Original Article

All-trans retinoic acid inhibits epithelial-to-mesenchymal transition (EMT) through the down-regulation of IL-6 in endometriosis

Luyi Li[1], Hui Gao[2], Liuliu Pan[1], Yu Zhao[1], Zongwen Liang[1], Qiong Zhang[1], Danhan Wang[1]

[1]Department of Obstetrics and Gynecology, the Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, China; [2]Key Laboratory of Fertility Preservation and Maintenance of Ministry of Education, Ningxia Medical University, Yinchuan, China

Contributions: (I) Conception and design: D Wang; (II) Administrative support: Q Zhang; (III) Provision of study materials or patients: Y Zhao, H Gao; (IV) Collection and assembly of data: L Li, H Gao, L Pan; (V) Data analysis and interpretation: L Li, Z Liang; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Qiong Zhang; Danhan Wang. Department of Obstetrics and Gynecology, the Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, China. Email: joan_zhang2002@sina.com; handan3711@163.com.

Background: Endometriosis (EMs) is a benign, but potential metastatic, gynecological disease. Our current study aims to examine whether all-trans retinoic acid (ATRA) inhibits the epithelial-to-mesenchymal transition (EMT) of endometriotic stromal stem cells, as well as to explore the mechanisms involved, especially the role of IL-6 played in.

Methods: Cell clonogenic capacity was examined by the low-density clonogenicity assay. Cell differentiation capacity was assessed by *in vitro* differentiation. The level of IL-6 was measured by the ELISA assay. Migration and invasion abilities were measured using the transwell assay. Western blot and RT-qPCR were performed to detect EMT-related genes and proteins.

Results: Large endometriotic stromal colony forming units (CFUs) could be regarded as the enrichment sets of endometriotic stromal stem cells. They maintained a higher potential for self-renewal, proliferation, invasion, and EMT, along with up-regulated IL-6. After ATRA treatment, the expression of IL-6 was significantly reduced, accompanied by a decrease in the migration, invasion, and EMT of large endometriotic stromal CFUs. In addition, the inhibition of ATRA was mediated by IL-6.

Conclusions: Our study showed that one of the therapeutic effects of ATRA on EMs through its modulation in EMT of large endometriotic stromal CFUs. ATRA may be a promising therapeutic strategy aimed at IL-6 for the stem-cell treatment of EMs.

Keywords: All-trans retinoic acid (ATRA); endometriosis (EMs); stem cell; epithelial-to-mesenchymal transition (EMT); interleukin-6 (IL-6)

Submitted Jul 19, 2021. Accepted for publication Oct 13, 2021.
doi: 10.21037/apm-21-2175
View this article at: https://dx.doi.org/10.21037/apm-21-2175

Introduction

Endometriosis (EMs) is a non-malignant, but potential metastatic, reproductive disorder that manifests as inflammatory endometriotic implants growing outside of the uterus, mainly occurring in women of childbearing age. The incidence of chronic pelvic pain and infertility in patients is as high as 45%, and around 10–12% of women worldwide suffer from this disease (1,2). However, its defined etiology and pathogenic mechanism remain obscure, and the current clinical therapeutic effect is dismal. Multiple hypotheses have been proposed to elucidate the initiation and development of EMs (3). Among, the most commonly accepted hypothesis is "Sampson's theory", postulating that endometrial cells are deposited into the peritoneal cavity via retrograde menstruation (4). However, nearly 90% of women experience retrograde menstruation while endometriotic lesions are observed in only 10%, suggesting that additional factors trigger the incidence (5). In the 1970s, Prianishnikov

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presented the concept of the "endometrial stem cell", positing that a small amount of endometrial stem cells, which reside in the endometrial functional layer, possess the characteristics of mesenchymal stem cells (6). It has been suggested that endometrial stem cells participate in EMs pathogenesis due to their features of cell regeneration, proliferation, and high developmental plasticity (7-9).

Epithelial-to-mesenchymal transition (EMT) is an established mechanism that drives the progression of the in-situ tumor towards metastasis through affecting cell morphology and polarization, which causes a disruption of the integrity of the intercellular cohesion (10). Although EMs is acknowledged to be a benign gynecological disorder, it has similar characteristics to malignant tumors such as cell infiltration, migration, and invasion, thereby infiltrating and developing new ectopic lesions (11). Recently, it has been verified that EMT plays a critical role in the initial formation and evolution of EMs (12,13). In addition, genic transition changes shown in endometriotic cell phenotypes were involved in the processes of mesenchymal-epithelial metastasis (MET) and EMT, which also strengthened the association between EMs and EMT (14,15).

