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## Endoglin promotes cell migration and invasion in endometriosis by regulating EMT

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**Endoglin promotes cell migration and invasion in endometriosis by regulating EMT**

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**ABSTRACT**

**Objectives:** This study aims to investigate the expression and role of Endoglin (ENG) in endometriosis (EM).

**Material and methods:** In this study, quantitative real-time polymerase chain reaction, western blot and immunohistochemistry were used to examine the expression of ENG in tissues. Cellular experiments were performed to evaluate the effect of ENG on cellular biological function. Western blot was used to examine the expression of epithelial to mesenchymal transition-related proteins.

**Results:** The expression of ENG was significantly higher in the ectopic endometriotic tissues than that in eutopic endometriotic tissues. Knockdown of ENG inhibited cell viability, migration and invasion, and induced cell apoptosis in hEM15A cells.

Additionally, silenced ENG caused increased levels of E-cadherin and decreased levels of N-cadherin, vimentin, MMP-2 and MMP-9.

**Conclusions:** These results confirmed that ENG may be involved in the development of endometriosis by promoting EMT process, revealing a new insight into the pathogenesis of endometriosis and contributing to the exploration of molecular therapeutic strategies against endometriosis.

**Key words:** endometriosis; endoglin; epithelial to mesenchymal transition

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**INTRODUCTION**

Endometriosis is an estrogen-dependent chronic gynecological disease. Menstrual cramps, pelvic pain, infertility and other problems caused by endometriosis seriously affect women's quality of life and fertility [1]. To date, 20% to 50% of postoperative recurrence rate and long-term side effects of medication lead to huge physical and mental pain and economic burden for patients [2, 3]. Therefore, in-depth study of the pathogenesis of endometriosis is crucial for intervention and treatment.

Endoglin (ENG, also known as CD105) is a 90 kDa type I transmembrane glycoprotein which is highly expressed in the vascular endothelium and plays an important role in the regulation of angiogenesis [4]. ENG has been proved to enhance tumor angiogenesis by acting as a coreceptor for transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling, and targeting ENG exhibits strong inhibition of tumor invasion, angiogenesis and tumor metastasis [5, 6]. The formation of endometriosis ectopic lesions is very similar to the process of tumor metastasis. ENG has been reported to be upregulated [in the eutopic and ectopic endometrium of women with endometriosis compared with control](#) endometrium and plays a vital role in endometriotic angiogenesis [7, 8]. However, the molecular mechanism of ENG in endometriosis remains unclear.

**Objectives**

In this study, we aim to investigate the expression, biological function and potential mechanism of ENG in endometriosis (EM).

## **MATERIAL AND METHODS**

### ***Patients and tissue samples***

In this study, we enrolled a total of 30 patients with advanced stage ovarian endometriosis (17 ASRM stage III and 13 stage IV) who underwent laparoscopic surgery in the department of gynecology of People's Hospital of Anji. The pathology of endometriosis was diagnosed with tissues analyzed by histopathology to confirm the presence of endometrial stroma and glands. The normal endometrial tissues were obtained from 25 healthy fertile women who underwent operative treatment for uterine myoma or cervical intraepithelial neoplasia (CIN) other than endometriosis. They had not received hormone therapy for at least three months before sampling, and none of them had visible endometrial hyperplasia or neoplasia and inflammatory disease at the time of clinical examination or laparoscopy. The study protocol was approved by the ethics committee of People's Hospital of Anji, and informed written consent was obtained from all participants.

### ***Quantitative real-time polymerase chain reaction (qRT-PCR)***

The expression of ENG mRNA in EM and normal endometrial (EN) tissues was examined by qRT-PCR. Total RNA was isolated from the ectopic and normal endometrial tissues using Trizol (Invitrogen, USA), and then reversely transcribed into cDNA using SuperScript™ III reverse transcriptase (Invitrogen, USA). qRT-PCR was performed using the Universal SYBR Green Master kit. The levels of ENG were normalized to GAPDH and calculated by  $2^{-\Delta\Delta C_t}$  method.

