets of miR-200c were identified through the TargetScan database. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used study the expression of miR-200c in the endometrial carcinoma cell lines, Ishikawa and JEC, *in vitro*. Cell migration was studied using transwell assays. Expression of the mesenchymal marker, N-cadherin, the epithelial marker, E-cadherin, the transcription factor, Slug, the *BMI-1* protein, AKT, and p-AKT were measured using Western blot. Small interfering RNA (siRNA) was used to silence the *BMI-1* gene to study the targeting effect.

Results: Over-expression of miR-200c in Ishikawa and JEC cells resulted in reduced cell migration and proliferation. Western blot showed that overexpression of miR-200c downregulated the expression of the *BMI-1* protein, p-AKT, N-cadherin and Slug, and the expression E-cadherin was upregulated; silencing miR-200c reversed these results. Silencing the *BMI-1* gene inhibited EMT and suppressed p-AKT in miR-200c-inhibited endometrial carcinoma cells by increasing E-cadherin expression, reducing the expression of N-cadherin and the EMT-associated transcription factor, Slug.

Conclusions: In endometrial carcinoma cells *in vitro*, miR-200c inhibited EMT by targeting the *BMI-1* gene through the p-AKT pathway.

MeSH Keywords: **Carcinoma, Endometrioid • Epithelial-Mesenchymal Transition • MicroRNAs • Proto-Oncogene Proteins c-akt**

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Background

Worldwide, endometrial carcinoma is one of the most common gynecologic malignancies and is increasing in incidence with 320,000 new cases of endometrial carcinoma reported in 2012 [1]. It has been estimated that, in the USA, 54,870 women will be diagnosed with endometrial carcinoma and 10,170 women will die from this malignancy in 2015 [2]. In the USA in 2017, it has been estimated that endometrial carcinoma will be ranked fourth among expected new cancer cases in women with an estimated 61,380 new cases and 10,920 deaths [3].

The prognosis of endometrial carcinoma is dependent on many factors, including the tumor stage, and grade [4]. Because of the potential for tumor recurrence and metastasis and the lack of response in some patients to hormone therapy, radiotherapy, and chemotherapy, the median overall survival time is for endometrial carcinoma is short [5]. Radical surgery can be an effective treatment for early-stage endometrial carcinoma, but this tumor metastasizes in 40% of cases [6]. To improve patient prognosis, when diagnosis at an early stage is not possible, the identification of the molecules and signaling pathways involved in tumor metastasis may provide biomarker targets for diagnosis and treatment.

In epithelial-mesenchymal transition (EMT), epithelial cells lose both cell-cell and cell-extracellular matrix contacts, undergo cytoskeletal remodeling, and acquire a migratory phenotype, which are factors involved in tumor metastasis [7]. EMT has been categorized into three types, based on different functional effects: type I EMT is involved in embryonic development; type II EMT participates in wound healing and fibrosis; type III EMT is associated with cancer progression and metastasis [8–10]. Specific signals induce type III EMT in carcinoma cells, leading to a characteristic reduction in E-cadherin expression and upregulation of mesenchymal proteins, including N-cadherin and vimentin. Also, EMT-inducing regulators inhibit the interaction of E-cadherin transcription with specific E-boxes of the proximal E-cadherin promoter [11-12]. Common EMT characteristics have been described in endometrial carcinoma, including the loss of E-cadherin expression, and other molecular changes consistent with the mesenchymal phenotype [13]. Recently published studies have shown that EMT regulation in endometrial carcinoma is associated with PI3K activity, estrogen signaling, and microRNA (miRNA) expression [14]. EMT has also been shown to be dependent on the expression of several common differentiation markers, including Snail/Slug, Twist, ZEB 1/2, and AKT/PI3K, and the PI3K/AKT/mTOR pathway is an important cell cycle regulator in EMT, cell proliferation, and cancer [15]. However, further studies are required to understand the complex relationships between these factors.

miRNAs are a group of endogenous, small, non-coding RNAs that influence multiple biological processes by post-transcriptional repression of gene expression or by inducing mRNA degradation via binding to the 3' untranslated region (UTR) of specific target genes [16,17]. Recently, miRNAs have received increasing global attention due to their known ability to transcriptionally silence genes and modulate multiple signaling cascades. Recent studies have shown that miRNA expression plays an important role in tumorigenesis and cancer progression [18,19]. Aberrant miRNA expression is associated with tumorigenesis, tumor metastasis, and disease progression due to its involvement in EMT, anti-apoptosis, and angiogenesis [20–22].

Abnormal expression of miRNA has been shown to be involved in the development of endometrial carcinoma, and miRNA regulates EMT in endometrial carcinosarcoma [23–26]. The most important miRNAs involved in EMT belong to the miR-200 family, which include miR-200a, miR-200b, miR-200c, miR-141, and miR-429, which is a key regulator of EMT. The miR-200 family members can target ZEB (also known as SIP1), which is a transcriptional inhibitor of E-cadherin [27,28]. Some reports have suggested that AKT/phosphorylated AKT (p-AKT) is involved in the regulation of miR-200 expression, although the mechanism of action has not yet been determined [29–31]. Also, the newly identified tumor suppressor roles of the miR-200 family, including inhibition of cancer stem cell self-renewal and differentiation, modulation of cell division and apoptosis, and reversal of chemoresistance, support the view that the miR-200 family members are important miRNAs in cancer research [32].

BMI-1 is a polycomb group (PcG) protein, which is an important class of transcriptional repressors that orchestrate changes in chromatin structures to regulate gene activity, and many PcG proteins demonstrate altered expression in human cancers [33,34], including in endometrial carcinoma [35]. Previously published studies have shown that certain miRNAs inhibit EMT in cancer cells by targeting the *BMI-1* oncogene [36–38]. In several types of cancer, the miR-200 family modulate EMT-associated factors, such as Bmi-1 and E-cadherin, preserving an epithelial phenotype [39].

