



Research Article

Inhibitory effect on endometrial cancer: Collagen type XII α 1 chain

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ABSTRACT

Objective: Endometrial cancer (EC) is one of the most common gynecological malignancies, and it poses a considerable threat to women's lives. Therefore, searching for EC inhibitors and exploring the potential mechanism of action is particularly important. This article aims to investigate the potential effect of collagen type XII α 1 chain (COL12A1) on macrophage polarization and its subsequent influence on the biological behavior of EC cells to further elucidate the underlying mechanisms of EC development.

Material and Methods: Quantitative real-time polymerase chain reaction and Western blot were used to detect the expression levels of COL12A1 messenger RNA and protein in EC cells. A subcutaneous tumor formation assay was performed in nude mice to evaluate the effect of COL12A1 on EC cell growth *in vivo*. Flow cytometry was utilized to assess the expression levels of macrophage surface markers under different treatments. Cell counting kit-8, Transwell assay, and Western blot experiments were conducted to investigate the effects of COL12A1 knockdown and various macrophage treatments on the biological behavior of EC cells.

Results: The expression of COL12A1 was upregulated in EC cells. Knockdown of COL12A1 significantly inhibited the viability, invasion, migration, and extracellular matrix abilities of EC cells and tumor growth *in vivo*. Overexpression of COL12A1 significantly promoted M2-type macrophage polarization, which enhanced the invasion, migration, and epithelial-mesenchymal transition abilities of EC cells.

Conclusion: The expression of COL12A1 is upregulated in EC, and COL12A1 promotes EC cell invasion and migration by activating macrophage M2 polarization.

Keywords: Collagen type XII alpha 1 chain COL12A1, Endometrial cancer, Invasion, Migration, Tumor-associated macrophage polarization

INTRODUCTION

Endometrial cancer (EC) is a gynecological malignant tumor that arises in the glandular epithelium of the endometrium. The incidence rate of EC has been increasing annually, significantly affecting women's quality of life.^[1] A study has identified postmenopausal women as the primary population at risk, with an increasingly younger age of onset.^[2] While the overall prognosis for this disease is favorable, patients with infiltration and metastasis exhibit poor outcomes. Recent research has confirmed that tumor development is not solely attributed to cellular changes within tissues but also results from interactions between tissue cells and the tumor microenvironment.^[3] Notably, infiltration by tumor-associated macrophages (TAMs) has been linked to myxoid infiltration and lymphatic metastasis in EC, showing a negative correlation with patient prognosis.^[4-6] Therefore, understanding its underlying mechanisms

holds great significance in providing a biological foundation for treatment.

TAMs are a crucial cell subpopulation within the tumor microenvironment.^[7] On the basis of differences in macrophage surface markers, activation status, and function, TAMs can be classified into M1 and M2 types. The classically activated M1 type is predominantly found in inflammatory environments, expressing epitope molecules such as cluster of differentiation (CD86/68) and major histocompatibility complex II. They secrete pro-inflammatory factors like interleukin (IL-6, IL-12, and IL-23) to mediate inflammatory responses. Meanwhile, alternatively, activated M2 type primarily exists in the tumor microenvironment and secretes anti-inflammatory factors IL-4 and IL-10. They participate in regulating tumorigenesis and development.^[8] Monocytes present in peripheral circulation are recruited to tumor tissues by chemokines secreted by stromal cells and tumors. Under stimulation from the local microenvironment, these monocytes polarize into TAMs with an M2 phenotype. These differentiated TAMs express phenotypic molecules, including Arg1, CD68, CD163, and CD206, while producing various growth factors and cytokines that facilitate tumor cell proliferation, infiltration metastasis, and neovascularization.^[9] Numerous studies have demonstrated that infiltration of M2-type macrophages, including EC, is linked to worsened prognosis,^[10,11] although the underlying mechanisms remain unclear.

Epithelial-mesenchymal transformation (EMT) allows otherwise quiescent epithelial cells to become more mobile and may detach from the primary tumor site, enter the circulatory system, and ultimately colonize distant organs to form metastatic foci.^[12] Cytoskeletal changes occur during EMT, with weakened adhesion between cells and increased expression levels of mesenchymal markers such as N-cadherin and Vimentin.^[13] Tanaka's study identified N-cadherin as an independent predictor of survival in patients with EC.^[14] Extracellular matrix (ECM) alterations are important during EMT. The ECM represents a major component of the tumor microenvironment, and abnormal collagen expression within the ECM has been observed in various cancers.^[15] This association is believed to modulate the interaction between collagen I fibrils and the surrounding matrix, thereby playing a pivotal role in regulating the tumor microenvironment.^[16,17]

Collagen type XII alpha 1 chain (COL12A1), a gene encoding XII type collagen genes of alpha chain, is a member of the protofiber-associated collagen family, whereas XII type collagens are important cellular scaffolding and support proteins in the ECM.^[18] Previous studies have found that dysregulation of COL12A1 expression is involved in various tumorigenesis, progression, and metastasis.^[19,20] COL12A1 expression is increased in pancreatic cancer and associated

with poor prognosis, and it can be used as an immune marker for pancreatic cancer.^[21] By performing bioinformatics analysis, Hauptman found that genes involved in ECM receptor interactions, such as COL12A1, COL1A2, and COL3A1, were upregulated in cancers compared with adenomas, suggesting that ECM organizing processes are involved in the progression of adenomas to carcinomas.^[22] The underlying mechanisms of COL12A1 in EC are still not well understood. This study seeks to explore how COL12A1 contributes to the progression of EC and uncover its potential mechanisms, thereby laying new theoretical groundwork for the treatment of EC.