### ***Western blot***

Western blot was conducted to examine the expression of ENG protein in EM and EN tissues. Total protein was extracted with radioimmunoprecipitation assay (RIPA) lysis buffer, separated using SDSsodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Co., USA). Immunoblotting was performed with primary antibodies. The PVDF membranes were incubated with secondary antibody, and then developed using ECL chemiluminescence assay kit (Proteintech, China) and analyzed by the ChemiDoc™

XRS imaging system. Anti-endoglin antibody was purchased from Abcam, anti-E-cadherin, anti-N-cadherin, anti-vimentin, anti-MMP-2, anti-MMP9 and anti-GAPDH antibodies were obtained from Proteintech (Shanghai, China).

### ***Immunohistochemistry (IHC)***

IHC was performed to detect the intracellular expression and location of ENG. The formalin-fixed, paraffin-embedded section samples were dewaxed in xylene and hydrated in graded ethanol series, followed by antigen repair with repair solution. The tissues were then blocked endogenous peroxidase activity using 3% H<sub>2</sub>O<sub>2</sub>. Subsequently, anti-endoglin antibody and secondary antibodies were added, followed by DAB staining and hematoxyline restaining. Finally, cells were observed under the microscope.

### ***Cell culture and transfection***

Human immortalized eutopic stromal cell line hEM15A was purchased from China Center for Type Culture Collection (Wuhan, China). Cells were cultured in DMEM medium (Gibco, USA) containing 10% FBS.

To knock down the intrinsic expression of ENG in cells, shRNA targeting ENG was used to transfect cells as follows. A total of  $3 \times 10^5$  hEM15A cells were seeded in six-well plates, cultured for 24 hours, and then transfected with shRNA targeting human ENG (sh-ENG) or shRNA negative control (sh-NC) by using Lipofectamine 3000 (Invitrogen, Carlsbad, CA) according to the instruction of the manufacturer.

### ***Cell counting kit-8 assay (CCK-8)***

The viability of hEM15A cells was examined using CCK8 assay. Briefly, a total  $1.0 \times 10^6$  of hEM15A cells were seeded into 96-well plates and subjected to indicated treatments. After 24, 48 and 72 hours, 10  $\mu$ L of CCK8 reagent was used and incubated for four hours at 37 °C. The absorbance value at 450 nm was detected by enzyme-labeled instrument.

### ***Cell apoptosis assay***

The [Annexin V-FITC/PI Apoptosis Detection Kit](#) (Yeasen, Shanghai, China.) was used to evaluate cell apoptosis. hEM15A cells were collected, washed with PBS, and

resuspended in binding buffer. Subsequently, cells were stained with FITC-labeled annexin V and propidium iodide (PI) for 15 min at room temperature in darkness. Finally, the stained cells were analyzed by the flow cytometry BD FACSDiva 6.1 software.

### ***Wound healing assays***

Wound healing assay was performed to assess the migratory capacity of hEM15A cells. Cells were seeded into a six-well plate. When the cells merged into a monolayer, the scratch was made with a 200- $\mu$ l sterile pipette tip. The cells were then washed for three times to remove the cell debris and cultured in complete DMEM for 24 hours. After the medium was removed, serum-free DMEM was added. The wound was observed and photographed at the indicated times with an inverted microscope (Olympus Corporation) and analyzed using ImageJ software. All assays were performed three times in our study.

### ***Transwell assay***

The migratory and invasive capacities of hEM15A cells were assessed using transwell assays. For migration assay, we added  $4 \times 10^4$  cells in 200  $\mu$ L of serum-free DMEM in the upper chamber and then placed 500  $\mu$ l of DMEM containing 30% FBS to the lower chamber. For invasion assays, we precoated the chamber inserts with 50  $\mu$ L of 1:6 mixture of Matrigel (BD Biosciences) and DMEM for about two hours in a 37 °C incubator. Then we seeded  $8 \times 10^4$  cells in the upper chamber. The lower chamber also added 500  $\mu$ l of DMEM containing 30% FBS. After cultured for 48 h, the migrated cells were fixed with 4% paraformaldehyde and stained with crystal violet. Five random 100 $\times$  microscopic fields were selected to count the stained cells by using an inverted microscope (Olympus Corporation).