The aims of this study were to investigate the role of miR-200c in cell migration and epithelial-mesenchymal transition (EMT) in endometrial carcinoma cells *in vitro*. By studying the mechanisms of miR-200c regulation of EMT in endometrial carcinoma cells *in vitro*, these findings of this study showed a direct association between the expression of miR-200c and, p-AKT, *BMI-1*, E-cadherin, N-cadherin, and Slug, indicating the capacity of endometrial carcinoma cells to proliferate, invade, and metastasize. In this study, we found that miR-200c suppressed EMT by targeting the *BMI-1* gene through the p-AKT pathway.

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Material and Methods

Cell culture

The human endometrial carcinoma cell lines, Ishikawa and JEC, were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Both cell lines were cultured in RPMI 1640 medium (HyClone, Utah, USA) with 10% fetal bovine serum (FBS) (Gibco, Australia), and 2.0 g/L NaHCO**3** and cultured at 37°C in 5% CO**2** and 95% air.

Cell transfection

The Ishikawa and JEC cells were seeded into a 6-well plate (a density of 5×10^5 cells/well) and grown to 50–80% confluence. The cells were then transfected with 100 nmol of RNA using Lipofectamine 2000 in accordance with the manufacturer's protocol (Invitrogen, USA). The cells were divided into three groups: 1) the control group or negative control (NC) group (transfected with miR-200c mimic NC or inhibitor NC); 2) the miR-200c mimic group (transfected with miR-200c mimic sequence), and; 3) the miR-200c inhibitor group (transfected with miR-200c inhibitor sequence to silence miR-200c) using different transfection sequences.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol (Invitrogen), treated with DNase I (Takara, Tokyo) to eliminate contaminating genomic DNA, and then reverse transcribed using a PrimeScript™ RT reagent Kit (Perfect Real Time) (Dalian TaKaRa Biotechnology Co., Ltd. China). qRT-PCR was performed in a reaction volume of 20µl containing SYBR green PCR mix according to the manufacturer's protocol. Each sample was run in triplicate. U6 snRNA was used as an endogenous control. All samples were normalized to internal controls, and fold changes were calculated through relative quantification.

The primer sequences for miR-200c and U6 snRNA were as follows:
miR-200c: Forward Primer UAAUACUGCCGGGUAAUGAUGGA
Reverse Primer CAUCAUUACCCGGCAGUAUUAUU
U6: Forward Primer CTCGCTTCGGCAGCACA
Reverse Primer AACGCTTCACGAATTTGCGT

MTT cell viability assay

Briefly, 24 h after the cells were seeded in a 96-well plate (200 µl/well; 2000 cells/well), with six wells for each group, the cells were transfected with miR-200c mimic, miR-200c inhibitor or NC. The plate was then placed in an incubator with a 5% CO**2** atmosphere at 37°C. After 6 h, the cells were incubated in complete medium as indicated. At 24 h, 48 h, 72 h, 96 h, 20 µl of 1 mg/ml MTT (Sigma, USA) was added to each well. After 4 h of incubation at 37°C in 5% CO_2 and 95% air, the medium was removed and precipitated formazan was dissolved in 200 µl of dimethyl sulfoxide (DMSO). After shaking for 15 min, the absorbance of the medium was measured at 495 nm with a microplate reader (Bio-Tek, USA). The effect of miR-200c on cell growth and cell viability was determined.

Transwell invasion assay

Briefly, 24-well transwell chambers of 8 µm pore size (Corning Costar, Cambridge, MA, USA) were used to compare the cell migration and invasion properties. At 48 h after transfection, the cells were seeded in the upper portion of the 24-well transwell chambers in 200 µl of serum-free medium (1×10^5 cells/mL). After 24 h of incubation with different treatments, non-invasive cells were removed. The cells on the underside of the chambers were fixed in methanol and 3.7% formaldehyde solution, each for 5 min. Then, the invasive cells were stained with crystal violet for 30 min and imaged using an Olympus IX51 (Olympus Optical, Melville, NY, USA) inverted microscope and cells in five individual fields were counted. Three independent experiments were performed for statistical analysis.

Western blotting

After transfection for 48 h, proteins were extracted, and Western blotting was performed. After different treatments, the proteins were extracted using RIPA lysis buffer (Beyotime, Jiangsu, China) with 1% phenyl methyl sulfonyl fluoride (PMSF) (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 1% NaF (Beyotime, Jiangsu, China). The proteins were then separated on a 10% or 12% polyacrylamide gel (PAGE) and transferred to a pure nitrocellulose blotting membrane. The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes, followed by incubation with primary rabbit monoclonal antibodies (1: 1000). The membranes were washed, incubated with secondary goat anti-rabbit IgG, horseradish peroxidase (HRP)-conjugated antibodies (1: 5000) (Abcam, Cambridge, MA, USA) for 2h. Primary antibodies targeting epithelial-mesenchymal transition (EMT) markers (E-cadherin, N-cadherin, and Slug) and the loading control (GAPDH) were purchased from Cell Signaling Technology (USA). p-AKT, AKT, and Bmi-1 antibodies were obtained from Abcam (Cambridge, MA, USA). The results were analyzed using ImageJ software. Three independent experiments were performed for statistical analysis.

BMI-1 gene siRNA transfection

The sequence targeting of the *BMI-1* gene and scrambled negative siRNA control were purchased from GenePharma (Shanghai, China).

