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MTA1, a Target of Resveratrol, Promotes Epithelial-Mesenchymal Transition of Endometriosis via ZEB2

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Endometriosis is a benign disease that shares some malignant features. Epithelial-mesenchymal transition (EMT) is involved in the pathogenesis of endometriosis. Metastasis-associated protein 1 (MTA1) plays an important role in various cancers by promoting EMT, yet there are no studies on its function in endometriosis. In the present study, we found that MTA1 was highly expressed in the ectopic endometrium of endometriosis patients and that the expression of MTA1 was related to the revised American Fertility Society stage. MTA1 facilitated endometrial stroma cell proliferation, migration, and invasion by inducing EMT, and the promotion function and MTA1 expression were suppressed by resveratrol, a natural polyphenol. Moreover, we revealed that MTA1 induced EMT through interaction with ZEB2. The findings in a mouse endometriosis model further showed that MTA1 and ZEB2 were upregulated in ectopic tissues and that resveratrol inhibited the growth of ectopic lesions and expression of MTA1 and ZEB2. Taken together, we demonstrate that MTA1 is a protein that promotes EMT via interacting with ZEB2 in the pathogenesis of endometriosis, and may be a target of resveratrol.

INTRODUCTION

Endometriosis is a common disease that affects 5%–10% of women at reproductive age. It is defined by the presence of functional ectopic endometrial glands and stroma within extra-uterine sites, primarily within the ovaries.[1,2] It is widely accepted that endometriosis is caused by endometrial tissue in menstruation retrograded into the peritoneal cavity through the fallopian tubes.[3] Approximately 90% of women have retrograde menstruation; however, the prevalence of endometriosis is just 10%–15%,[4] indicating that some other mechanism is involved in the pathogenesis of endometriosis.

Metastasis-associated protein 1 (MTA1), an essential component of nucleosome remodeling and deacetylase (NuRD), plays an important role in transcriptional regulation via histone deacetylation and chromatin remodeling,[5] and it contributes to the process of cancer progression and metastasis.[6] Endometriosis shares some features of malignancy, such as invasiveness and metastasis.[7] Previously, we demonstrated that MTA1 is overexpressed in endometrial cancer cells and that MTA1 promotes the progression of endometrial cancer by facilitating cell proliferation, migration, and invasion.[8,9] Therefore, we hypothesized whether MTA1 plays a role in the pathogenesis of endometriosis. Recently, immunochemical staining showed the overexpression of MTA1 in ectopic tissues of patients with endometriosis in the advanced revised American Fertility Society (r-AFS) stage.[10] However, the mechanism of MTA1-inducing endometriosis has not yet been studied.

Resveratrol, a natural polyphenol mainly isolated from grapes, is effective in treating various cancers.[11–14] It is also effective in treating endometriosis.[15–22] Resveratrol not only increases antioxidant capacity and decreases lipid peroxidation,[15] but it also exhibits anti-inflammatory activity by suppressing nuclear factor κB (NF-kB), tumor necrosis factor (TNF)-α, and inflammatory cytokines in endometriosis.[20] More importantly, resveratrol has the activities of anti-proliferation and anti-invasion in endometriosis through inhibiting the expressions of VEGF, MMP-2, and MMP-9.[17–19] Studies have shown that resveratrol

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enhances cells apoptosis and inhibits prostate cancer progression via suppressing the MTA1/NuRD complex.[23,24] Thus, we speculated whether resveratrol has therapeutic effects in endometriosis through interaction with MTA1.

The pathogenesis of endometriosis involves epithelial-mesenchymal transition (EMT), which is a complex process of epithelial cells transforming into mesenchymal cells.[2,25–31] Transcription factors, such as ZEB1, ZEB2, and the Snail/Slug family, drive the process of EMT, along with the decreased expression of epithelium-related E-cadherin and increased expression of mesenchymal-related vimentin.[32] The ectopic endometrium of endometriosis patients shows significantly increased vimentin and remarkably decreased E-cadherin, compared with eutopic endometrium.[33] Moreover, in the epithelial cells of endometriotic lesions, E-cadherin is downregulated, while the N-cadherin is upregulated.[34] These studies have demonstrated that EMT occurs in the pathogenesis of endometriosis. Studies have revealed that MTA1 contributes to metastasis by promoting EMT in various cancers.[35–40] However, whether MTA1 promotes EMT in endometriosis remains unclear.

In the present study, we revealed that MTA1 was highly expressed in the ectopic endometrium of endometriosis patients and was significantly upregulated in the advanced r-AFS stage. MTA1 promoted the proliferation, migration, and invasion of endometrial stroma cells (ESCs), which were suppressed by resveratrol. Moreover, it was demonstrated that MTA1 induced EMT through interaction with ZEB2. The findings favor the understanding of endometriosis pathogenesis and provide a potential therapeutic target for endometriosis patients.

RESULTS

MTA1 Is Overexpressed and Correlated with Clinical Features in Endometriosis

To analyze the difference in the expression of genes between ectopic and normal endometrium in women with and without endometriosis, we collected human gene expression datasets in the Gene Expression Omnibus (GEO) database and found the expression of MTA1 to be upregulated in the patients (Figures 1A and 1B). We further compared the expression of MTA1 in ectopic endometrium samples from 33 patients with endometriosis and in endometrium samples from 20 controls without endometriosis by detecting MTA1 protein with immunohistochemistry (IHC). The patients' characteristics are presented in Table 1. Representative results of IHC are shown in Figure 1E; the composite scores for measuring MTA1 expression in ectopic endometrium were significantly higher than those in eutopic endometrium and normal endometrium (Figure 1F). Moreover, MTA1 expression was significantly upregulated in the advanced r-AFS stage (Table 2, $p = 0.016$). The results of western blotting also showed the increased expression of MTA1 in endometriosis (Figures 1C and 1D). In addition, we assessed the MTA1 expression in ectopic endometrium from the mouse endometriosis model and found that MTA1 was overexpressed in the ectopic endometrium (Figure 1G).

MTA1 Promotes Proliferation, Migration, and Invasion of ESCs

To investigate the biological functions of MTA1 in ESCs, we isolated ectopic ESCs (EcESCs) from ectopic samples and normal ESCs (NESCs) from normal samples, followed by downregulation of MTA1 in EcESCs and upregulation of MTA1 in NESCs by transfection of different plasmids (see Materials and Methods). As expected, the MTA1 protein levels were decreased in EcESCs (Figure S1A), leading to decreased proliferation, migration, and invasion of the cells (Figures 2A–2E), and the MTA1 protein levels were increased in NESCs (Figure S1B), which enhanced the proliferation, migration, and invasion of the cells (Figures 2A–2E).

MTA1 Promotes Endometriosis via Inducing EMT and the Process Is Suppressed by Resveratrol

Since EMT is involved in the pathogenesis of endometriosis,[2,22–28] we measured the expression of E-cadherin, an epithelial marker, and vimentin, a mesenchymal marker, by western blotting, and revealed lower E-cadherin expression and higher vimentin as well as MTA1 expression in EcESCs than those in NESCs (Figures 3A and 3D). Moreover, in a mouse endometriosis model, vimentin expression was higher and E-cadherin expression was lower in ectopic tissues than in eutopic tissues in IHC (Figure 1F). Furthermore, we found that silencing MTA1 in EcESCs upregulated E-cadherin and downregulated vimentin, and overexpressed MTA1 in Ishikawa cells downregulated E-cadherin and upregulated vimentin (Figures 3B and 3E). Alternatively, overexpression of MTA1 in EcESCs reduced the E-cadherin level and increased the vimentin level, and silencing MTA1 in Ishikawa cells increased E-cadherin and decreased vimentin (Figures 3C and 3F).