### ***Statistical analysis***

All experiments were performed in triplicate. data were expressed as means  $\pm$  SD and analyzed by Student's t-test, one- or two-way analysis of variance (ANOVA) using SPSS 21.0 software. The bar or scatter chart were showed using GraphPad Prism software version 8.0 (San Diego, CA). P value < 0.05 was taken to indicate statistical significance.

## RESULTS

Our data showed that the expression of ENG mRNA in EM tissues (n = 30) was significantly higher than that in EN tissues (n = 25) ( $p < 0.001$ ) (Fig. 1A), while there was no difference in ENG expression between EM of different stage (Fig. 1B). Consistently, western blot analysis showed that ENG protein levels were upregulated in EM tissues compared with the EN group (Fig. 1C) ( $p < 0.001$ ). Further, we observed that ENG was mainly located in the glandular and the luminal stromal cells in the endometriosis tissues (Fig. 1D). These data suggest that ENG may be involved in the development and progression of endometriosis.

To further explore the biological function of ENG in endometriosis, hEM15A cells were transfected with ENG-shRNA or control shRNA. As shown in Figure 2A, transfection of cells with ENG-shRNA efficaciously decreased the expression of ENG compared with the control group. CCK-8 assay showed that down-regulated ENG significantly inhibited hEM15A cells proliferation at 48 and 72 hours compared with the control group, whereas there was no significant difference between sh-NC and control group ( $p < 0.05$ ) (Fig. 2B). Further, results of flow cytometry analysis showed that down-regulated ENG markedly induced hEM15A cell apoptosis by 9.03% compared with the control group ( $p < 0.001$ ) (Fig. 2C). Moreover, western blot analysis showed that down-regulated ENG could remarkably reduce bcl-2 expression and elevated the levels of bax and cleave-caspase-3 proteins (Fig. 2D), which confirmed the promoting effect of down-regulated ENG on apoptosis. These data suggest ENG knockdown can inhibit proliferation and promote apoptosis of hEM15A cells.

Further, the results of wound healing assay showed that down-regulated ENG noticeably decrease the migratory rate by  $29.63\% \pm 0.63\%$  compared with the control group ( $p < 0.05$ ) (Fig. 3A). Furthermore, transwell assays showed that down-regulated ENG significantly inhibited ectopic endometrial stromal cell migratory and invasive capacities compared with the control groups, whereas there was no significant difference between sh-NC and control groups (Fig. 3B and C). These data suggest ENG knockdown can inhibit migration and invasion of hEM15A cells.

The epithelial to mesenchymal transition (EMT) has been implicated as a key regulator of cell proliferation, apoptosis, and metastasis and is activated in endometriosis,

suggesting the involvement of EMT in the disease. As described in Figure 3D, ENG knockdown significantly inhibited the expression of N-cadherin, vimentin, matrix metalloproteinase (MMP)-2 and MMP-9 and increased E-cadherin expression in ectopic endometrial stromal cells compared with the control groups, whereas sh-NC transfection had no effects on EMT-related proteins compared with the control groups (Tab. 1). These data indicate ENG knockdown can inhibit EMT process in endometriosis.

## DISCUSSION

Endometriosis is manifested by ectopic implantation of endometrial cells tissue outside the uterine cavity and may cause symptoms, which include chronic pelvic pain, bleeding, infertility, and an increased susceptibility to development of adenocarcinoma [9]. However, the pathogenesis of endometriosis has not been fully elucidated. In this study, we found that ENG was upregulated in ectopic endometrial tissues from patients with endometriosis compared with normal endometrial tissues, suggesting that ENG plays a vital role in the development of endometriosis. Subsequently, a series of cell experiments showed that ENG knockdown could induce cell apoptosis and inhibit cell proliferation, migration, invasion in hEM15A cells. Furthermore, ENG knockdown led to the changes of EMT-related proteins, indicating ENG may be involved in endometriosis through regulating EMT process.