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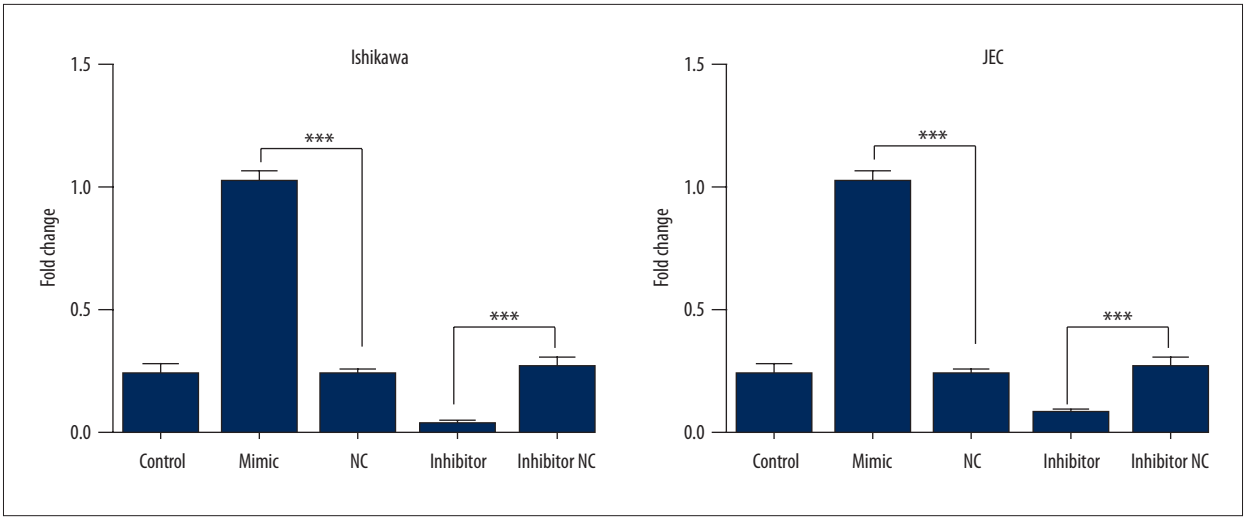

Figure 1. Quantitative real-time polymerase chain reaction (qRT-PCR) detection of the transfection efficiency. Data are presented mean ±S.D. The symbol *** denotes the statistically significant difference of P<0.001 by an independent t-test. The results are representative of three separate experiments.

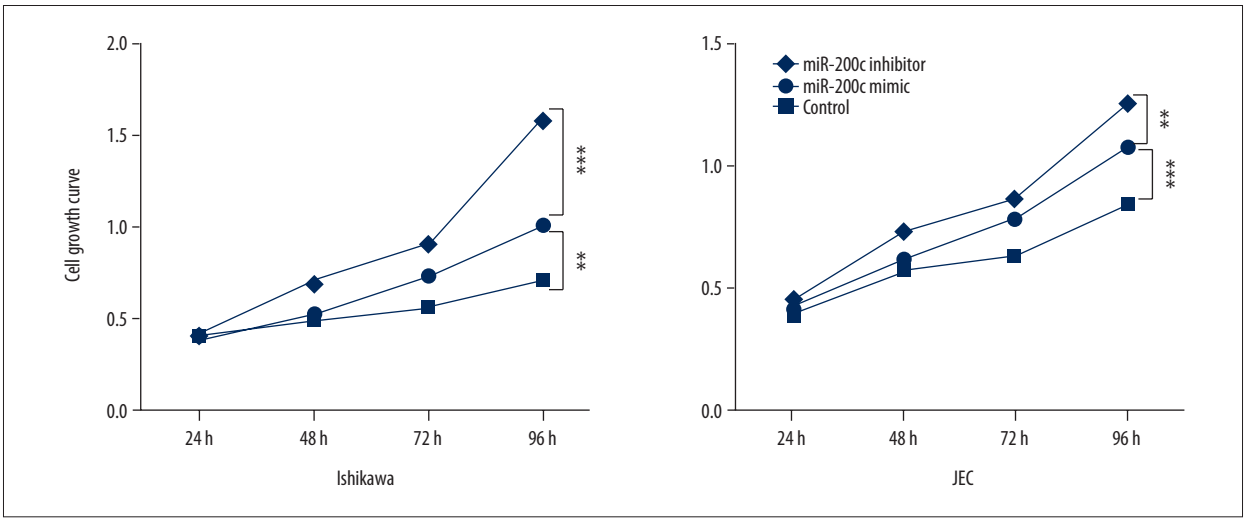

Figure 2. Growth curves of Ishikawa and JEC cells after transfection of miR-200c mimic or miR-200c inhibitor for 96 hours. Data are presented the mean ±S.D. The symbols ** and *** denote the statistically significant difference of P<0.01 and P<0.001 (versus control). The results are representative of three separate experiments.

The *BMI-1* gene siRNA sequences were as follows:
Forward Primer: 5'-CCAGAUUGAUGUCAUGUAUTT
Reverse Primer: AUACAUGACAUCAAUCUGGTT-3'

The cells were seeded into 6-well plates overnight, to reach 50–60% confluence, and transfected with *BMI-1* siRNA/NC and the miR-200c inhibitor sequence according to the manufacturer's instructions. After 6 h, the cells were incubated in complete medium, as indicated. Then, proteins were extracted, and Western blotting was performed after 48 h. Three independent experiments were performed for statistical analysis.

Statistical analysis

All experiments were performed at least three times for statistical analysis. The results are presented as the mean ±SEM. The data were analyzed with GraphPad Prism Version5.01 (GraphPad Software, Inc., La Jolla, CA, USA) using two-tailed Student's t-test. P<0.05 was considered to be statistically significant.