Besides, emerging evidence has demonstrated that the abnormal immune response in the peritoneal microenvironment emerges as a hinge for endometriotic cells which escape from the immunosurveillance of the peritoneal cavity (16). Endometriotic implant growth was accompanied by the recruitment of various immune cells and severe inflammation caused by increased pro-inflammatory cytokines, growth factors, and angiogenesis. There was solid evidence that the function of almost all types of immune cells was abnormal in women with EMs (17). Amongst all the inflammatory cytokines, interleukin-6 (IL-6), involved in many biological processes including immune processes (T- and B-cell activation and macrophage differentiation), bone metabolism, arthritis, tumors, and reproduction (18), has attracted significant attention in EMs research. Prior studies revealed that endometriotic cells were resistant to IL-6 (19-21), and its secretion was significantly up-regulated (22,23). Additional reports have also described a significant increase in IL-6 levels both in the peritoneal fluid and serum of EMs patients (23,24). Furthermore, a previous study uncovered that the increased expression of IL-6 enhanced the migration and invasion abilities of endometriotic stromal cells (25). Considering that an increased level of IL-6 is associated with tumor development and progression via prompting EMT in a variety of cancers, we propose a hypothesis that abnormal IL-6 stimulates EMs progression by improving the EMT process.

Additionally, a link between abnormal retinol metabolism and EMs has recently been reported. Some researchers pointed out that abnormal retinoic acid (RA) signal transduction pathways had a certain effect on endometriotic stromal cell survival, and there was growing evidence that treatments for the path of retinal metabolism could be therapeutic for EMs (26-28). As a small-molecule derivative of vitamin A, all-trans retinoic acid (ATRA), a well-known anti-leukemic drug, has been clinically used in the treatment of certain tumor types. For the past few years, its importance in EMs studies has amplified (29,30). IL-6 level has been found to be inhibited after RA treatment (31). Consequently, we assume that ATRA inhibits the EMT capability of endometriotic cells. The current study attempts to explore whether ATRA inhibits the EMT of endometriotic stromal stem cells by targeting IL-6.

We present the following article in accordance with the MDAR reporting checklist (available at https://dx.doi.org/10.21037/apm-21-2175).

Methods

Cell isolation and culture

Endometriotic samples were obtained from the ovarian endometrioma of patients who underwent laparoscopic surgery for EMs, while control endometrial samples were obtained from the endometrium of patients who underwent laparoscopic surgery without EMs. Only patients who had not taken exogenous hormones for 3 months before surgery were included in the research. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). Informed written consent was acquired from each patient, and the Second Affiliated Hospital of Wenzhou Medical University provided ethical approval (No. 2014-28).

Hepes-buffered Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (DMEM-F12) (Gibco, USA) containing 5% fetal bovine serum (FBS) (Gibco, USA) and 1% antibiotics (Gibco, USA) (32) was used to collect endometrial and endometriotic samples. After dissociation, 300 μg/mL of collagenase type 3 (Worthington Biochemical Corporation, USA) and 40 μg/mL of deoxyribonuclease type I (Roche Diagnostics, Germany) were added to the dissociated tissue to digest. After digestion and filtration, purified stromal cell suspensions were acquired

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and subsequently cultured in DMEM-F12 medium supplemented with 10% FBS and 1% antibiotics. The culture medium was replaced every 3–4 days.

Low-density cell clonogenicity

Low-density endometrial and endometriotic stromal cells were inoculated onto fibronectin (10 mg/mL)-coated plates (Thermo Fisher Scientific, USA) to test cell clonogenicity. Briefly, cells at the 3rd passage were treated with 0.05% trypsin/0.2% EDTA (Gibco, USA) to obtain single-cell suspensions, adjusted to a clonal density of 500 cells/cm^2, and seeded in triplicate in plates (32). Then, these cultures were incubated for 15 days and the cells were microscopically controlled daily to ensure the single-cell origin of the clusters. The clusters were then rinsed 3 times using phosphate buffered saline (PBS, pH =7.2) and stained with Giemsa solution at the end of the incubation period. Clusters of cells which contained more than 50 cells were considered as clones or colony forming units (CFUs). To determine cloning efficiency (CE), the following formula was applied: cloning efficiency (CE%) = (number of clones/number of cells seeded) × 100.