In this study, we found up-regulated ENG in endometriotic tissues. Further, we showed that down-regulated ENG by shRNA could induce cell apoptosis and promote cell viability, migration and invasion in hEM15A cells. It is well known that ENG is involved in [tumor microvessel growth](#), metastasis, drug-resistance in many solid tumors, whereas targeting ENG exhibits a significant anti-tumor effect [10–13]. Similar to our findings, few studies have reported that the expression of ENG was significantly increased in endometriotic tissues and has been identified as an active player in the process of endometriotic angiogenesis [7, 14, 15]. However, the biological function and underlying mechanism of ENG in endometriosis has not been clearly clarified. It has been reported that ENG plays an important biological role in EMT process. Mercado-Pimentel et al. [16], showed that ENG directly participated in the process of EMT through interacting with both TGF $\beta$ -regulated activation and invasion pathways



during cardiac valve formation, whereas inhibition of ENG expression could directly perturb EMT process and decrease the expression of EMT markers including slug, runx2, RhoA, and latrophilin-2. Hu et al. [17] found that ENG positive renal tumor-initiating cells distinctly exhibit upregulated N-cadherin and vimentin and negligible E-cadherin in comparison with the unselected parental tumor cells. Silencing ENG could increase E-cadherin level and decrease the levels of vimentin and N-cadherin and inhibit cell invasion and motility. Consistent with these previous studies, our results showed that knockdown of ENG could increase epithelial marker E-cadherin expression and decrease the expression of mesenchymal markers N-cadherin and vimentin in hEM15A cells. In addition, we also found silencing ENG attenuated the levels of MMP2 and MMP9 that are the major proteases in extracellular matrix degradation. EMT endows cells with migratory and invasive properties, which are also considered to be prerequisites for the original establishment of endometriotic lesions. The mesothelial cells that have undergone EMT no longer provide a lamellar protective barrier between the basal layer and the lumen. In the absence of the mesothelial barrier, endometrial cells readily adhere to the subperitoneum and form endometriosis. Many studies on endometriosis invasion and fibrosis have also shown the importance of EMT [18-21]. Therefore, based on all findings, we speculate that ENG may promote cell migration and invasion by regulating EMT phenotype in endometriosis.

## **CONCLUSIONS**

In conclusion, we observe that ENG expression is upregulated in endometriosis tissues and silencing ENG inhibits proliferation, migration, invasion and induces apoptosis through modulation of EMT in endometriosis. These results reveal new insights into the pathogenesis of endometriosis and contribute to the exploration of molecular therapeutic strategies against endometriosis.

### ***Compliance with Ethical Standards***

The study was approved by the Institute Research Ethics Committee of People's Hospital of Anji. All protocols and methods were in accordance with the guidelines and regulations. Written informed consent was obtained from all patients.

### ***Consent for publication***

All authors approved publication of the manuscript.

### ***Availability of data and material***

The datasets used and/or analyzed during this study are available from the corresponding author on reasonable request.

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### ***Conflict of interest***

The authors state that there are no conflicts of interest to disclose.