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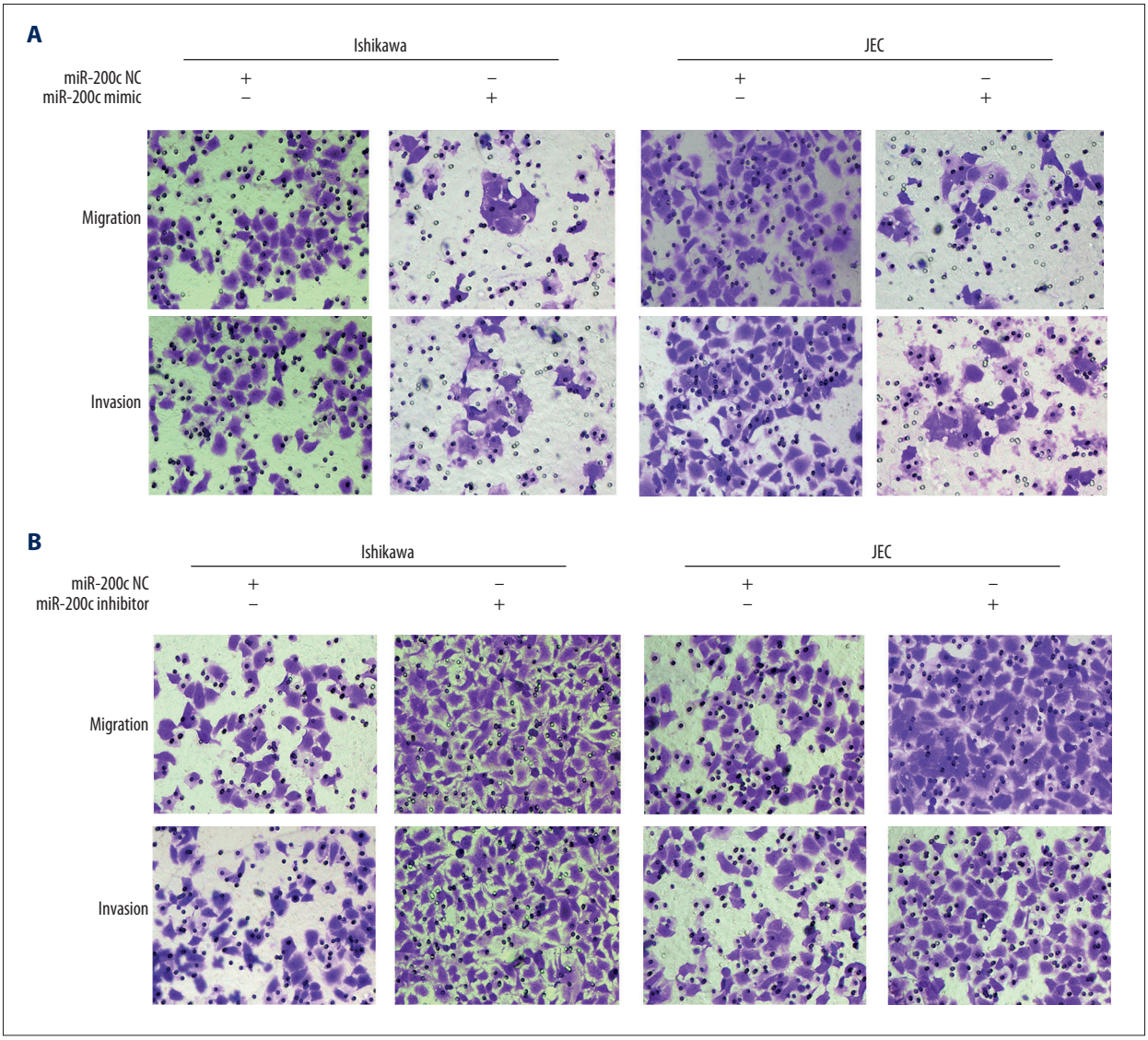
Results

miR-200c suppressed endometrial carcinoma cell growth and proliferation *in vitro*

To determine the potential functional roles of miR-200c in the Ishikawa and JEC endometrial carcinoma cell lines, transfection was performed with the miR-200c mimic to over-express miR-200c. Also, both cell lines were transfected with the miR-200c inhibitor to silence miR-200c expression. The transfection efficiency was assessed with qRT-PCR. The results showed that the miR-200c mimic significantly activated miR-200c expression, while the miR-200c inhibitor suppressed miR-200c expression (P<0.001) (Figure 1). The MTT assay results showed that cell growth and proliferation were lower in miR-200c mimic-transfected Ishikawa and JEC cells compared with the controls, and cell growth and proliferation increased after the cells were transfected with miR-200c inhibitor (Figure 2).

Over-expression of miR-200c reduced migration and invasion of Ishikawa and JEC cells and silencing miR-200c increased migration and invasion of Ishikawa and JEC cells

After the cells were transfected for 48 h, the effects of miR-200c on cell migration and invasion using transwell assays were assessed. The transwell assay results showed that that over-expression of miR-200c significantly reduced the migration and invasion ability of Ishikawa and JEC cells (P<0.001) (Figure 3A, 3C). Conversely, inhibiting miR-200c significantly promoted the cell migration and invasion ability of both cell lines (P<0.001) (Figure 3B, 3D).