MATERIAL AND METHODS

Cell culture and transfection

Human monocyte THP-1 cells (cat. SCSP-567) were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (cat. 11875093, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, cat. A5256701, Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin (cat. C0222, Beyotime Biotech, Shanghai, China). Human endometrial mesenchymal stromal cells (HESCs) and EC cells Ishikawa (cat. SC0202), AN3CA (cat. SC0199), HEC-1A (cat. SC0204), and RL-95 (cat. SC0200) were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM, cat. C11960500BT, Gibco, Grand Island, NY, USA). All EC cells were purchased from Yuchi Biological Technology Co., Ltd. (Shanghai, China), with correct STR identification and negative mycoplasma detection. All cell lines were incubated at 37°C with 5% carbon dioxide (CO₂). The EC cells in the logarithmic growth phase were enzymatically dissociated and seeded into six-well plates.

Construction and transfection of small interfering RNA (siRNA) and overexpressed plasmid

Si-NC and si-COL12A1 were designed using the online GenScript siRNA Target Finder tool (GenScript, Piscataway, NJ, USA) and synthesized by Ribo Bio (Guangzhou, China). The sequence is si-NC: 5'-UUC UCC GAACGU GUC ACG UTT-3', si-COL12A1: 5'-GAT CGG CAA TAC TCT CAC AGG CAT GGC TCG AGC CAT GCC TGT GAG AGT ATT GCT TTT TTG GAA TTC-3'. The overexpressed plasmid pcDNA-COL12A1 was completed by Feng Hui Biological Company (Hunan, China). An empty vector was used as a negative control. When the cell density reached 60–80%, the culture medium was replaced with serum-free DMEM. LipofectamineTM2000 (Invitrogen, Carlsbad, CA, USA) was used to transfect si-NC and si-COL12A1 into EC cells and transfect pc DNA-COL12A1 and Vector into macrophages

according to the manufacturer's instructions. After 48 h, the transfected cells were collected for subsequent studies.

Induced differentiation of THP-1 cells

The THP-1 cell suspension was collected, and THP-1 monocytes were stimulated with Phorbol 12-myristate 13-acetate (PMA, cat. HY-18739, 100 ng/mL, Med Chem Express, New Jersey, USA) for 24 h to induce their differentiation into M0-type (inactivated state) macrophage.^[23] Subsequently, lipopolysaccharides (LPS, cat. HY-D1056, 10 ng/mL, Med Chem Express, New Jersey, USA) and interferon-gamma (IFN- γ , cat. HY-P7025, 20 ng/mL, Med Chem Express, New Jersey, USA) were added and treated for 48 h to promote their polarization toward M1-type macrophages (M1 group).^[24] IL-4 and IL-13 at a concentration of 20 ng/mL were applied for 48 h to stimulate the cells' differentiation into M2-type macrophages (M2 group).^[25] Then, Vector or pcDNA-COL12A1 plasmids were transfected into THP-1 differentiated M2-type macrophages using Lipofectamine 2000 according to the manufacturer's instructions. After incubation for an additional 48 h, the cells were collected for subsequent experiments.

Macrophages co-cultured with EC cells

The transfected THP-1 cells were seeded at a density of 1×10^5 cells/well in the upper chamber (0.4 μ m diameter, Corning, NY, USA) of the co-culture system, with 200 μ L per well. EC cells were then resuspended in a DMEM medium containing 10% FBS at a concentration of 2500 cells/mL, and 800 μ L of cell suspension was added to the lower chamber of the co-culture system. Care should be taken during this process to prevent the formation of air bubbles between the upper and lower chambers. Following this, the co-culture system was incubated in a cell culture incubator at 37°C with 5% CO₂ for a duration of 48 h. Finally, the cells were harvested for subsequent analysis.

RNA extraction and quantitative real-time polymerase chain reaction (RTqPCR)

The cells were subjected to TRIzol-based (cat. 15596026CN, Invitrogen, Carlsbad, CA, USA) total RNA extraction followed by an assessment of its purity. Reverse transcription was performed in accordance with the instructions provided with the reverse transcription kit (cat. N8080234, Invitrogen, Carlsbad, CA, USA). The resulting cDNA was diluted using RNase-free water (cat. R0021, Beyotime Biotech, Shanghai, China) and used as a template for PCR amplification on a fluorescent quantitative PCR instrument, employing the SYBR Green quantitative PCR kit (Qiagen, Germantown, MD, USA). The relative expression levels of the target genes were determined using the 2^{- $\Delta\Delta$ Ct} method. The primer

sequence is as follows: *COL12A1*, forward, 5'-CCA CAG GTT CAA GAG GTC CC-3' and reverse, 5'-TGT GTT AGC CGG AAC CTG GA-3', glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*); forward, 5'-ACA ACT TTG GTA TCG TGG AAG G-3' and reverse, 5'-GCC ATC ACG CCA CAG TTT C-3'.

Western blot

The cells were lysed, and the total cellular proteins (cat. 89900, Thermo Scientific, Waltham, MA, USA) were subsequently extracted and quantified spectrophotometrically. A denatured protein sample of 35 μ g was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene fluoride membrane (cat. 1620177, Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% bovine serum albumin at 37°C for 2 h. Specific primary antibodies *COL12A1* (cat. ab121304, Abcam), E-cadherin (cat. 3195, Cell signaling Technology), N-cadherin (cat.13116, Cell signaling Technology), Vimentin (cat. ab92547, Abcam, all diluted at 1:1000), and *GAPDH* (cat. ab181602, 1:5000, Abcam) were incubated with the membrane overnight at 4°C. Subsequently, an HRP-labeled secondary antibody (cat. ab205718, 1:5000, Abcam) was added and incubated at 37°C for 1 h. Finally, an enhanced chemiluminescence luminescent solution (cat. WBKLS0500, Millipore, Billerica, MA, USA) and an imaging system (iBright FL1000, Thermo Fisher Scientific, USA) were used for imaging. The gray value of the bands was determined using ImageJ (version 1.53k, NIH, Bethesda, MD, USA).