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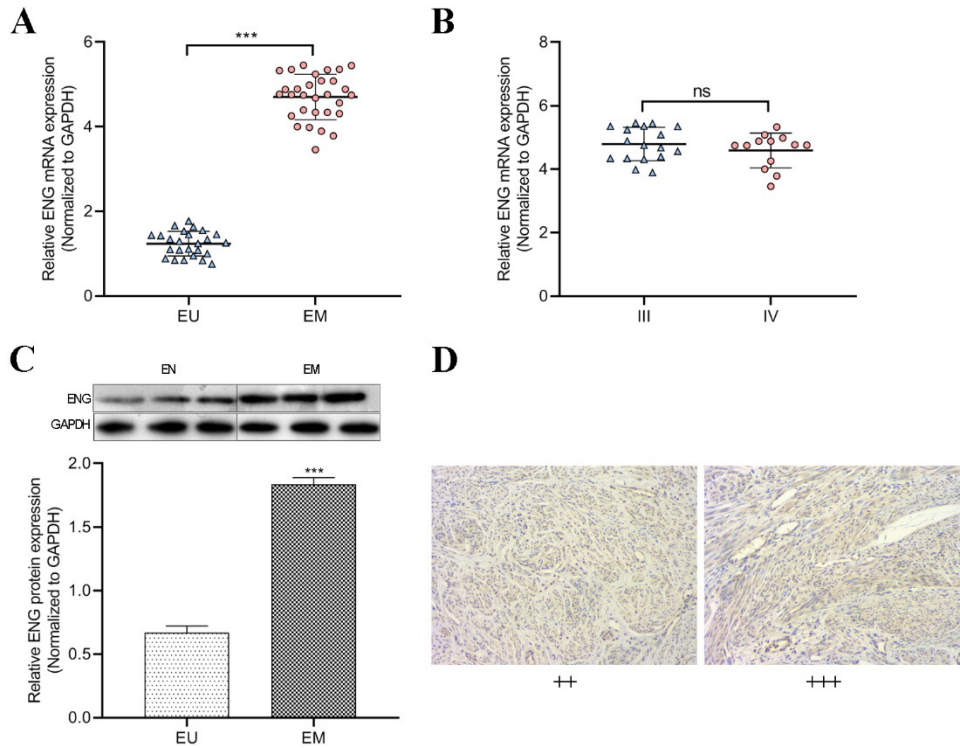
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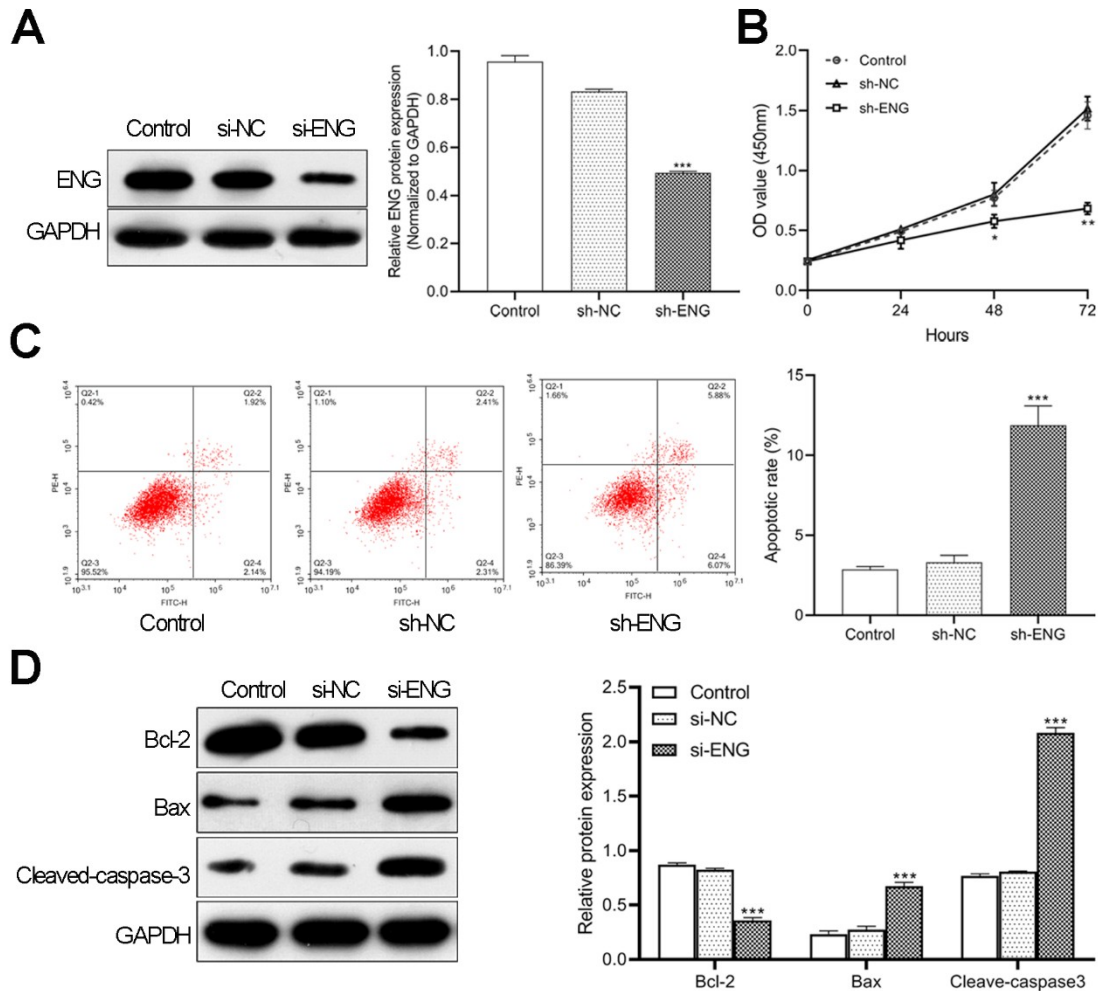
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**Table 1.** The expression of EMT-related proteins in hEM15A cells after knockdown of ENG