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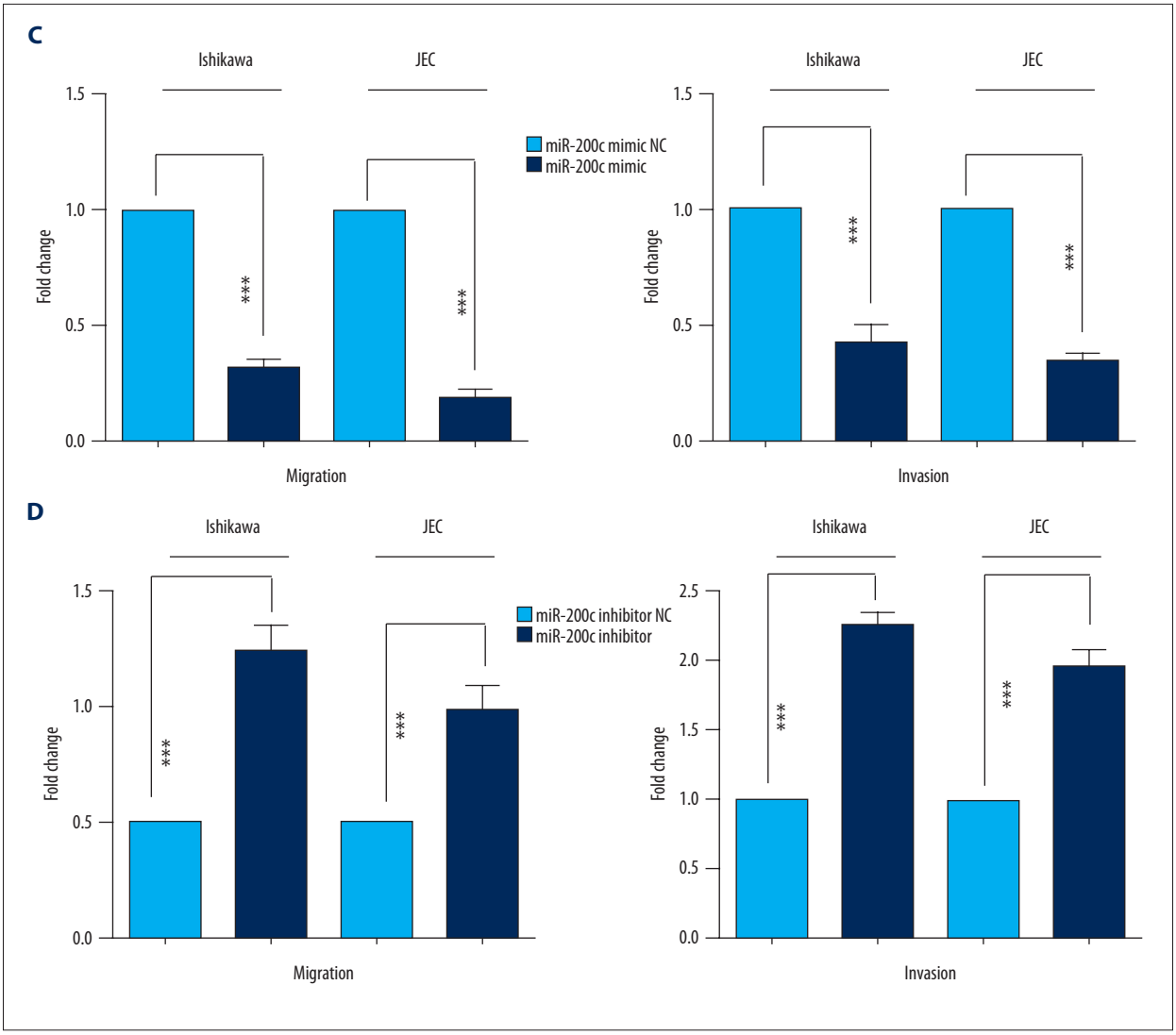

Figure 3. The effects of miR-200c on cell migration and invasion. (**A, B**): Representative transwell migration and invasion assay of Ishikawa and JEC cells (magnification ×200). (**C, D**): Quantification of migration and invasion abilities of Ishikawa and JEC cells versus negative control (NC).*** P<0.001.

miR-200c inhibited EMT in Ishikawa and JEC cells by downregulating the *BMI-1* gene through the p-AKT pathway

miRNAs exert their function by regulating their target genes, and through the bioinformatics prediction software TargetScan (*http://targetscan.org*), a common set of candidate genes were identified that included the *BMI-1* gene. To further investigate the mechanisms of miR-200c in the migration and invasion and to validate the *BMI-1* gene as a target gene of miR-200c in Ishikawa and JEC cells, we transfected Ishikawa and JEC cells with miR-200c mimic, miR-200c mimic negative control (NC), miR-200c inhibitor, miR-200c inhibitor NC or *BMI-1* siRNA to examine the expression of EMT-associated proteins and *BMI-1* protein. The Western blot results showed that, when the cells were transfected with miR-200c mimic, the expression of the mesenchymal marker N-cadherin and the downstream EMT transcription factor, Slug, were decreased, while the expression of the epithelial marker E-cadherin was increased relative to the control cells. Also, we tested whether miR-200c inhibited EMT by regulating p-AKT and *BMI-1*, and the results showed that the expression of p-AKT and Bmi-1 were lower in both cell lines compared with the control after transfection with miR-200c mimic (Figure 4A, 4B). However, all the results were reversed in the two cell lines after transfection with miR-200c inhibitor (Figure 4C, 4D).