As resveratrol is effective in treating endometriosis,[9–16] we treated EcESCs with resveratrol to explore whether MTA1 is a target. The 50% inhibition concentration (IC_{50}) of resveratrol was determined by a Cell Counting Kit-8 (CCK-8) assay and calculated using GraphPad Prism 7.0 (Figure S2). We evaluated the inhibition effect of resveratrol in EcESCs and found that resveratrol suppressed their proliferation (Figure 4A), migration (Figure 4C), and invasion (Figure 4E). Moreover, we restored MTA1 expression by transfection of pCDH-MTA1 in EcESCs, which offset the inhibitory effects of resveratrol (Figures 4B, 4D, and 4F). Furthermore, we found that, after EcESCs were treated with resveratrol, MTA1 expression was reduced, and vimentin expression was decreased while E-cadherin expression was increased (Figure 3G). Rescue experiments with upregulation of MTA1 by transfection of EcESCs with pCDH-MTA1 reversed the expression of vimentin and E-cadherin (Figure 3H).

MTA1 Interacts with ZEB2

To further explore the mechanism of MTA1 in the pathogenesis of endometriosis, we used Ishikawa cells and shMTA1-Ishikawa cells (see Materials and Methods) to perform RNA sequencing (RNA-seq) analysis. We compared the downregulated genes following MTA1 knockdown in the present study with the upregulated genes

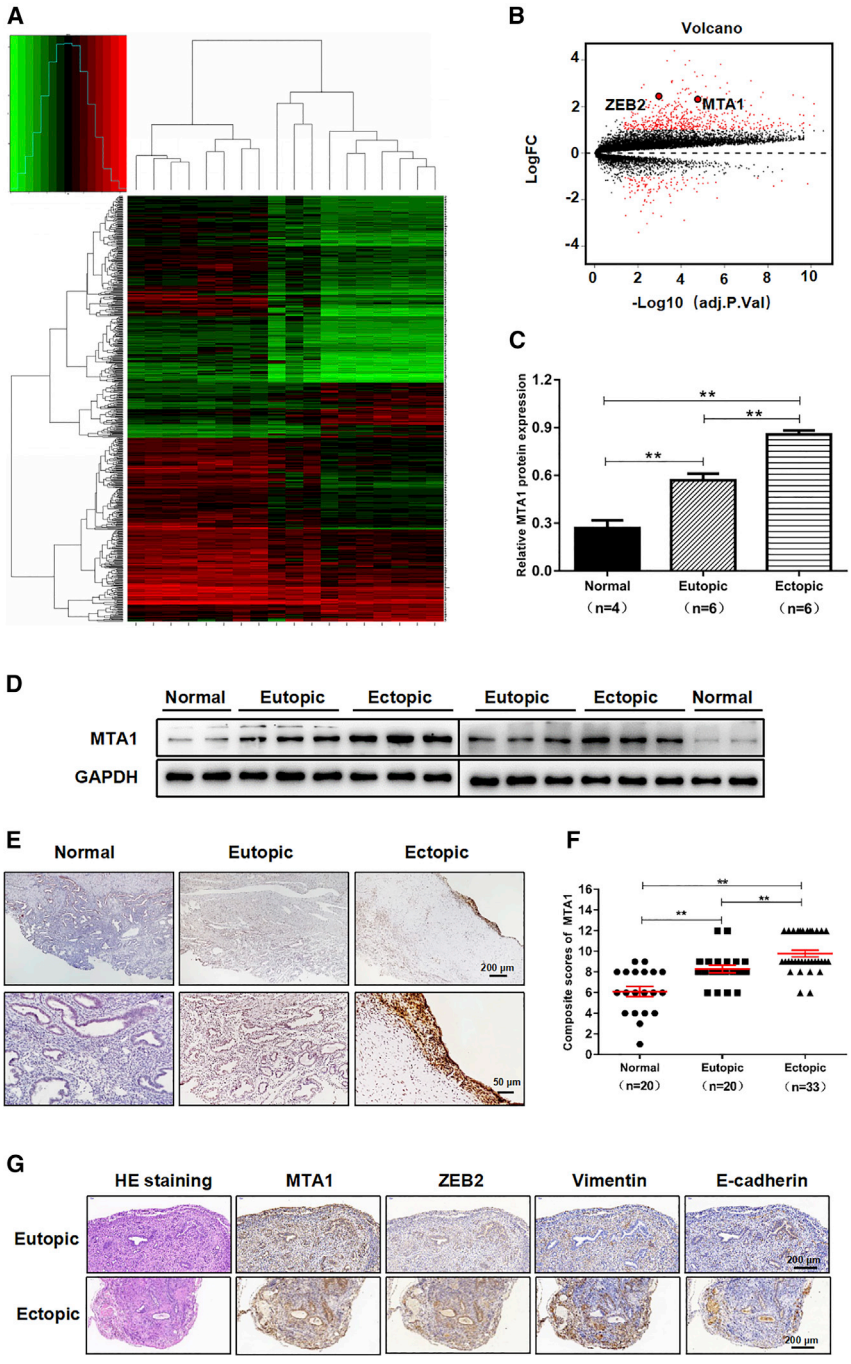

Figure 1. MTA1 Is Overexpressed in Ectopic Endometrium of Endometriosis

(A) Heatmap shows all differentially expressed genes. (B) Volcano plot shows the high expression of MTA1 and ZEB2 in endometriosis. (C and D) Quantitative graph of MTA1 protein levels (C) and western blots (D) showing MTA1 in normal, eutopic, and ectopic endometrium. (E) Representative images of immunohistochemistry (IHC) staining for detecting MTA1 in normal, eutopic, and ectopic endometrium. (F) Composite scores of MTA1 staining in (E) (see Materials and Methods). (G) Representative hematoxylin and eosin staining (H&E) and IHC of MTA1, ZEB2, E-cadherin, and vimentin in eutopic and ectopic endometrium from an endometriosis mouse. **$p < 0.01$.

in endometriosis in the GEO database (Figures 1A and 1B), and found that 38 genes were overlapped. Of them, ZEB2 plays a pivotal role in EMT-induced processes such as development, differentiation, and malignant mechanisms.[41] Thus, we further studied the role of ZEB2 in the pathogenesis of endometriosis. In western blotting and qRT-PCR, we revealed the decline of ZEB2 expression after MTA1 knockdown, as well as increased ZEB2 expression following MTA1 overexpression in Ishikawa cells (Figures 5A and 5B). Immunoprecipitation showed the interaction between ZEB2 and MTA1 (Figure 5C). In addition, ZEB2 also precipitated HDAC1 and MBD3.

ZEB2 Is Related to Migration and Invasion of Endometrial Cells via EMT

Since ZEB2 was upregulated in ectopic tissues from endometriosis patients in the GEO database, we also validated the high expression of ZEB2 in ectopic endometria from the mouse endometriosis model (Figure 1F). To investigate the role of ZEB2 in the pathogenesis of endometriosis, we downregulated the expression of ZEB2 in Ishikawa cells by transfection with siRZEB2 (Figures S1C and S1D) and found an increased expression of E-cadherin and decreased expression of vimentin (Figure 6A). Moreover, the downregulation of ZEB2 in Ishikawa cells inhibited cell migration and invasion (Figures 6B and 6D). To further validate the interaction between MTA1 and ZEB2, we co-transfected Ishikawa cells with siRZEB2 and pCDH-MTA1 and found that the expression of ZEB2 in the cells was upregulated, and E-cadherin was decreased while vimentin was increased (Figure 6A), indicating the occurrence of EMT. Moreover, the inhibited cell migration and invasion were reversed (Figures 6C and 6E). Interestingly, we also revealed that ZEB2 expression declined in EcESCs after the cells were cultured in the presence of resveratrol, and the inhibited expression of ZEB2 was reversed by MTA1 overexpression (Figures 3G and 3H).