CCK-8 assay

The EC cells were routinely digested, centrifuged, and then resuspended. The cell concentration was 3×10^4 cells/mL in complete medium, and 100 μ L per well was seeded in 96-well plates. After the cells were incubated for 0, 24, 48, and 72 h, each well was added with 10 μ L CCK-8 solution (cat. 96992, Sigma-Aldrich, St. Louis, MO, USA) and further incubated for an additional 2 h. The absorbance at a wavelength of 450 nm was measured using a microplate reader (Synergy HT, BioTek, Winooski, VT, USA) to record the optical density value for plotting the cell growth curve.

Transwell assay

The cell invasion assay involved the even distribution of the diluted Matrigel gel solution onto the basement membrane of the upper chamber of the Transwell, followed by overnight incubation in a cell incubator to allow for semi-solidification of the matrix gel. On the next day, the EC cells were prepared as a single-cell suspension with a cell density of 4×10^4 cells/mL. Subsequently, 300 μ L of the treated cell suspension from each group was added to the upper layer

of the chambers, and 600 μ L of medium containing 10% FBS was added to the lower layer. After 24 h, the cells in the lower layer were fixed using a solution consisting of 4% paraformaldehyde. Following fixation, crystal violet staining (0.1%, cat. C0121, Beyotime Biotech, Shanghai, China) was performed for 5 min on these cells. Five randomly selected non-overlapping fields were photographed under an inverted microscope (Olympus IX71, Tokyo, Japan) for counting and taking the average value. The cell migration assay did not necessitate the preparation of Matrigel matrix gel, and the remaining experimental procedures remained consistent with those employed in the invasion assay.

Subcutaneous tumor formation in nude mice

All 12 of 4-week-old BALB/c nude mice were purchased from Animal Laboratory (Shanghai, China). The mice were maintained at a temperature of 20–27 °C, a relative humidity of 40–60%, and a light/dark cycle of approximately 12 h and fed and watered ad libitum. After 1 week of adaptive feeding, they were randomly divided into two groups: si-NC and si-COL12A1 groups. The EC cells in the logarithmic phase were selected, trypsinized, and collected by centrifugation. Subsequently, the cells were resuspended in serum-free DMEM at a cell density of 1×10^6 cells/mL. Afterward, EC cell suspensions stably transfected with short hairpin sh-NC, and sh-COL12A1 were subcutaneously injected into the back of the nude mice at a volume of 200 μ L each. The nude mice were euthanized through cervical dislocation at the end of a 5-week period, and subsequently, tumor tissues were excised. The length (a, mm) and width (b, mm) were measured with calipers to calculate the tumor volume (mm^3) using the following formula: $a \times b^2 \times 0.5$. Tumor volume growth curves and tumor weights were then measured for each group of nude mice. The animals used in this study have been approved by the Fuzhou First General Hospital Affiliated with Fujian Medical University Ethics Committee (No. 202210015).

Immunohistochemical Ki-67 staining

Tumor tissues were collected from nude mice, fixed, and embedded in paraffin. The paraffin sections were then deparaffinized. Subsequently, they were repaired using sodium citrate buffer and incubated with the primary antibody Ki-67 (1:100, cat. GB111141, Servicebio, Wuhan, China) at 37°C for 1 h. Afterward, they were washed 3 times with phosphate-buffered saline (PBS) for 3 min each time. Then, the secondary antibody (1:200, cat. GB23303, Servicebio, Wuhan, China) was added and incubated for 30 min before being washed again with PBS three times. Finally, a light-absorbing diaminobenzidine chromatography solution (cat. ZLI 9017, Zsgb-bio, Beijing, China) was used to develop color, followed by restaining with hematoxylin for

3 min. The tissues were then observed under a microscope (Eclipse 50i, Nikon fluorescence microscope, Tokyo, Japan), and photographs were taken. The Ki-67-positive cell rate was quantitatively analyzed by ImageJ, and the positive cell rate was calculated as follows: number of positive tumor cells/total number of tumor cells \times 100.

Flow cytometry

The single cell suspension was obtained by washing the macrophages treated with different conditions 2 times with PBS, followed by resuspending the cells in 100 μ L of PBS. By following the instructions provided for the antibodies, fluorescein 5-isothiocyanate-anti-CD68 (cat. 333805, BioLegend; San Diego, CA), PE-anti-CD206 (cat. 321105, BioLegend; San Diego, CA), APC-anti-CD86 antibodies (cat. 374207, BioLegend; San Diego, CA), and 5 μ L of the corresponding fluorescent-labeled antibody were added to each tube, gently mixed, and incubated at 4°C for 30 min in darkness. After two washes with PBS, the cells were resuspended in 300 μ L of PBS. The Beckman Coulter Gallios flow cytometer (Danaher, Washington, USA) was used to detect the expression of surface differentiation antigens in macrophages.

Statistics and analysis

The results of each experiment were statistically analyzed using the Statistical Package for the Social Sciences (version 21.0) software (IBM, Chicago, IL, USA). All experiments were independently repeated 3 times. Data are presented as mean \pm standard deviation; differences between two groups were compared using an independent samples *t*-test; and comparisons among multiple groups were analyzed using analysis of variance, followed by the least significant difference test for *post hoc*. $P < 0.05$ was considered statistically significant.

RESULTS

COL12A1 was upregulated in EC cells

The expression of COL12A1 in HESCs and EC cells Ishikawa, AN3CA, HEC-1A, and RL-95 was assessed using RT-qPCR. A significant upregulation in COL12A1 messenger RNA (mRNA) expression was found in EC cell lines compared with HESCs ($P < 0.001$, Figure 1a). Western blot experiments confirmed a substantial increase in COL12A1 protein levels in EC cells ($P < 0.01$, Figure 1b and c). The two EC cell types exhibiting the highest COL12A1 expression were selected for subsequent investigations. The results collectively indicated a pronounced upregulation of COL12A1 across all EC cells, suggesting its potential involvement in the pathogenesis and progression of EC.