Self-renewal of stromal cells was assessed by serial cloning of individual large and small clones. Cells from individual clones were reseeded at clonal density to generate secondary clones. Two types of stromal clones were harvested per primary clone. Each clone was sub-cloned into duplicate culture plates until the 5th passage if more than 600 cells could be harvested.

Immunocytochemistry (ICC)

Endometrial and endometriotic clonogenic stromal cells generated from the large stromal CFUs were inoculated at 5,000–10,000 cells/cm^2 onto 25-mm Thermanox coverslips (Nalge Nunc International, USA) in 6-well plates. After culturing for nearly 3 days, these coverslips were rinsed with PBS and then fixed with 10% formalin (Sigma-Aldrich, USA) before being treated with 0.3% hydrogen peroxide (Merck, Germany) to quench the endogenous peroxidase, followed by 10% blocking serum (Sigma-Aldrich, USA). Then, the primary antibodies were diluted with 10% serum and incubated overnight at 4 ℃. The primary antibodies were vimentin (Abcam, USA), CD90 (Abcam, USA), and CD146 (Abcam, USA). Subsequently, the coverslips were rinsed and incubated with the corresponding secondary antibody for 1 hour, followed by incubation with the ABC regent (Vector Laboratories, USA) for 30 min. Then, positive immunoreactivities were visualized after incubation with DAB chromogen (Sigma-Aldrich, USA).

In vitro differentiation

To assess multipotency, the cells of large endometriotic stromal CFUs were induced to undergo osteogenic, chondrogenic, and adipogenic differentiation. When cells reached more than 80% confluence, large endometriotic stromal CFUs were isolated, expanded in culture medium, and seeded at 5,000 cells/cm^2. Subsequently, they were cultured in differentiation induction medium for 4 weeks using standard methods. Meanwhile, undifferentiated control cells were concurrently cultured in low serum medium (DMEM-F12/1%FBS/1% antibiotics). Osteogenic differentiation was detected using Alizarin Red, chondrogenic differentiation was detected using Alcian Blue, and adipogenic differentiation was detected using Oil Red O.

Transwell assay

Upon reaching 80–90% confluence with treatment for 48 hours, the cells of large endometriotic stromal CFUs were digested and assessed for migration and invasion abilities by the transwell assay, which are the main manifestations of EMT as previously described (33). For the migration and invasion assay, cells were seeded into wells averagely and incubated for 24 h. Then, these cells were randomly counted in 5 fields of each chamber. Then the average cell numbers in 3 duplicate wells for each patient sample were calculated statistically.

Quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted from each treatment group of large endometriotic stromal CFUs with TRIzol reagent (Invitrogen, USA). A spectrophotometer (Thermo Fisher Scientific, USA) was used to assess the quality of the RNA. SuperScript First-Strand Synthesis System (Invitrogen, USA) was used to convert RNA into cDNA. RT-qPCR was performed in triplicate using the FastStart Universal SYBR Green Master (Roche, Germany). The gene-specific primers used for the present study were as follows: PCNA: sense 5'-GGCCGAAGATAACGCGGATAC-3', antisense 5'-GGCATATACGTGCAAATTCACCAG-3'; Caspase-3: sense 5'-CGTCGATTTTTGTGAT

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GCTCGTCAG-3', antisense 5'-GAAGCATTTA TCAGGGTTATTGTCTCATG-3'; IL-6: sense 5'-GGACAUGACAACUCAUCUCTT-3', antisense 5'-GAGAUGAGUUGUCAUGUCCTG-3'; Vimentin: sense 5'-CCAGGCAAAGCAGGAGTC-3', antisense 5'-GGGTATCAACCAGAGGGAGT-3'; N-cadherin: sense 5'-ATGTGCCGGATAGCGGGA-3', antisense 5'-TCAGTCATCACCACCACCAT-3'; Snail: Sense 5'-GCCUUCAACUGCAAAUACUTT-3', antisense 5'-AGUAUUUGCAGUUGAAGGCTT-3'; Slug: sense 5'-CAAGGACACATTAGAACTCACAC-3', antisense 5'-CTACACAGCAGCCAGATTC-3'; Twist: sense 5'-TCCTCTATGGGGTCGT-3', antisense 5'-ACCACCGCATCTCTAC-3'.