High Expression of MTA1-ZEB2 Promotes Endometriosis in a Mouse Model and Resveratrol Alleviates Endometriosis

In a mouse endometriosis model (Figure 7A), we found that the expression of MTA1 and ZEB2 in ectopic tissues was higher than that in eutopic tissues by IHC and western blotting (Figures 1F and

Table 1. Clinical Characteristics of Normal Controls and Endometriosis Patients

	Endometrium for Immunohistochemistry Analysis			Endometrium for Western Blotting Analysis			Endometrium for Primary Cell Cultures	
	Normal (n = 20)	Eutopic (n = 20)	Ectopic (n = 33)	Normal (n = 4)	Eutopic (n = 6)	Ectopic (n = 6)	Normal (n = 6)	Ectopic (n = 8)
Age,[a] years	31.7 ± 8.13	36.6 ± 8.60	34.3 ± 8.23	30.5 ± 6.03	31.7 ± 5.35	31.7 ± 5.35	32.1 ± 4.75	30.8 ± 6.33
r-AFS								
II	NA	NA	3	NA	NA	NA	NA	2
III	NA	9	17	NA	4	4	NA	3
IV	NA	11	13	NA	2	2	NA	3

All samples were collected in the menstrual cycle proliferative phase. No patient was in r-AFS stage I. In immunohistochemistry analysis, ectopic samples were collected from 33 patients, and eutopic samples were collected from 20 of these 33 patients. In western blotting analysis, eutopic and ectopic samples were collected from the same six patients. r-AFT, revised American Fertility Society stage; NA, not applicable.
[a]Mean ± SD.

7C). Compared to the mice without resveratrol treatment, the mice treated with resveratrol had lower ectopic tissue volume, indicating the lower growth rate (Figure 7B). Moreover, in mice treated with resveratrol, the ectopic tissues had decreased expression of MTA1, ZEB2, and vimentin, as well as increased E-cadherin expression (Figures 7C and 7D).

DISCUSSION

In the present study, we showed that the expression of MTA1 was upregulated in the ectopic endometrium tissues in humans and mice, and higher MTA1 expression was associated with the advanced r-FAS stage. MTA1 enhanced the proliferation, migration, and invasion of ESCs, whereas resveratrol treatment downregulated the expression of MTA1, inhibited the proliferation, migration, and invasion of ESCs, and suppressed the growth of ectopic endometrium tissues. Moreover, we found that MTA1 induced EMT through interaction with ZEB2. Taken together, these findings demonstrate the important role of MTA1 in the pathogenesis of endometriosis.

MTA1 acts as an oncogene in various cancers,[6] and promotes the process of cancer progression and metastasis by inducing EMT in epithelial cancers,[35] hepatocellular cancer,[36] non-small-cell lung cancer,[37] and other tumors.[38–40] Our previous studies have demonstrated that MTA1 is overexpressed in endometrial cancer and facilitates endometrial cancer cell proliferation, migration, and invasion.[8,9]

Table 2. The Relationship between MTA1 Expression and r-AFS Stage

		MTA1 IHC Expression			
r-AFS	n	Low	High	χ^2	p
II	3	3	0		
III	17	11	6	6.172	0.016
IV	13	3	10		

$p < 0.05$ was considered to indicate a statistically significant difference. r-AFS, revised American Fertility Society stage; IHC, immunohistochemistry.

Since endometriosis has similar characteristics to cancers, we investigated the role of MTA1 in endometriosis in the present study. We found that downregulation of MTA1 inhibited cell proliferation, migration, and invasion in EcESCs, and overexpression of MTA1 enhanced cell proliferation, migration, and invasion in NESCs (Figure 2). Moreover, we found that upregulated MTA1 induced EMT and downregulated MTA1 expression reversed EMT in both EcESCs and Ishikawa cells (Figures 3B and 3C). These findings indicate that MTA1 induces EMT in the pathogenesis of endometriosis.

Having demonstrated the role of MTA1 in the pathogenesis of endometriosis, we further investigated how MTA1 works in this process. It is better to use endometrial epithelium cells (EECs) for EMT research; however, due to the difficulties in the isolation and culture of pure EECs, limited passage, and poor transfection, the use of EECs is limited in various studies. Ishikawa cells, a well-differentiated human endometrial epithelial adenocarcinoma cell line, have been used to illuminate the mechanisms of EMT in endometriosis by many researchers.[25,26,29,42–45] Our previous studies also showed that the upregulation of MTA1 in Ishikawa cells enhanced metastatic invasions.[8,9] Thus, we used the Ishikawa cell line as a surrogate of human EECs. The datasets in the GEO database showed that many genes are overexpressed in endometriosis (Figure 1A). Among these genes, we selected ZEB2 for further study since it was downregulated following silencing MTA1.

ZEB2 is a DNA-binding transcriptional regulator, which dimerizes with the E-box motif in different promoters.[46] Studies have shown that ZEB2 acts as a key regulator of EMT by repressing E-cadherin expression, not only in cancer progression but also in body development such as nervous system development and preterm birth.[41] There is a multi-regulatory network of signaling molecules that regulate ZEB2 expression. For instance, many reports have highlighted that ZEB2 is targeted by microRNAs (miRNAs) in different tumors,[41] while miR-200s are aberrantly expressed in endometriosis and play important roles in the onset of EMT through targeting ZEB2.[43] These findings, together with the overexpression of ZEB2 in endometriosis demonstrated in our study, suggest that ZEB2 is a critical factor in