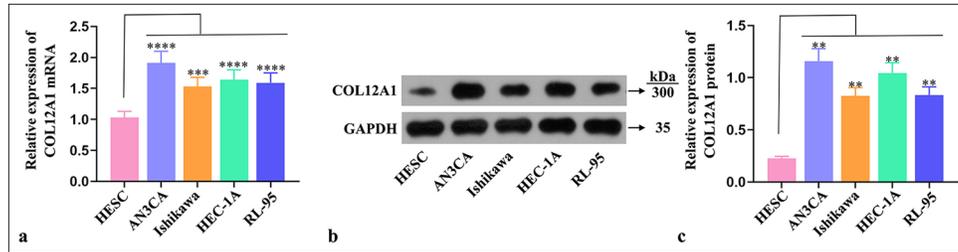


Figure 1: COL12A1 upregulation in EC cells. (a) RT-qPCR detection of the expression of COL12A1 mRNA in EC cells. (b and c) Western blot analysis of the protein expression of COL12A1 in EC cells. $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$ compared with HESC group. Statistical analysis of significance using one-way ANOVA. COL12A1: Collagen type XII alpha 1 chain, EC: Endometrial cancer, mRNA: Messenger RNA, ANOVA: Analysis of variance, RT-qPCR: Quantitative real-time polymerase chain reaction.

Knockdown of COL12A1 suppressed the effects of invasion, migration, and EMT in EC cells

Si-COL12A1 was transfected into two types of EC cells to investigate the effect of COL12A1 on EC progression, and the successful transfection efficiency of COL12A1 was confirmed ($P < 0.05$, Figure 2a-c). CCK-8 assay was employed to assess the effect of COL12A1 on the proliferative capacity of both types of EC cells, revealing a significant inhibition in their proliferation on knockdown of COL12A1 ($P < 0.05$, Figure 2d and e). Cell migration assay results demonstrated a notable reduction in migration capabilities following COL12A1 knockdown in both cell types ($P < 0.05$, Figure 2f-h). Transwell assay showed that the invasive ability of both cell types was significantly reduced after COL12A1 knockdown ($P < 0.05$, Figure 2i-k). Western blot analysis detected alterations in the expression levels of EMT-related proteins N-cadherin, Vimentin, and E-cadherin in both types of EC cells. Notably, downregulation of COL12A1 significantly upregulated E-cadherin protein expression while downregulating N-cadherin and Vimentin protein expression levels ($P < 0.05$, Figure 2l-n). Collectively, these findings suggested that suppressing COL12A1 inhibited *in vitro* proliferation, migration, invasion, and EMT abilities in EC cells.

Knockdown of COL12A1 inhibited EC tumor growth *in vivo*

EC cell suspensions that were stably transfected with si-NC and si-COL12A1 were subcutaneously injected into the nude mice to investigate the effect of COL12A1 on the *in vivo* growth of EC tumors. After a period of 5 weeks, the mice were euthanized, and a significant reduction in tumor volume and weight was observed following the knockdown of COL12A1 ($P < 0.05$, Figure 3a-c). Immunohistochemical analysis revealed a notable decrease in Ki-67 positive expression within the tumor tissues on COL12A1 suppression ($P < 0.05$, Figure 3d and e). These findings

provided compelling evidence that silencing COL12A1 effectively inhibited the growth ability of EC tumors in an *in vivo* setting.

Overexpression of COL12A1 promoted macrophage M2 polarization

Light microscopy was employed to observe the morphological characteristics of macrophages. The THP-1 cells underwent a transition from suspension agglomerative growth to adherent growth with protruding pseudopods following PMA treatment [Figure 4a]. Flow cytometry experiments revealed that LPS + IFN- γ treatment significantly augmented the proportion of CD86+ cells, a marker for M1 macrophages, and IL-4 + IL-13 treatment notably increased the proportion of CD206+ cells, a marker for M2 macrophages ($P < 0.05$, Figure 4b-d), thereby confirming successful induction of macrophage polarization. COL12A1 mRNA expression was significantly higher in M2-type macrophages than in M1-type macrophages ($P < 0.05$, Figure 4e). The overexpressed COL12A1 was transfected into THP-1-derived macrophages treated with IL-4 and IL-13 ($P < 0.05$, Figure 4f-h), and flow cytometry was performed to assess the proportion of M2-type macrophages. The results demonstrate that the IL-4 + IL-13 + pcDNA-COL12A1 group had a significant increase in the percentage of CD206+ cells compared with the IL-4 + IL-13 + vector group ($P < 0.01$, Figure 4i and j), indicating that overexpression of COL12A1 effectively promoted M2 macrophage polarization.

Effect of COL12A1 on EC cell invasion, migration, and EMT by inducing macrophage M2 polarization

To investigate the impact of differently treated macrophage supernatants on the proliferation, invasion, migration, and EMT abilities of EC cells, we observed through CCK-8 assay, Transwell assay, and Western blot experiments that co-cultivation of IL-4- and IL-13-treated macrophages with