The transcript level for each specific gene was normalized to the level of the β-actin gene, and the comparative threshold cycle (Ct) method ($2^{-\Delta\Delta Ct}$) was used to calculate.

Western blot

Total protein was extracted from each treatment group of large endometriotic stromal CFUs for the western blot, as previously described (33). The protein samples were loaded on 8–12% sodium dodecyl sulfate polyacrylamide gels equally to carry out electrophoresis. The primary antibodies were anti-IL-6 (Abcam, USA), anti-N-cadherin (Abcam, USA), anti-Vimentin (Abcam, USA), anti-Snail (Abcam, USA), anti-Slug (Abcam, USA), anti-Twist (Abcam, USA), anti-PCNA (Abcam, USA), and anti-Caspase-3 (Abcam, USA). GAPDH was used as the loading control. The results were quantified by densitometry by using ImageJ software (NIH, USA).

ELISA assay

According to the manufacturer's instructions, the Human IL-6 Quantikine ELISA Kit (R&D Systems, USA) was used to measure the IL-6 level in the liquid culture supernatants. For each treatment group, the cell number was basically the same in each well, and culture medium was collected.

Statistical analysis

GraphPad Prism Version 7 was applied for statistical analysis. The data are shown as the mean of mean ± standard error. The differences between groups and among groups were analyzed by student *t*-test and one-way analysis of variance respectively. P value <0.05 was considered significant statistically.

Results

Primary culture of endometrial and endometriotic stromal cells and cell clonogenicity

Both endometrial stromal cells from the endometrium and endometriotic stromal cells from ovarian endometrioma were characteristically flat and spindle shaped. The low-density clonogenicity assay was conducted under microscopy during the whole culture process. The purified individual endometrial and endometriotic stromal cells both attached within 24 hours, and small clusters of 3 to 4 flattened fibroblasts began to form on the 4th day. During the first week of culture, 2 clone types appeared in different sizes. Large stromal clone was defined as comprising more than 4,000 cells with a dense center of tightly-packed cells, while small stromal clone comprised less than 4,000 loosely-packed cells (32). Around day 11 to day 12, small clones ceased proliferation and maintained their sizes, while large clones kept increasing dramatically and contained as many as 15,000 cells which could form CFUs until day 15 (*Figure 1A*). Then, the CE between endometrial and endometriotic stromal clones showed no statistically significant difference regardless of the large or small size (*Figure 1B*).

Next, the serial clonogenicity assay was performed to evaluate the self-renewal capacity of stromal cells. The numbers for serial cloning of large and small clones or CFUs were shown as serial cultivation. Large stromal clones could produce large and small clones until passage 4, while small clones at all times produced no large clones and eventually failed to form clones, with loosely dispersed cells attached on the culture plate (*Figure 1C,1D*). These results suggested that the large stromal clones or CFUs, not the small stromal clones, were endowed with a high capacity for self-renewal.

Expression of stem cell markers of large endometriotic stromal CFUs

Heretofore, no specific marker for endometrial/endometriotic stem/progenitor cells has been identified. In prior studies, cultured endometrial cells, which expressed the typical markers previously used to label bone marrow stem cells (34,35), were considered as endometrial stem cells. In our assay, large endometriotic stromal CFUs were not