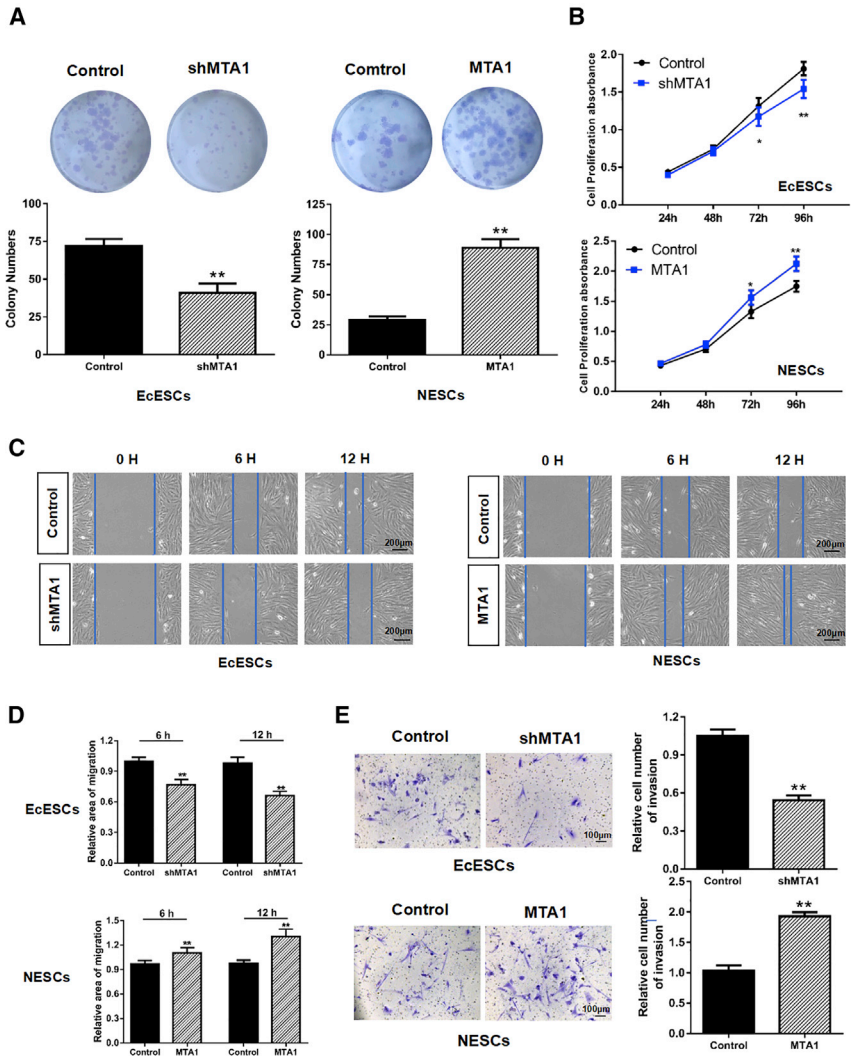

Figure 2. MTA1 Promotes Proliferation, Migration, and Invasion of Endometrial Stroma Cells

(A) Colony formation after knockdown of MTA1 in ectopic endometrial stroma cells (EcESCs) and overexpression of MTA1 in normal endometrial stroma cells (NESCs) respectively. (B) Cell proliferation assay after knockdown of MTA1 in EcESCs and overexpression of MTA1 in NESCs respectively. (C) Scratch wound assay after knockdown of MTA1 in EcESCs and overexpression of MTA1 in NESCs, respectively. (D) Graphical presentation of scratch wound assay data. (E) Transwell assay after knockdown and overexpression of MTA1 in EcESCs and NESCs, respectively. $^{**}p < 0.01$.

endometriosis. In our study, we proved the high expression of ZEB2 in mouse ectopic tissues and the downregulation of ZEB2 along with MTA1 knockdown in endometrial cells. Furthermore, we showed that ZEB2 silencing inhibited endometrial cell migration and invasion (Figures 6B and 6D), and it resulted in the increased expression of E-cadherin and decreased expression of vimentin (Figure 6A). The inhibition of cell invasiveness and EMT by siRZEB2 could be reversed by MTA1 overexpression (Figure 6C and 6E). Moreover, we also found an interaction between MTA1 and ZEB2. It was noteworthy that ZEB2 could recruit not only MTA1, but also HDAC1 and MBD3 (Figure 5C), indicating that it is via the NuRD complex that MTA1 exerts its effect by regulating ZEB2 or by interaction with ZEB2, which remains to be explored in the future. Thus, these observations reveal a crucial function of MTA1-ZEB2 in inducing EMT of endometriosis.

Resveratrol is considered to be effective in preventing and treating endometriosis.[15–22] In the present study, we showed that resveratrol inhibited the proliferation, migration, and invasion of ESCs in cell cultures (Figures 4A, 4C, and 4E) and suppressed the growth of ectopic endometrium in the mouse endometriosis model (Figure 7B). Then, we demonstrated that resveratrol decreased MTA1 expression and suppressed EMT induced by MTA1-ZEB2 in endometrial cells (Figure 3G) and endometriosis mice (Figures 7C and 7D). Furthermore, rescue experiments with MTA1 overexpression led to reversing the inhibition of resveratrol on cell function (Figures 4B, 4D, and 4F) and the regulation on expression of vimentin and E-cadherin (Figure 3H). These results suggest that MTA1 is a target of resveratrol, which helps to understand the mechanism of the therapeutic effect of resveratrol.

In conclusion, our present study shows that the upregulation of both MTA1 and ZEB2 induces EMT in endometriosis, and resveratrol downregulates the expression of MTA1 and ZEB2, leading to the inhibition of EMT. The findings indicate that MTA1 plays a crucial role in the pathogenesis of endometriosis and may serve as a target for treatment.

MATERIALS AND METHODS

Ethical Approval and Consent to Participate

The study was approved by the Ethics Committee of The Affiliated Drum Tower Hospital of Nanjing University Medical School. Written informed consent was obtained by all patients. All experiments in nude mice were performed in accordance with protocols approved by the Institutional Experimental Animal Review Board of The Affiliated Drum Tower Hospital of Nanjing University Medical School.

Patient Characteristics and Tissue Samples

Paraffin-embedded tissue samples were obtained from 55 patients who underwent surgeries in The Affiliated Drum Tower Hospital of Nanjing University Medical School from January 2018 to January 2019 for endometriosis or other non-hormone-related benign diseases. The

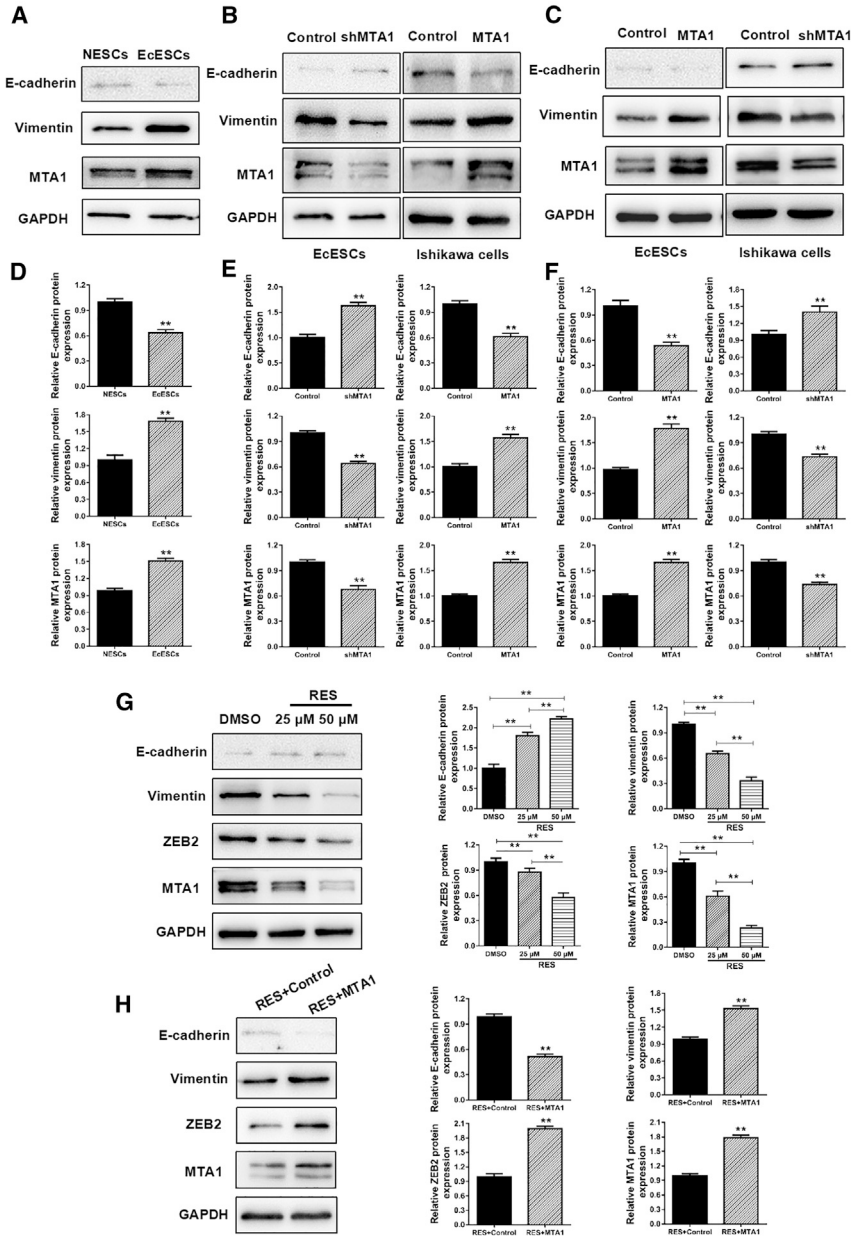

Figure 3. MTA1 Induces EMT in Endometriosis and the Process Is Inhibited by Resveratrol