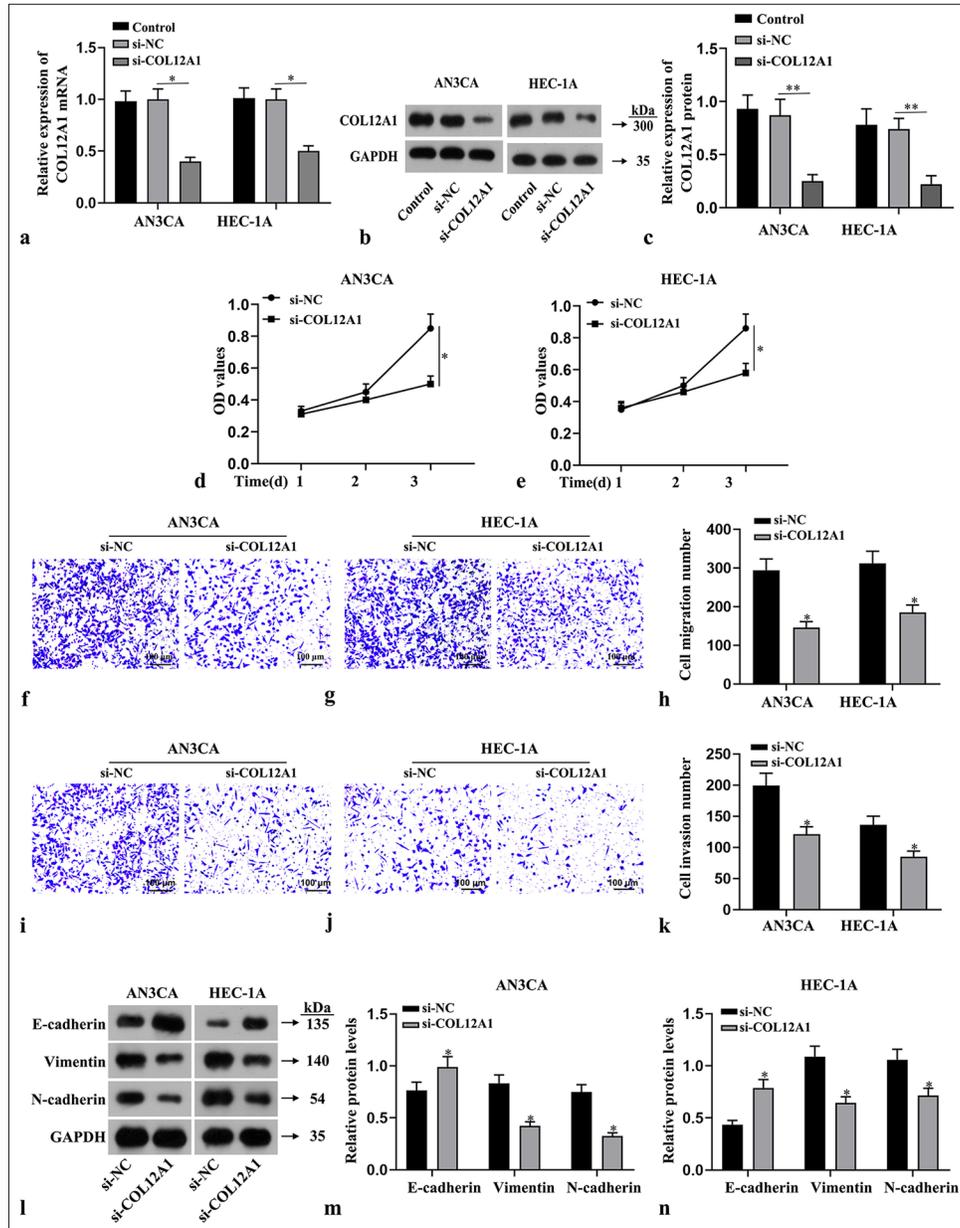


Figure 2: Suppression of the effects of invasion, migration, and EMT in EC cells by knockdown of COL12A1. (a-c) Assessment of the transfection efficiency of COL12A1 by RT-qPCR and Western blot. (d and e) CCK-8 assay evaluation of the viability of EC cells. (f-h) Cell migration assay detection of the migratory capacity of EC cells (Magnification 40 \times). (i-k) Transwell assay measurement of the invasion capabilities of EC cells (Magnification 40 \times). (l-n) Western blot analysis of the protein expression levels of N-cadherin, Vimentin, and E-cadherin in EC cells. * $P < 0.05$ compared with si-NC group. Scale bar = 100 μ m. Statistical analysis of significance using one-way ANOVA or Student's *t*-test. COL12A1: Collagen type XII alpha 1 chain, EMT: Epithelial-mesenchymal transformation, EC: Endometrial cancer, ANOVA: Analysis of variance, RT-qPCR: Quantitative real-time polymerase chain reaction.

EC cells significantly enhanced their proliferation ($P < 0.01$, Figure 5a), invasion, migration ($P < 0.01$, Figure 5b-d), and EMT abilities ($P < 0.01$, Figure 5e-h). These results suggested that a higher proportion of M2-type macrophages induced

stronger proliferation, invasion, migration, and EMT abilities in co-culture conditions with EC cells. Therefore, COL12A1 promoted EC cell invasion, migration, and EMT ability by facilitating M2 polarization in macrophages.