	Control	sh-NC	sh-ENG	p value
E-cadherin	0.3587 ± 0.0070	0.3403 ± 0.0131	0.8683 ± 0.0140	< 0.001
N-cadherin	0.5483 ± 0.0192	0.5380 ± 0.0181	0.1993 ± 0.0125	< 0.001
Vimentin	0.8637 ± 0.0141	0.8220 ± 0.0217	0.3183 ± 0.0133	< 0.001
MMP2	0.4747 ± 0.0119	0.4597 ± 0.0059	0.2203 ± 0.0180	< 0.001
MMP9	0.7690 ± 0.0167	0.7470 ± 0.0191	0.2327 ± 0.0075	< 0.001

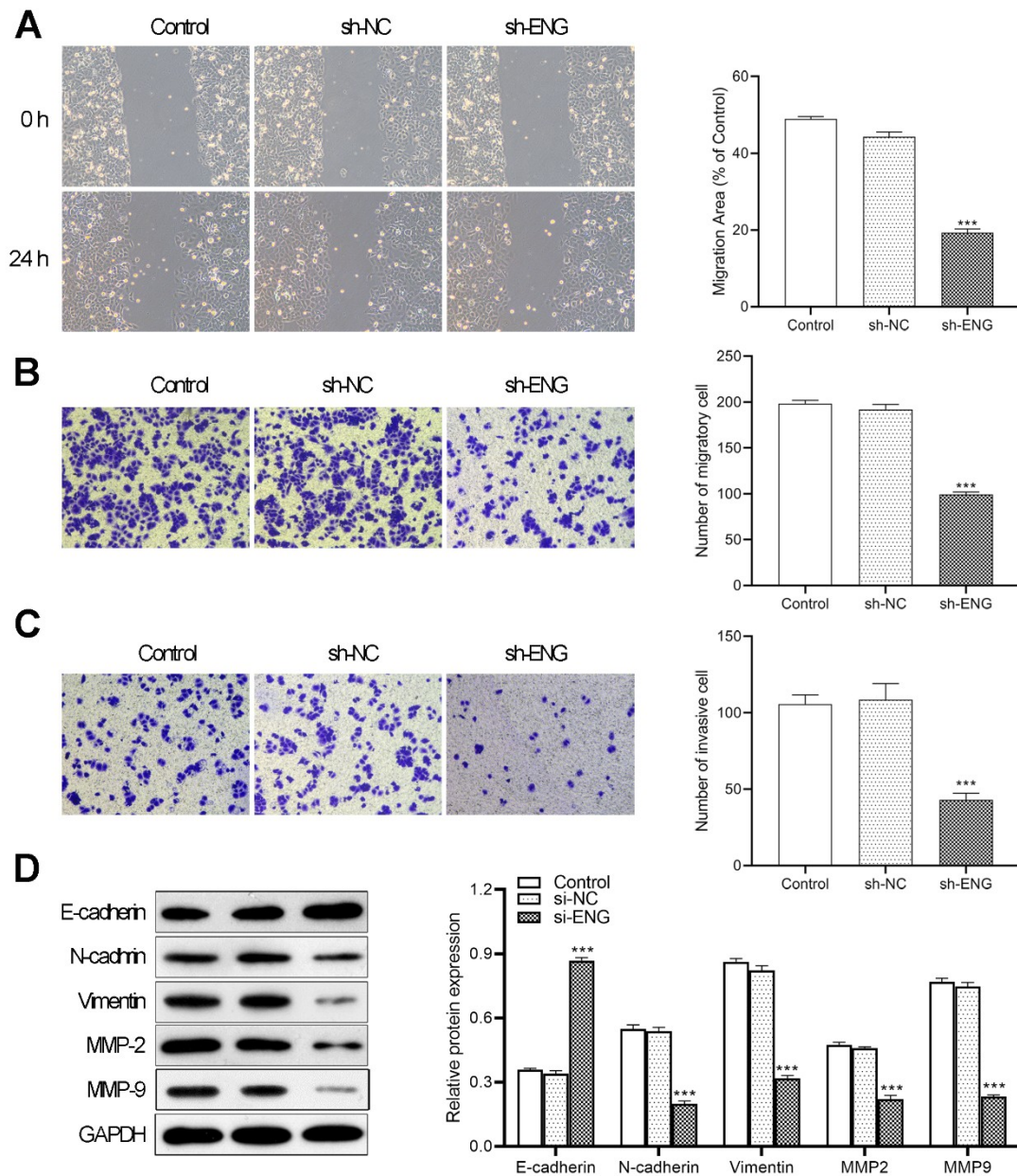


**Figure 1.** ENG expression was increased in ectopic endometrial tissues; **A.** qRT-PCR was used to detect ENG mRNA expression in endometriosis and normal endometrial tissues; **B.** The expression of ENG in stage III and IV endometriosis, revealed by qRT-PCR. **C.** Western blot was performed to measure ENG protein level. IHC was carried out to examine the intracellular expression and location of ENG, and the most representative results were shown; EN — normal endometrium; EM — ectopic endometrium; \*\*\*  $p < 0.001$  as compared with EU groups; ns — no significance



**Figure 2.** ENG knockdown inhibited proliferation and induced apoptosis of hEM15A cells; **A.** qRT-PCR and western blotting were used to detect ENG expression in hEM15A cells transfected with sh-ENG or sh-NC. The effects of ENG knockdown on cell proliferation **B.** and apoptosis **C.** were measured by CCK8 and flow cytometry analysis; **D.** Cell apoptosis-related proteins were detected by western blot; \* $p < 0.05$ ; \*\* $p < 0.01$  and \*\*\* $p < 0.001$  as compared with the control groups





**Figure 3.** ENG knockdown regulated migration and invasion and EMT-related proteins of hEM15A cells; **A.** Wound healing was performed to assess cell migratory ability. Transwell assay were used to asses **B.** cell migratory and **C.** invasive ability; **D.** Western blot was used to measure the expression of EMT-related proteins, including N-cadherin, Vimentin, MMP-2, MMP-9 and E-cadherin; \*\*\* $p < 0.001$  as compared with the control groups