The protein levels of MTA1, E-cadherin, vimentin, and ZEB2 were determined by western blotting. (A and D) Relative expression of MTA1, E-cadherin, and vimentin in EcESCs and NESCs. (B and E) Relative expression of MTA1, E-cadherin, and vimentin after MTA1 knockdown in EcESCs and after MTA1 overexpression in Ishikawa cells, respectively. (C and F) Relative expression of MTA1, E-cadherin, and vimentin after MTA1 overexpression in EcESCs and after MTA1 knockdown in Ishikawa cells, respectively. (A–C) Western blots. (D–F) Graphical presentation of western blot data. (G and H) Relative expression of MTA1, ZEB2, E-cadherin, and vimentin in EcESCs after 25 and 50 μM resveratrol (RES) treatment (G) and 25 μM RES co-treatment with MTA1 overexpression by transfection of plasmid pCDH-MTA1 (H). **$p < 0.01$.

ectopic endometrial tissues were obtained from patients (n = 33) who were diagnosed with ovarian endometriosis cyst pathology. In addition, there were 20 patients who provided the eutopic endometrium tissues in the meantime (n = 20). The patients were classified according to the r-AFS classification: 3 patients were in early-stage (stage II), 17 patients were in stage III, and 13 patients were in stage IV. Normal endometrium tissues were gained from hysterectomy specimens of patients (n = 20) who were suffering from benign non-endometrial pathologies such as premalignant disease of the cervix and uterine prolapse.

In addition, six paired ectopic and eutopic endometrium tissues obtained from ovarian endometriosis cyst patients and four normal endometrium tissues obtained from infertility patients were frozen and prepared for protein extraction. Another eight ectopic endometrium tissues and six normal endometrium tissues were obtained for primary endometrial cell isolation. None of the patients had received hormone treatment for at least 3 months before surgery. All specimens were at the proliferative phase of the regular menstrual cycle when the surgery was performed. All of the participants' characteristics are listed in Table 1.

IHC Analysis

Paraffin tissue sections were deparaffinized, and dehydration steps were then followed through a graded ethanol series. The slides were incubated in 3% H_2O_2 at room temperature for 10 min to block endogenous peroxidase activity. Antigen retrieval was performed by boiling in a pressure cooker at 120°C in 0.01 M citrate buffer solution for 10 min. The samples were incubated with primary antibodies, including MTA1 (1:200, #5646, Cell Signaling Technology), ZEB2 (1:200, ab138222, Abcam), E-cadherin (1:200, #14472, Cell Signaling Technology), and vimentin (1:200, #5741, Cell Signaling Technology), at 4°C overnight. After washing with phosphate-buffered saline (PBS), the slides were incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:1,000, Invitrogen, Carlsbad, CA, USA) at room temperature for 30 min. After that, the sections were stained with diaminobenzidine (DAB) per the manufacturer's protocols and imaged using a microscope and the expression levels were analyzed. All of the samples were evaluated independently by two experienced pathologists who did not have knowledge regarding the patients' identities.

The degree of staining was evaluated based on the percentage of positive staining and the intensity of staining.[47] Based on the percentage

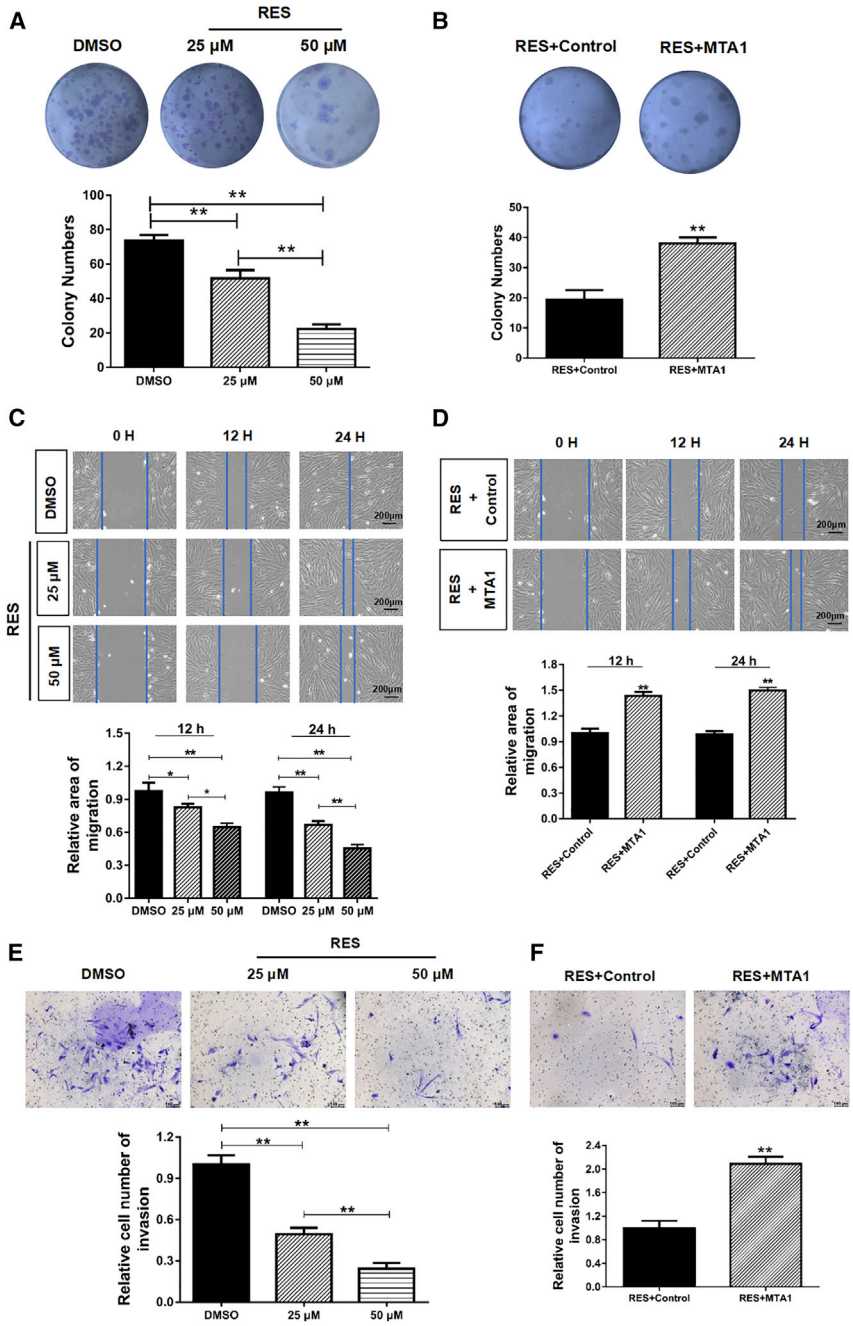

Figure 4. RES Suppresses Proliferation, Migration, and Invasion of Endometrial Cells through Downregulation of MTA1