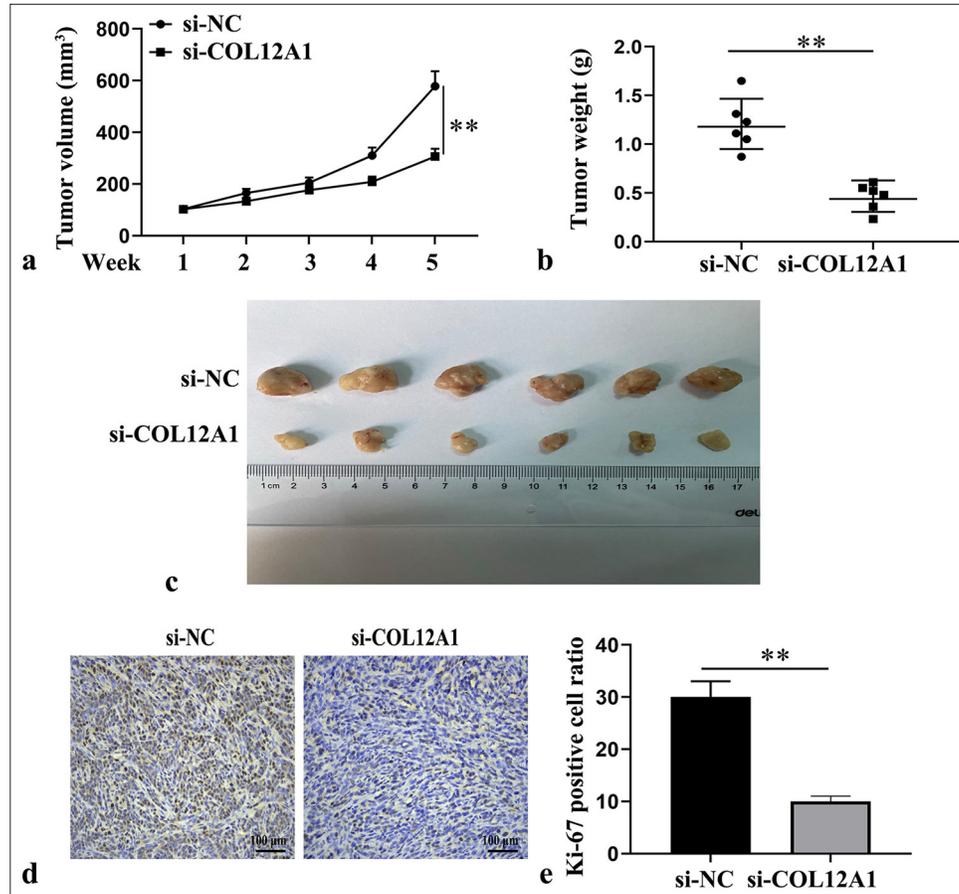


Figure 3: Inhibition of EC tumor growth *in vivo* by knockdown of COL12A1. (a) Assessment of tumor growth kinetics of nude mice in each group ($n = 6$). (b) Quantification of tumor mass of nude mice in each group ($n = 6$). (c) Representative images of transplanted tumor in nude mice at 5 weeks. (d and e) Immunohistochemical analysis of Ki-67 expression in tumor tissue (Magnification 40 \times). * $P < 0.05$. Scale bar of Ki μ m. Statistical analysis of significance using one-way ANOVA or Student's *t*-test. EC: Endometrial cancer, COL12A1: Collagen type XII alpha 1 chain, ANOVA: Analysis of variance.

DISCUSSION

COL12A1 is highly expressed in several types of tumors, including gastric,^[26] colorectal,^[27] and pancreatic adenocarcinomas.^[28] Knockdown experiments targeting COL12A1 demonstrated its ability to inhibit malignant phenotypes of cancer cells. Jiang *et al.*^[20] found that COL12A1 expression in gastric cancer tissue increased significantly, proving that COL12A1 is a prognostic factor in patients with gastric cancer and a potential therapeutic target. In the present study, knockdown experiments were conducted on COL12A1 to investigate its biological function in EC cells. Specifically, its effect on EC cell invasion and migration and EMT were assessed. The results revealed that knocking down COL12A1 significantly suppressed the invasion, migration, and EMT abilities of EC cells *in vitro* and *in vivo*. Collectively, the findings suggested that COL12A1 exerted pro-cancer effects in EC.

The tumor microenvironment of EC comprises immune cells, non-immune stromal cells, and ECM proteins, which encompass immunosuppressive cell populations such as regulatory T-cells, M2-type macrophages, and myeloid-derived suppressor cells. This environment fosters the progression of EC.^[29] Among these components, TAMs exert the most significant influence on tumor progression and play a crucial role in evading immune surveillance mechanisms. Their presence often indicates a worsened prognosis.^[30-32] Macrophages can be categorized into M1-type macrophages (classically activated), which possess antitumor effects, and M2-type macrophages (alternatively activated), which exhibit pro-tumor effects.^[33] Under *in vitro* conditions, THP-1 cells can be polarized to M1-type macrophages by INF- γ combined with LPS or to M2-type macrophages by IL-4 combined with IL-13 after induction with PMA.^[34] In the tumor microenvironment, TAMs predominantly display an M2 phenotype.^[35] Studies have demonstrated that TAMs