To further confirm that miR-200c inhibited EMT by targeting the *BMI-1* gene, we knocked down *BMI-1* expression in miR-200c inhibited cells (after transfection of Ishikawa and JEC cells

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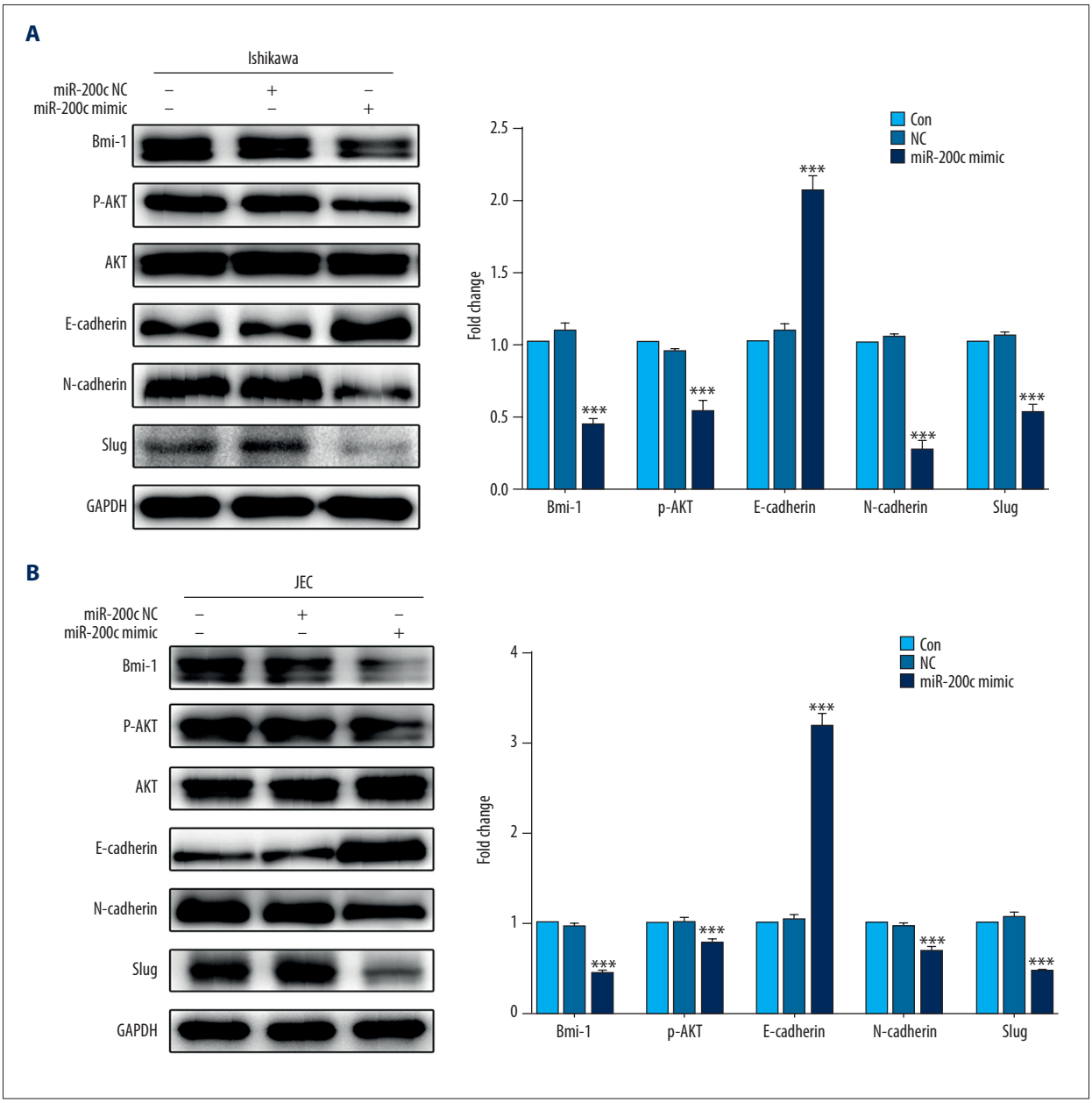
with the miR-200c inhibitor for 24 h). The expression of EMT-associated proteins, by Western blotting showed that the expression of N-cadherin and Slug decreased, while the expression of E-cadherin increased compared with the *BMI-1* siRNA NC group (Figure 5A, 5B).

Discussion

Recently, several studies have reported that miRNAs may inhibit epithelial-mesenchymal transition (EMT) via target genes in cancer cells and cancer stem cells [26–30]. miRNAs can target approximately 20–30% of genes [40]. A single miRNA can target many genes, and a single gene can be regulated by many miRNAs [40]. Therefore, the mechanisms of miRNAs in the inhibition of EMT are highly complex, and the role of miRNAs in EMT of endometrial carcinoma is poorly understood. miR-200c is important in cancer progression because it acts as a key modulator in E-cadherin upregulation, and in downregulation of the *BMI-1* gene [28,36]. Transfection of endometrial carcinoma cells with miR-194 has shown that miR-194 also directly targets the *BMI-1* gene and can reverse the EMT signature [36], but the mechanisms remain unclear. This is the first study to demonstrate that miR-200c inhibits EMT by targeting the *BMI-1* gene through the p-AKT pathway in endometrial carcinoma cells *in vitro*.