(A and B) Colony formation assay after 25 and 50 μM RES treatment (A) and 25 μM RES co-treatment with MTA1 overexpression by transfection of plasmid pCDH-MTA1 (B) in EcESCs. (C and D) The migration ability of EcESCs determined by a scratch wound assay. (C) EcESCs were treated with RES (25 and 50 μM) at 12 and 24 h, respectively. (D) EcESCs with MTA1 overexpression by transfection of plasmid pCDH-MTA1 were treated with 25 μM RES for 12 and 24 h, respectively. (E and F) The invasion abilities of EcESCs determined by a Transwell assay. (E) EcESCs were treated with RES (25 and 50 μM) for 48 h. (F) EcESCs with MTA1 overexpression by transfection of plasmid pCDH-MTA1 were treated with 25 μM RES for 48 h. *$p < 0.05$, **$p < 0.01$.

of positive staining, IHC staining results were scored as follows: 0, the proportion of glandular epithelial cells or stromal cells with positive staining <5%; 1, 5% to <25%; 2, 25% to <50%; 3, 50% to <75%; 4, ≥75%. Based on the staining intensity, IHC staining results were scored as follows: 0, negative staining; 1, weak positive, light yellow; moderate positive, yellow; 3, strong positive, brown. Ultimately, a composite score of staining was calculated by multiplying the scores obtained by the staining percentage and intensity, which ranged from 0 to 12 points. The composite scores 0–6 were defined as low expression, and scores of 7–12 were defined as high expression.

Cell Isolation and Culture

The isolation and culture of primary EcESCs and NESCs were performed as described previously.[48] In brief, endometrium tissues were immediately placed into culture medium and washed in PBS three times, then minced into ~1-mm pieces and digested with 0.1% (w/v) collagenase I (Worthington Biochemical, Freehold, NJ, USA) for 30 min at 37°C. Next, tissue pieces were filtered through 150-μm steel mesh sieves to remove debris and then through 38-μm stainless steel to roughly remove epithelial cells and to obtain ESC preparations. The prepared ESCs were then centrifuged at 1,200 × *g* for 5 min and re-suspended in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, Paisley, UK) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, USA), penicillin (100 U/mL), and streptomycin (100 mg/mL) (Gibco), and seeded into culture dishes. The cultured stromal cells were 95% pure, as determined by E-cadherin and vimentin staining (Figure S3). Usually, ESCs between passage 3 and passage 6 were used to obtain enough cells for subsequent experiments. In addition, the Ishikawa cell line, which is a well-differentiated endometrial adenocarcinoma cell line, was used as a surrogate of EECs. Ishikawa cells were kindly provided by Prof. L.H. Wei (Peking University People's Hospital, China), and three types of Ishikawa cells (Ishikawa, MTA1-Ishikawa, and shMTA1-Ishikawa)[8] were cultured in DMEM (Gibco) supplemented with FBS and antibiotics as previously mentioned. All cells were incubated at 37°C in 5% CO_2.

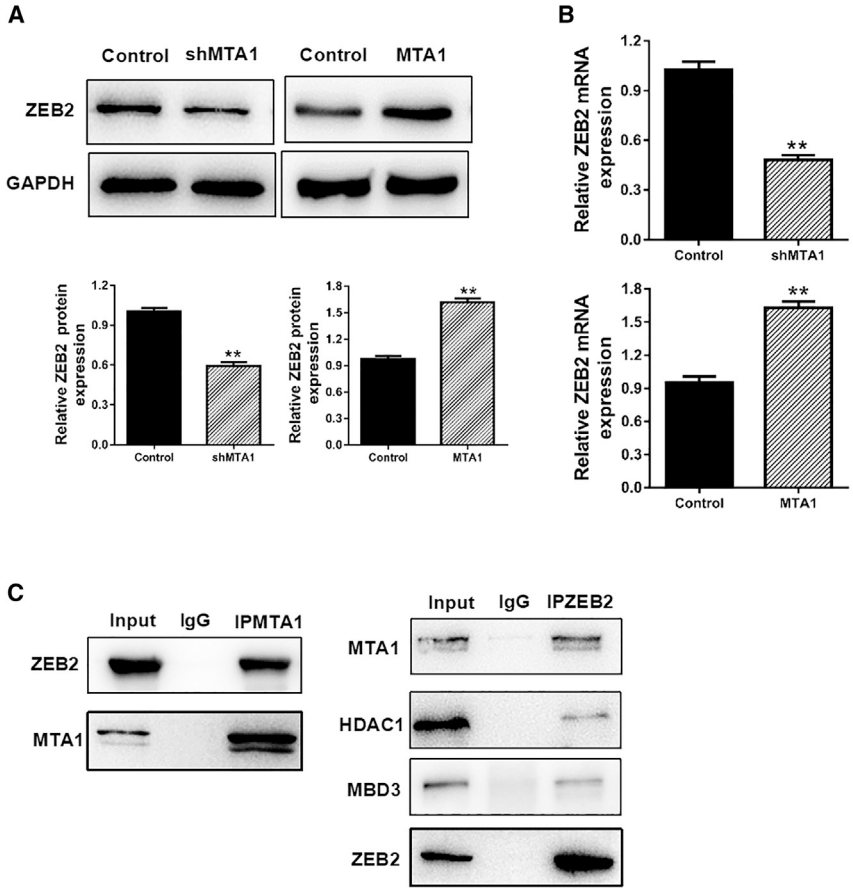

Figure 5. MTA1 Interacts with ZEB2

(A) Western blotting showing the change of ZEB2 protein expression after MTA1 knockdown and overexpression in Ishikawa cells. (B) Quantitative RT-PCR showing the ZEB2 mRNA expression following MTA1 knockdown and overexpression in Ishikawa cells. (C) The interaction between MTA1 and ZEB2 tested by immunoprecipitation. $^{**}p < 0.01$.

Cell Transfection

The downregulated plasmid of MTA1 (pMagic-shMTA), overexpressed plasmid of MTA1 (pCDH-MTA1), and their controls were made as described in our previous study.[8] The siRZEB2$_1$, siRZEB2$_2$, siRZEB2$_3$ and their control siRNAs (siRN) were synthesized by Obio Technology (Shanghai, China). Cell transfection was performed with Lipofectamine 3000 (Invitrogen, USA).

Immunoprecipitation and Western Blotting

Cell extracts were prepared by incubating the cells in lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% Nonidet P-40 [NP40]) for 30 min at 4°C, followed by centrifugation at 13,000 rpm for 15 min at 4°C. The protein concentrations of the total cellular lysates were measured using the micro bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). For immunoprecipitation, 500 μg of protein was incubated with specific antibodies (2–3 μg) for 12 h at 4°C with constant rotation, followed by incubation with 30 μL of 50% protein A/G agarose beads for 2 h. Beads were then washed five times with PBS. The precipitates were boiled in 2× SDS-PAGE loading buffer for 10 min, and then resolved in 10% SDS-PAGE gels. The proteins in the gel were transferred to polyvinylidene fluoride membranes. For western blotting analysis, membranes were incubated with antibodies, including MTA1 (1:1,000, #5646, Cell Signaling Technology), ZEB2 (1:1,000, ab138222, Abcam), E-cadherin (1:1,000, #14472, Cell Signaling Technology), vimentin (1:1,000, #5741, Cell Signaling Technology), MBD3 (1:1,000, ab157464, Abcam), and HDAC1 (1:1,000, ab19845, Abcam), overnight at 4°C followed by incubation with a secondary antibody. Immunoreactive bands were visualized using western blotting luminol reagent (Santa Cruz Biotechnology).