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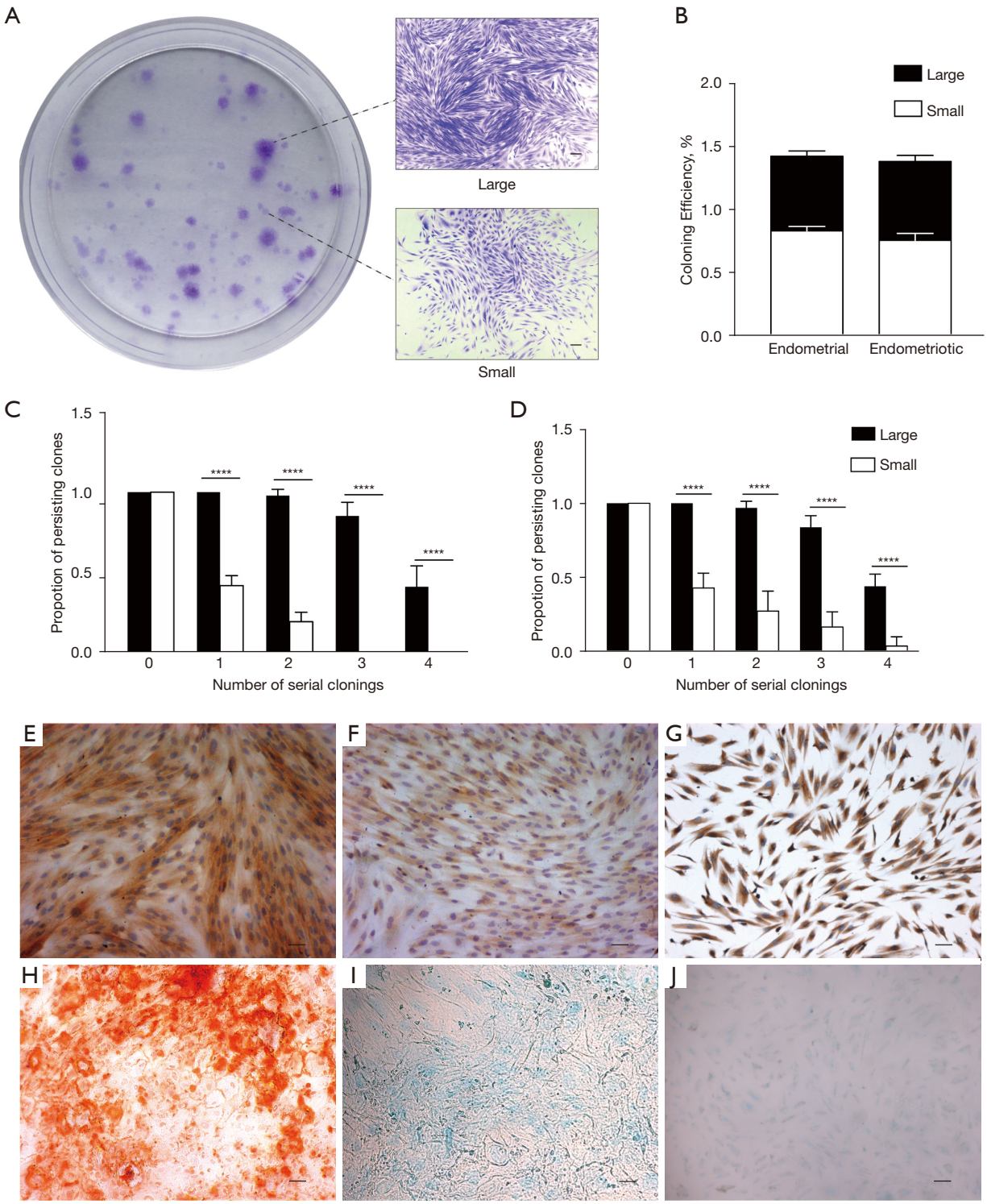

Figure 1 Isolation, identification, and stemness evaluation of primary stromal cells. (A) Clonogenicity of stromal cells stained by Giemsa. (B) Cloning efficiency of endometrial and endometriotic stromal cells. (C,D) Serial clonogenicity of endometrial (C) and endometriotic (D) stromal cells. (E-G) ICC for vimentin (E), CD90 (F), and CD146 (G) in the large endometriotic stromal CFUs. (H-J) Alizarin Red staining for osteogenic differentiation (H), Alcian Blue staining for chondrogenic differentiation (I), Oil Red O staining for adipogenic differentiation (J) in the large endometriotic stromal CFUs. ****P<0.0001. Bar =100 μm. CFUs, colony forming units; ICC, immunocytochemistry.

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only positive for the stromal marker vimentin (*Figure 1E*), but also positive for the stem cell markers CD90 (*Figure 1F*) and CD146 (*Figure 1G*), confirming that large endometriotic stromal CFUs were endometriotic stem cell enrichment groups, which was in accordance with previous experiment (36).