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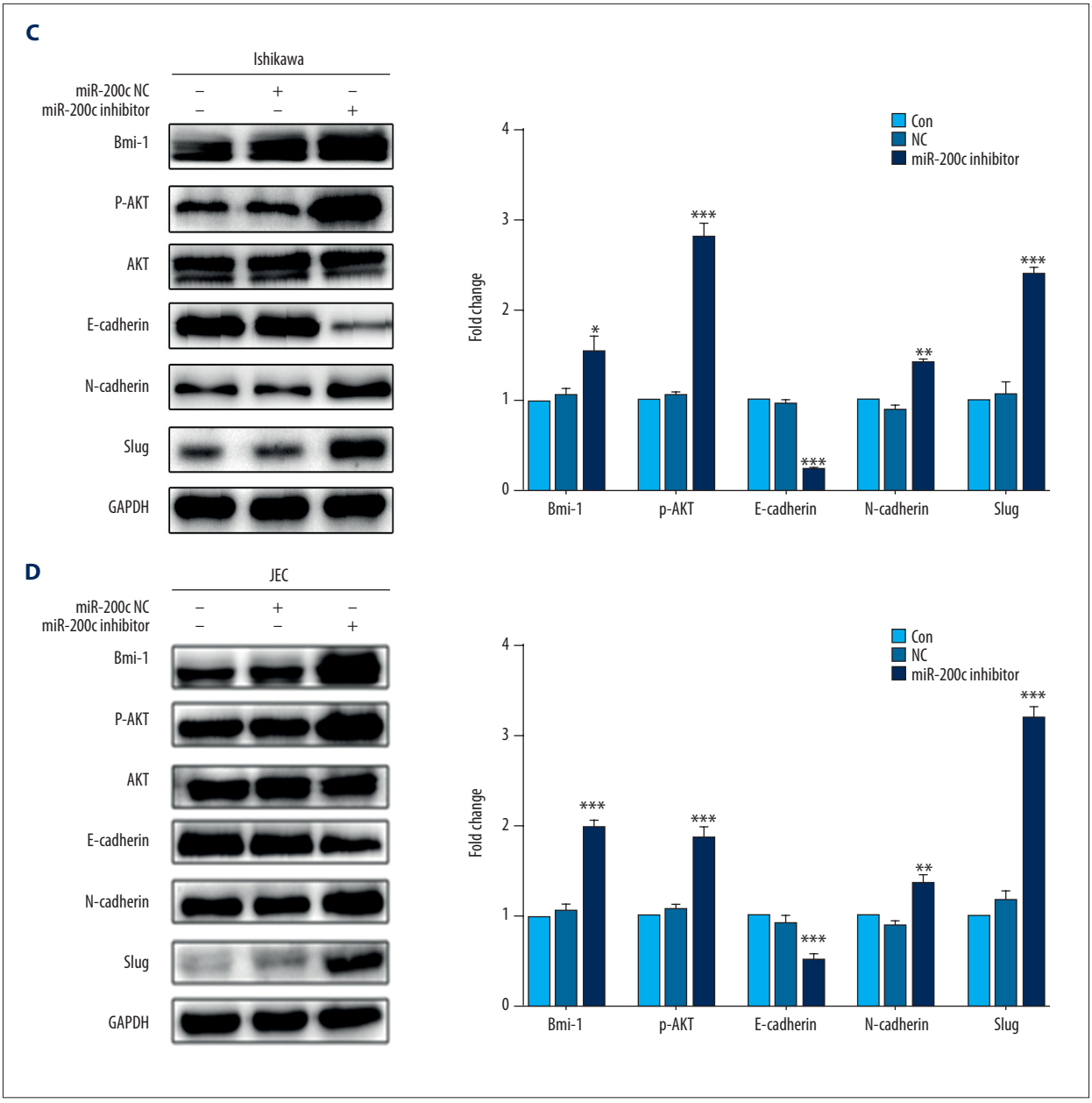

Figure 4. The expression of epithelial-mesenchymal transition (EMT) markers, *BMI-1* and p-AKT after the cells were transfected with miR-200c mimic or miR-200c inhibitor. (**A, B**): Western blotting showing the quantitative analysis of the expression of epithelial-mesenchymal transition (EMT) markers, *BMI-1,* and p-AKT after the cells were transfected with miR-200c mimic. (**C, D**): Western blotting showing the quantitative analysis of the expression of EMT, *BMI-1,* and p-AKT after the cells were transfected with miR-200c inhibitor. EMT – epithelial-mesenchymal transition. ** P<0.01; *** P<0.001.

By miRNA TargetScan analysis (*http://www.targetscan.org*) and recently published studies, we confirmed that the *BMI-1* oncogene is a direct target of miR-200c. We found that upregulation of miR-200c expression in the endometrial carcinoma cell lines, Ishikawa and JEC, *in vitro* resulted in reduced expression of Bmi-1 and p-AKT, and reduced invasion, migration, and cell proliferation, whereas silencing miR-200c further upregulated the *BMI-1* gene and p-AKT and promoted progression, which is consistent with previous studies [31,39]. As a polycomb gene family member, the *BMI-1* gene plays a key role in cell cycle regulation, cell oncogenesis, and cell senescence. The *BMI-1* gene has previously been reported to be upregulated in human cancers and plays important oncogenic roles in cancer initiation and progression [41–43].