Quantitative RT-PCR

Total RNA was extracted from tissue or cells with isolator reagent (Vazyme, China). After measurement of the RNA concentration, cDNAs were generated from reverse transcription with the HiScript II first-strand cDNA synthesis kit (Vazyme, China). The mRNA levels were analyzed with ChamQ Universal SYBR qPCR master mix kit (Vazyme, China). The levels of mRNA were normalized to GAPDH. The primers for MTA1 were forward, 5′-AGCTACGAGCAGCACAACGGGGT-3′ and reverse, 5′-CACGCTTGGTTTCCGAGGAT-3′; for GAPDH, forward, 5′-CGTGGGCCGCCCTAGGCACCA-3′ and reverse, 5′-TTGGCTTAGGGTTCAGGGGGG-3′; and for ZEB2, forward, 5′-GGAGACGAGTCCAGCTAGTGT-3′ and reverse, 5′-CCACTCCACCCTCCCTTATTTC-3′. The SYBR Green qPCR was performed using the ABI 7500 Fast real-time PCR system, and fold changes (FCs) were calculated using the $2^{-\Delta\Delta Ct}$ method. Each sample was run in triplicate in three independent experiments.

Resveratrol

Resveratrol was purchased from Sigma (St. Louis, MO, USA), dissolved in DMSO to create 10 mM stock solution for cell treatment and in ethanol to create 50 mg/mL stock solution for treatment of mice, and then stored at −20°C. For cell experiments, resveratrol was diluted to 25 and 50 μM, freshly prepared in culture medium prior to use, and 0.1% DMSO was used as vehicle control. For mouse experiments, the dose of resveratrol was 25 mg/kg, which was dissolved in 10% ethanol, and PBS was used as a vehicle control.

Cell Proliferation Assay

Cell proliferation was analyzed using a CCK-8 assay (Vazyme, China). Transfected or resveratrol-treated cells were cultured in

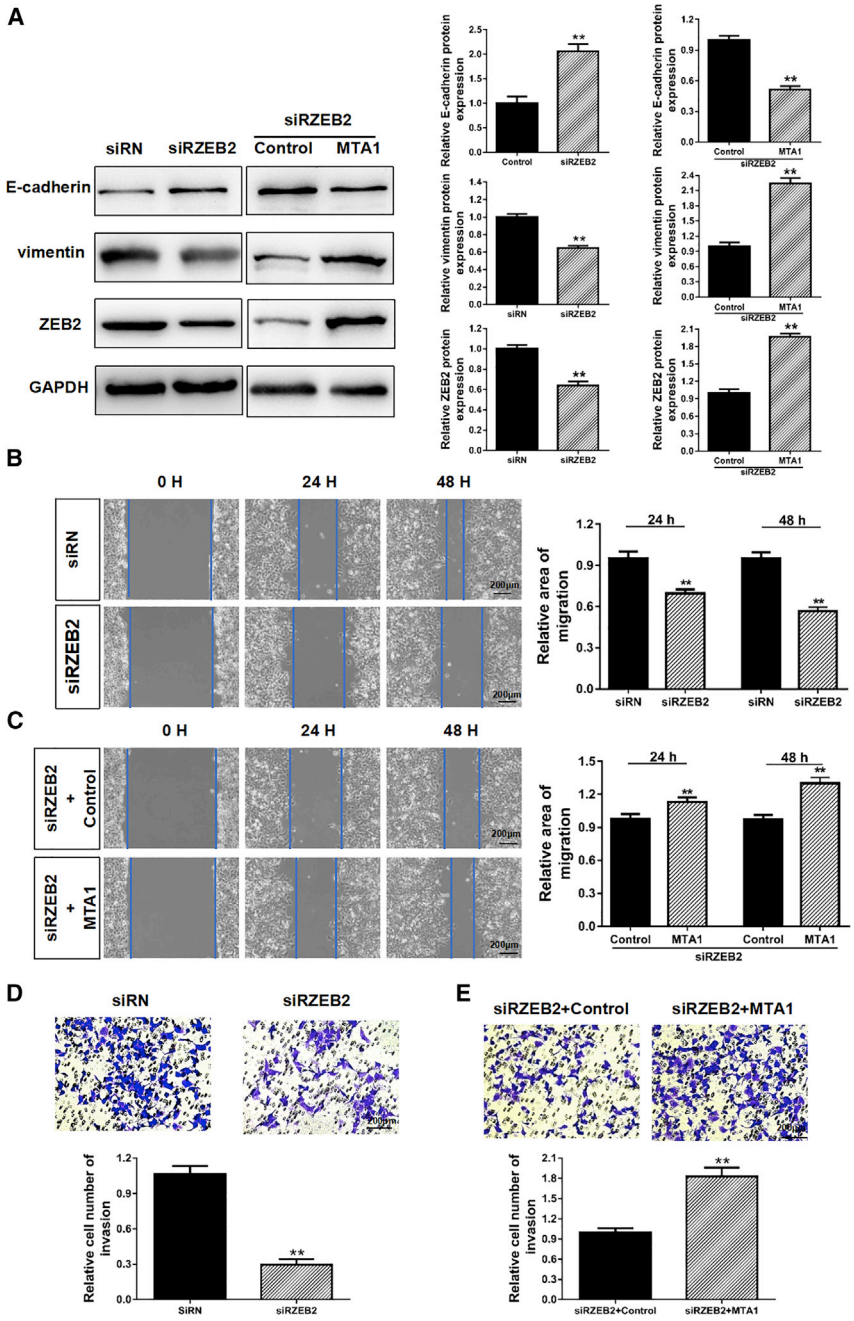

Figure 6. ZEB2 Inhibits Migration and Invasion of Endometrial Cells via Suppressing EMT

(A) Relative expression of ZEB2, E-cadherin, and vimentin in siRZEB2-transfected Ishikawa cells (left) and reversion of ZEB2, E-cadherin, and vimentin expression in co-transfected Ishikawa cells with pCDH-MTA1 (right), analyzed by western blotting. (B and C) The migration abilities of Ishikawa cells tested with a scratch wound assay. (B) The inhibition of migration abilities after knockdown of ZEB2 in Ishikawa cells. (C) Reversal of cell migration abilities following MTA1 overexpression. (D and E) The invasion abilities of Ishikawa cells measured with a Transwell assay. (D) The inhibition of invasion abilities after knockdown of ZEB2 in Ishikawa cells. (E) Reversal of cell invasion abilities following MTA1 overexpression. **$p < 0.01$.

four 96-well plates with 1×10^3 cells in each well. Each plate was used to analyze the cell proliferation by adding 10 μL of CCK-8 solution per well at an interval of 24 h, up to 96 h. Absorbance at 450 nm was measured using a spectrometer reader.

Colony Formation Assay

The cells were seeded at a density of 500 cells/mL (1,000 cells/well) into six-well plates and cultured for 14 days in 2 mL of DMEM with 10% FBS. Then, cell colonies were fixed with methanol and stained with Giemsa. Colony numbers were grossly counted.