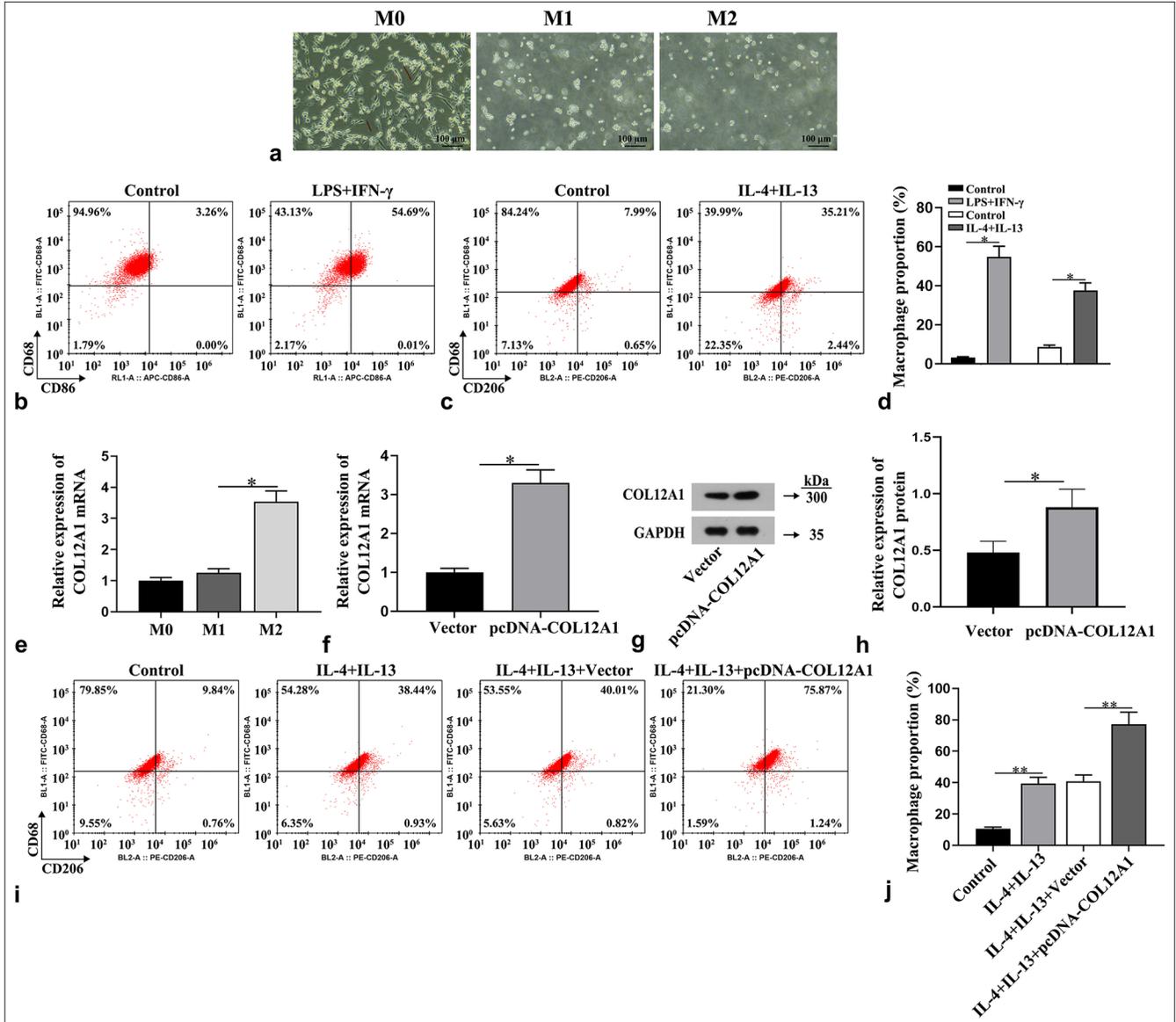


Figure 4: Promotion of macrophage M2 polarization by overexpression of COL12A1. (a) Examination of the morphology of macrophages using optical microscopy (Magnification 40 \times). (b-d) Flow cytometry used to determine the proportion of M1 and M2 macrophages. (e) Detection of COL12A1 mRNA expression by RT-qPCR. (f-h) Detection of COL12A1 overexpression efficiency. (i and j) Flow cytometry used to determine the proportion of M2 macrophages. * $P < 0.05$ and ** $P < 0.01$. Scale bar = 100 μ m. CD: Cluster of differentiation, IL: Interleukin. Statistical analysis of significance using one-way ANOVA or Student's *t*-test. COL12A1: Collagen type XII alpha 1 chain, mRNA: Messenger RNA, ANOVA: Analysis of variance, RT-qPCR: Quantitative real-time polymerase chain reaction.

can enhance the malignant biological behavior of tumor cells by secreting a diverse array of mediators capable of remodeling the tumor microenvironment.^[36] The protumorigenic properties exhibited by TAMs during tumor progression render them an important therapeutic target in cancer treatment.^[37] The current therapeutic strategies for TAMs aim to either directly reduce their abundance or polarize them toward an antitumor phenotype.^[38] Therefore, reducing the proportion of M2-type macrophages within the tumor microenvironment remains a valuable direction in

the field of tumor therapy. A recent study reported that the knockdown of COL12A1 suppressed the infiltration of M2-type macrophages in breast cancer.^[39] Similarly, the present study showed a high expression of COL12A1 in M2-type macrophages. The overexpression of COL12A1 significantly enhanced the proportion of IL-4 combined with IL-13-induced production of M2-type macrophages, indicating that COL12A1 promotes monocyte-macrophage polarization toward the M2 phenotype. Therefore, the pro-carcinogenic effect of COL12A1 on EC may involve its regulation of