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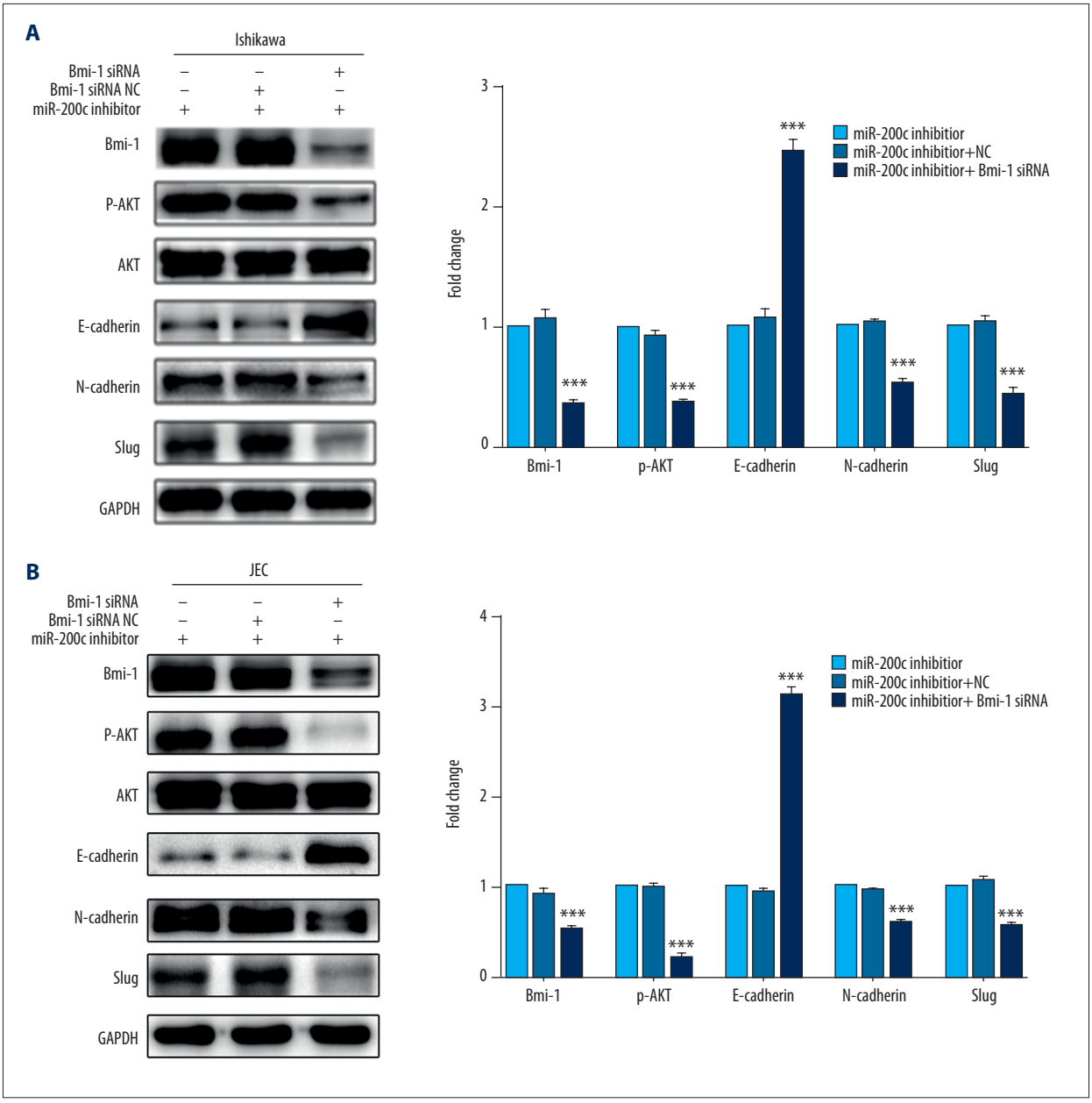

Figure 5. The expression of epithelial-mesenchymal transition (EMT) markers, *BMI-1* and p-AKT after knockout of *BMI-1* in miR-200c inhibitor cells. (**A, B**): Western blotting showing quantitative analysis of the expression of EMT, *BMI-1* and p-AKT, after knockout of *BMI-1* in miR-200c inhibitor cells, following Ishikawa and JEC cells transfection with miR-200c after 24 hours. EMT – epithelial-mesenchymal transition. ** P<0.01; *** P<0.001.

miR-200c has been reported to directly bind to the 3'UTR of the *BMI-1* gene, decreasing *BMI-1* mRNA and protein expression levels [39]. In our study, we found that silencing miR-200c upregulated Bmi-1 and p-AKT and promoted EMT while silencing the *BMI-1* gene inhibited EMT and suppressed p-AKT in the miR-200c-inhibited endometrial carcinoma cells. In a previous study, the *BMI-1* gene was found to regulate AKT phosphorylation in human cancers [44,45]. The findings of this study are consistent with the results of these previous studies and demonstrate that miR-200c directly targets the *BMI-1* gene in the endometrial carcinoma cell lines, Ishikawa and JEC, *in vitro*.

Molecular changes during EMT are characterized by loss of the epithelial marker, E-cadherin, and increased expression of the mesenchymal markers, vimentin and N-cadherin, and activation of the related pathways, leading to an increase in migratory and invasive behavior [9]. The *BMI-1* gene has been reported to induce EMT by reducing E-cadherin expression [46]. In this study,

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we showed that upregulation of miR-200c expression increased E-cadherin expression and decreased N-cadherin and Slug expression, whereas down-regulation of miR-200c expression decreased E-cadherin expression and increased N-cadherin and Slug expression. These data suggest that miR-200c down-regulates Bmi-1 expression and then modulates EMT by stimulating E-cadherin expression and suppressed N-cadherin expression through degradation of the EMT transcription factor Slug. Also, p-AKT is involved in this process. Therefore, we suggest that miR-200c may inhibit EMT by targeting the *BMI-1* gene through the p-AKT pathway. However, the process is complex, and the mechanism needs to be studied further.

Conclusions

The findings of this *in vitro* study, using the endometrial carcinoma cell lines, Ishikawa and JEC, showed that upregulation of miR-200c reduced the migratory and invasive capacities of endometrial carcinoma cells, potentially through the miR-200c targeting the *BMI-1* gene by the p-AKT pathway, leading to a decrease in the expression of the transcription factor Slug, a decrease in the expression of mesenchymal marker N-cadherin, and an increase in the expression of the epithelial marker E-cadherin. These findings indicate that further studies are required to determine whether targeting miR-200c may be an effective treatment method for inhibiting metastasis of endometrial carcinoma *in vivo*.

Conflicts of interest

None.

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