Cell Scratch Wound Assay

Cell migration ability was examined by a cell scratch assay. Briefly, cells were seeded on six-well plates and incubated to almost full confluence after treatment as mentioned previously. Scratching was performed with a 200-μL plastic pipette tip, and the cells were cultured in serum-free medium. The initial gap width and the residual gap width at 6 and 12 h for transfected cells, 12 and 24 h for resveratrol-treated EcESCs, and 24 and 48 h for treated Ishikawa cells after scratching were observed and imaged under the inverted microscope.

Cell Transwell Assay

We conducted transwell invasion assays using transwell chambers (24 wells, 8-μm pore size). Briefly, 200 μL of suspension containing 5×10^5 ESCs or 2×10^4 Ishikawa cells was seeded into the Matrigel-precoated upper transwell chambers with serum-free medium, and lower chambers were filled with 600 μL of culture media containing 10% FBS. The chambers were incubated at 37°C for 24 h. The invaded cells on the lower side were fixed with paraformaldehyde, stained with 1% crystal violet, and then counted in five random fields under a microscope.

Establishment of the Mouse Endometriosis Model

A mouse model of endometriosis was established as described.[49,50] Twenty female nude mice (6 to 8 weeks old) were purchased. Before invasive procedures, the mice were anesthetized with isoflurane. Endometriotic lesions were experimentally induced by

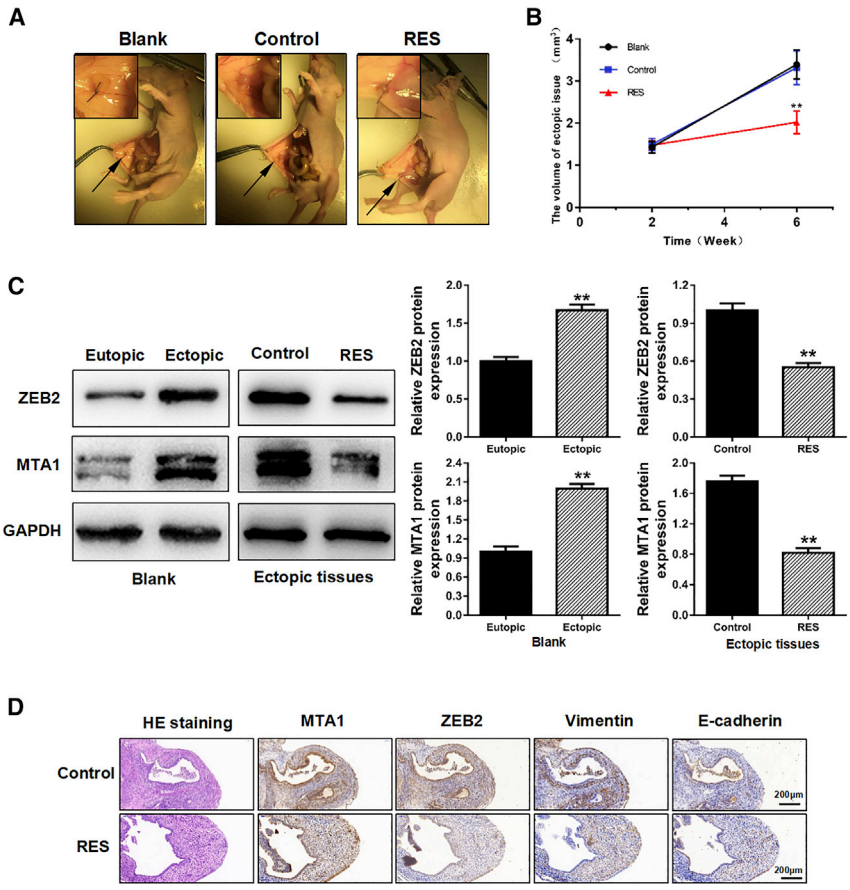

Figure 7. High Expression of MTA1 and ZEB2 Promotes Endometriosis in Mice and RES Alleviates Endometriosis

(A) Mouse model with endometriosis. The arrows indicate the ectopic endometrium. (B) Curves of the ectopic endometrium growth. Each group had six mice. (C) Relative expression of MTA1 and ZEB2 in eutopic and ectopic tissues of normal mice in blank group (left) and in ectopic tissues without and with RES (25 mg/kg) treatment (right) measured by western blotting. (D) Representative H&E staining and IHC of MTA1, ZEB2, E-cadherin, and vimentin expression in ectopic tissues of control group (upper) and RES group (lower) samples of endometriosis mice (n = 6). **p < 0.01.

autotransplantation of the right uterine horn onto the peritoneal wall. A mid-ventral incision was made and then the right uterine horn was excised and placed in serum-free medium. The uterine horn was opened longitudinally and then cut into square pieces measuring 4 × 4 mm^2. Each of the endometrial fragments of equal size was sutured onto both sides of the peritoneal wall. The abdomen was then closed. One mouse died 2 days later after the surgery. Two weeks later, we performed the second surgery to identify that the endometriosis model was successfully established in 18 mice and measured the size of the ectopic tissues. Then, 18 mice were randomly assigned to three groups (n = 6/group), that is, the resveratrol treatment group, the PBS group, and the blank group. Mice in the resveratrol treatment group were daily intraperitoneally injected with 100 μL of resveratrol (25 mg/kg) dissolved in 10% ethanol for 4 consecutive weeks, those in PBS group were daily intraperitoneally injected with 100 μL of PBS in 10% ethanol for 4 consecutive weeks, and those in the blank group received nothing. Finally, the mice were sacrificed by cervical dislocation under anesthesia 1 day after the last treatment. Lesions were measured in two perpendicular diameters (r < R) using a caliper. Ectopic tissue volume (V) determined using the formula $V = (4/3)\pi r^2 R$ (r and Rare the radii, r < R).[50] The lesion volume of each mouse was the mean of the two side lesions. Then, both sides of the endometriotic lesions were collected, the left side lesions were frozen for protein extraction, and the right side lesions were fixed in 10% formaldehyde for histological analysis.

Data Availability

Human gene expression datasets of endometriosis and non-endometriosis (GEO: GSE11691)[51] were downloaded from the GEO. The probe-level information was converted into the corresponding gene symbol according to the explanation data downloaded from platform GPL96 (Affymetrix Human Genome U133 Plus 2.0 array). Differentially expressed genes were screened by comparing the differences in gene expression between the two conditions (endometriosis versus non-endometriosis) using R language (limma package, R version 3.5.0). The adjusted (adj.) p <0.05 and $|\log_2 FC|$ >1 were considered as the cutoff values.

Statistical Analysis

Data are shown as the mean ± SEM, and statistical analyses were performed using SPSS 24.0 software (IBM, Chicago, IL, USA). Composite scores of MTA1 staining of IHC in different groups were examined using the Kruskal-Wallis test, followed by the Mann-Whitney test for pairwise comparison. A χ^2 test was used to evaluate the associations between expression levels with related clinicopathological factors. A Student's t test or one-way ANOVA was used to analyze differences between experimental groups. p <0.05 was considered a statistically significant difference.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.09.013.

AUTHOR CONTRIBUTIONS

X.K., X.X., X.Z., Y.G., and H.Z. conceived and designed the experiments. X.K., X.X., L.Z., M.Z., S.Y., Y.D., J.W., T.L., and Y.W. performed the experiments. J.L., R.L., X.T., Y.Z., X.Z., and H.Z. collected

clinical specimens. X.K. and X.X. prepared the manuscript. X.Z., Y.G., and H.Z. critically revised the manuscript. All authors read the manuscript and approved the final version.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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