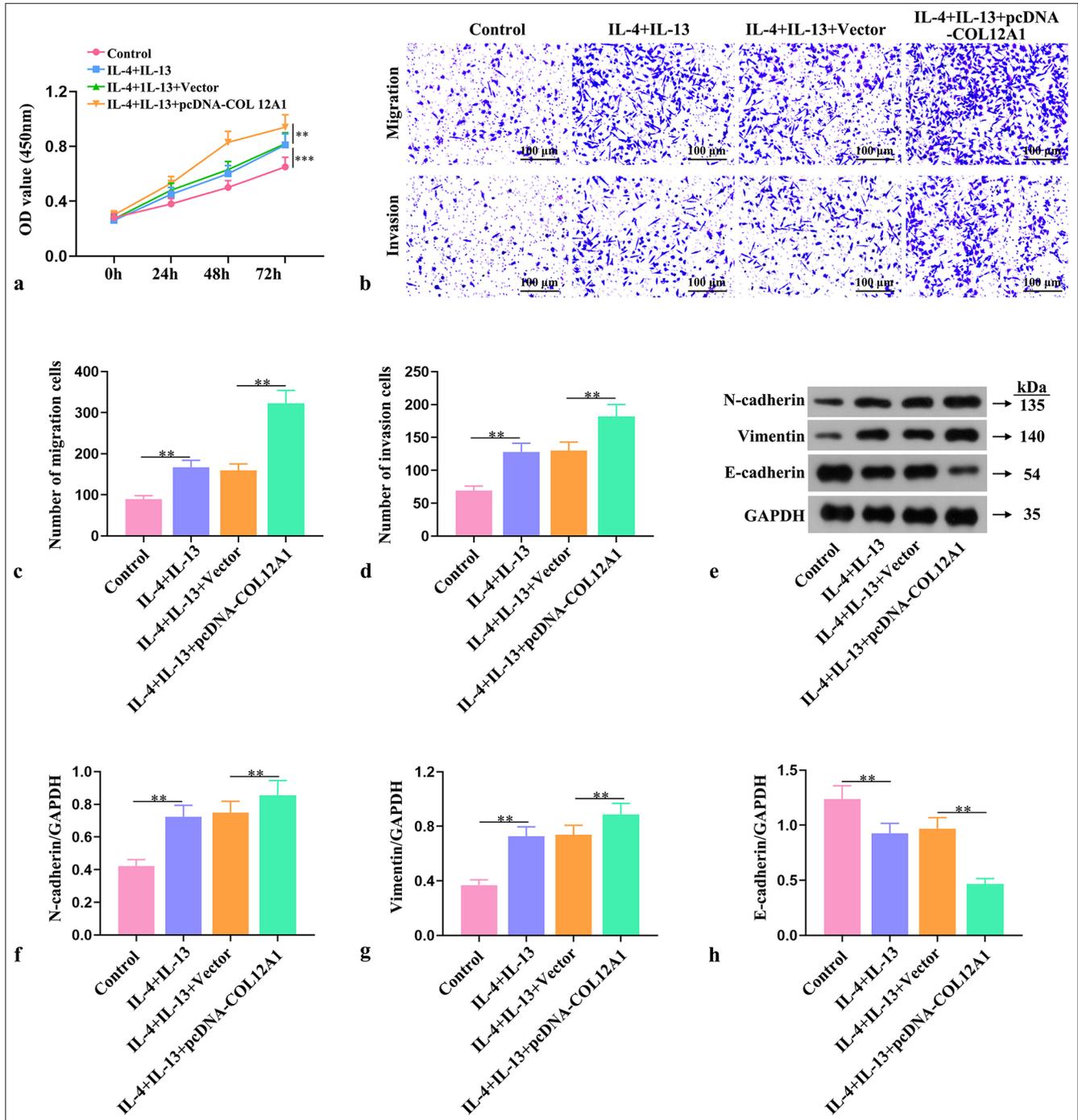


Figure 5: Effect of COL12A1 on EC cell invasion, cell migration, and EMT by inducing macrophage M2 polarization. (a) Assessment of EC cell viability through CCK-8 assay. (b-d) Evaluation of the invasive and migratory abilities of EC cells by Transwell assay (Magnification 40 \times). (e-h) Western blot analysis of the protein expression levels of N-cadherin, Vimentin, and E-cadherin in EC cells. $**P < 0.01$ and $***P < 0.001$. Scale bar = 100 μ m. OD: Optical density. Statistical analysis of significance using one-way ANOVA. COL12A1: Collagen type XII alpha 1 chain, EMT: Epithelial-mesenchymal transformation, EC: Endometrial cancer, ANOVA: Analysis of variance.

macrophage M2 polarization. CCK-8, Transwell migration and invasion, and EMT assays were conducted to assess the effects of COL12A1-treated macrophages on proliferation,

migration, invasion, and EMT in EC cells. The results revealed that overexpression of COL12A1 further augmented the pro-growth and invasive properties of macrophages toward

tumors. COL12A1 not only exerted its promotional effect on EC cells but also regulated the polarization of macrophages within the tumor microenvironment. Liu's study determined that the genes *COL1A1*, *COL4A1*, and *COL12A1* were significantly associated with macrophage M2 infiltration in gastric cancer.^[26] These experiments collectively illustrated that COL12A1 enhances growth and metastasis in EC cells by promoting M2-type polarization in macrophages.

This study has some limitations. It verified the cell performance characteristics of COL12A1 on macrophage polarization only, and a detailed study of the mechanism was not conducted. In addition, only specific EC cell lines were studied, which may not be representative of the characteristics of all types and subtypes of EC cells, so sample bias may be present. In future studies, multi-omics and bioinformatics analyses regarding EC and COL12A1 could contribute to a deeper understanding of the underlying mechanisms.

CONCLUSION

This study revealed that the expression of COL12A1 was upregulated in EC cells. Knockdown of COL12A1 significantly inhibited EC cell proliferation, migration, invasion, and EMT abilities. Overexpression of COL12A1 may enhance the proliferation and metastatic potential of EC cells by modulating M2-type macrophage polarization. These findings provide experimental and theoretical evidence supporting the utilization of COL12A1 as a biological marker and therapeutic target for EC.

AVAILABILITY OF DATA AND MATERIALS

The data analyzed are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

ZS and QJ: Designed the research study; MH and JL: Performed the research; SW: Collected the data and made statistical analysis; QJ: Help and advice on the experiments; ZS and QJ: Analyzed the data and drafted the manuscript. All authors contributed to important editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

ABBREVIATIONS

CCK-8: Cell counting kit-8
 CD86/68: Cluster of differentiation 86/68
 COL12A1: Collagen type XII α 1 chain
 DMEM: High-glucose Dulbecco's modified eagle medium
 EC: Endometrial cancer
 ECM: Extracellular matrix

EMT: Epithelial-mesenchymal transformation
 GAPDH: Glyceraldehyde- 3-phosphate dehydrogenase
 HESCs: Human endometrial mesenchymal stromal cells
 IFN- γ : Interferon-gamma
 IL: Interleukin
 LPS: Lipopolysaccharides
 PMA: Phorbol 12-myristate 13-acetate
 TAMs: Tumor-associated macrophages

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The animals used in this study have been approved by the Fuzhou First General Hospital Affiliated with Fujian Medical University Ethics Committee (No.202210015).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

EDITORIAL/PEER REVIEW

To ensure the integrity and highest quality of CytoJournal publications, the review process of this manuscript was conducted under a **double-blind model** (authors are blinded for reviewers and vice versa) through an automatic online system.

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