Differentiation of large endometriotic stromal CFUs

The mesenchymal features of endometrial stem/progenitor cells have been clearly demonstrated through the detection of *in-vitro* chondrogenic, osteogenic, and adipogenic differentiation (34,36-38). By cultivating large endometriotic stromal CFUs in osteogenic, chondrogenic, and adipogenic inducing medium for 4 weeks, the potential of large endometriotic stromal CFUs to differentiate toward mesenchymal cell lineages was examined in our study. For osteogenic differentiation, cells cultured in osteogenic inducing medium showed positive staining for Alizarin Red (*Figure 1H*). For chondrogenic differentiation, cells cultured in chondrogenic inducing medium showed positive staining for Alcian Blue (*Figure 1I*). For adipogenic differentiation, cells cultured in adipogenic inducing medium showed positive staining for Oil Red O (*Figure 1J*). Considering these findings, we could reasonably regard the large endometriotic stromal CFUs as a set of endometriotic stem cells.

Expression and effect of IL-6 in endometriotic stem cells

We aimed at the large endometriotic stromal CFUs in the next step of preliminarily discussing the relationship between endometriotic stem cells and EMs pathogenesis. Herein, we attempted to examine the role of IL-6 in the induction of the EMT process in the large endometriotic stromal CFUs. The large endometrial and endometriotic stromal CFUs were selected to examine the IL-6 level and EMT status. Compared with the large endometrial stromal CFUs, the large endometriotic stromal CFUs maintained a higher expression level of IL-6 (*Figure 2A*), along with a greater ability to invade in the transwell assay (*Figure 2B,2C*). Further, the large endometriotic stromal CFUs possessed higher levels of IL-6, PCNA, and the mesenchymal-related markers N-cadherin, snail, slug, and twist, showing a much stronger tendency of EMT and proliferation (*Figure 2D-2F*), indicating a relevant link between the upregulation of IL-6 and the invasive growth of endometriotic stromal stem cells. Considering that IL-6 may act as a functional regulator in EMT-like endometriotic stem cells, we presumed that IL-6 was a potential therapeutic target for EMs as aiming to reverse the EMT state in the large endometriotic stromal CFUs through inhibiting IL-6.

ATRA inhibited the EMT of endometriotic stem cells and IL-6 expression

Given that endometriotic stromal stem cells maintain a stronger ability for EMT, and RA has been reported to inhibit EMT in tumor development (39,40), we chose ATRA and AM580 [a selective retinoic acid receptor alpha (RARα) agonist] to examine the inhibitory effect of ATRA on IL-6 and EMT ability in the large endometriotic stromal CFUs. Our results revealed that ATRA inhibited the IL-6 level of the large endometriotic stromal CFUs in a dose-dependent manner after 48-hour treatment, with significant inhibition at 10^{-6} M (*Figure 3A,3B*). Then, we examined the effect of ATRA on the migration and invasion abilities of the large endometriotic stromal CFUs. As expected, the migration and invasion abilities of the large endometriotic stromal CFUs were both significantly decreased after treatment with ATRA for 48 h (*Figure 3C,3D*).

To explore whether ATRA reduced the migration and invasion abilities by preventing EMT, we measured the expression of EMT markers in the large endometriotic stromal CFUs. The proliferation ability, vimentin, and N-cadherin were decreased significantly, accompanied by decreased IL-6 expression after treatment with ATRA or AM580 for 48 h (*Figure 3E-3G*). Therefore, we confirmed the efficient inhibitory effect of ATRA on the EMT of the large endometriotic stromal CFUs.

ATRA inhibited the EMT of endometriotic stem cells by targeting IL-6

To investigate the role of IL-6 in the EMT status of the large endometriotic stromal CFUs and ATRA inhibition, additional IL-6 was added. After being cultured in IL-6-supplemented ATRA-conditioned medium, IL-6 expression of the large endometriotic stromal CFUs increased, and the migration and invasion abilities were also increased reversely (*Figure 4A-4C*). In addition, compared with ATRA treatment, the levels of vimentin and N-cadherin were increased after exposure to exogenous IL-6 (*Figure 4D-4F*). These data confirmed that ATRA inhibited EMT-related processes by effectively decreasing IL-6.

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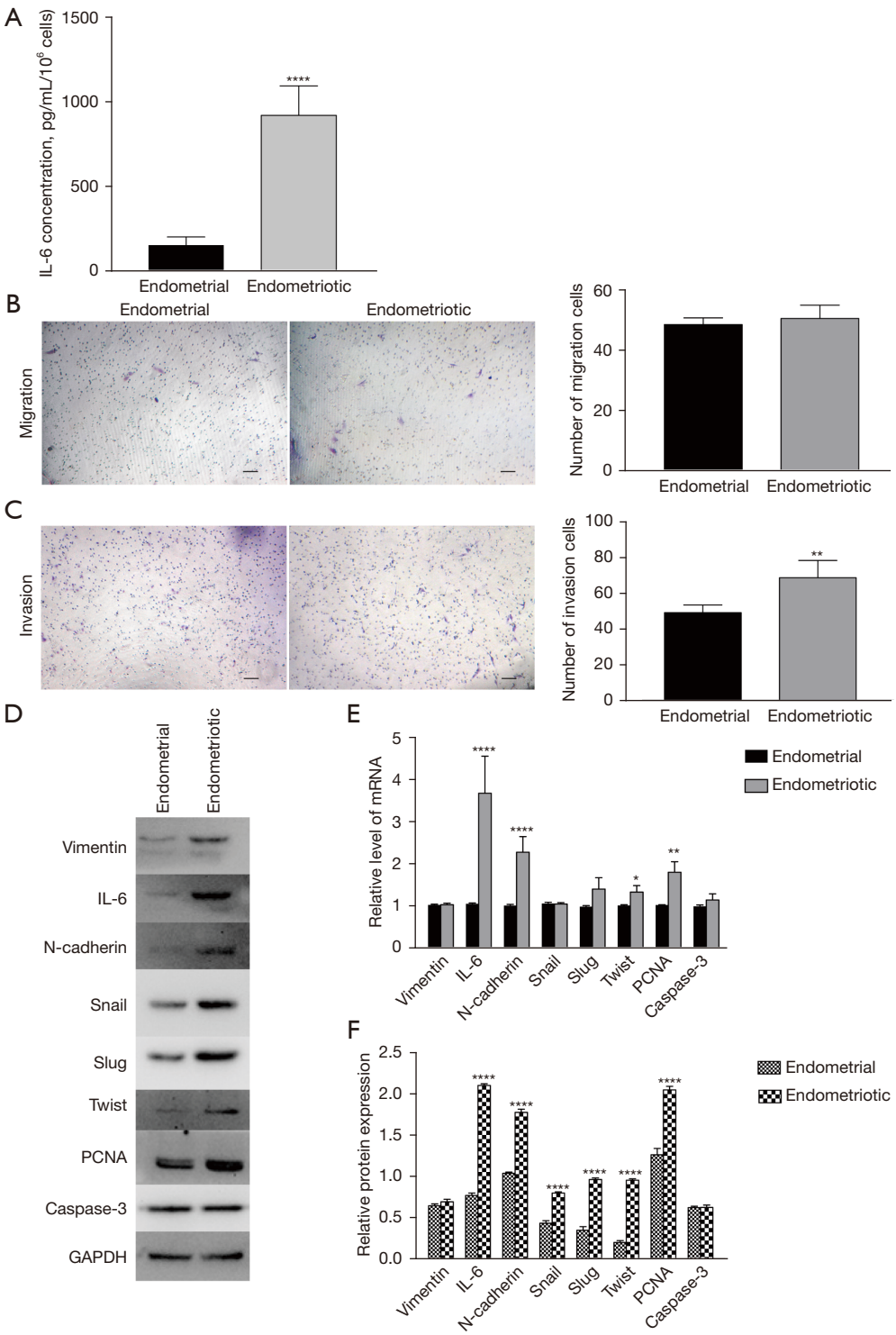

Figure 2 Expression of IL-6 and EMT status in the large endometrial and endometriotic stromal CFUs. (A) The expression of IL-6 was measured by ELISA. (B,C) The cell migration (B) and invasion (C) abilities were measured by the transwell assay and the cells were stained by crystal violet. (D-F) The expression levels of IL-6, vimentin, N-cadherin, snail, slug, twist, PCNA, and caspase-3 were measured by western blot (D,F) and RT-qPCR (E). *P<0.05, **P<0.01, ****P<0.0001. CFUs, colony forming units; EMT, epithelial-to-mesenchymal transition. Bar =100 μm.

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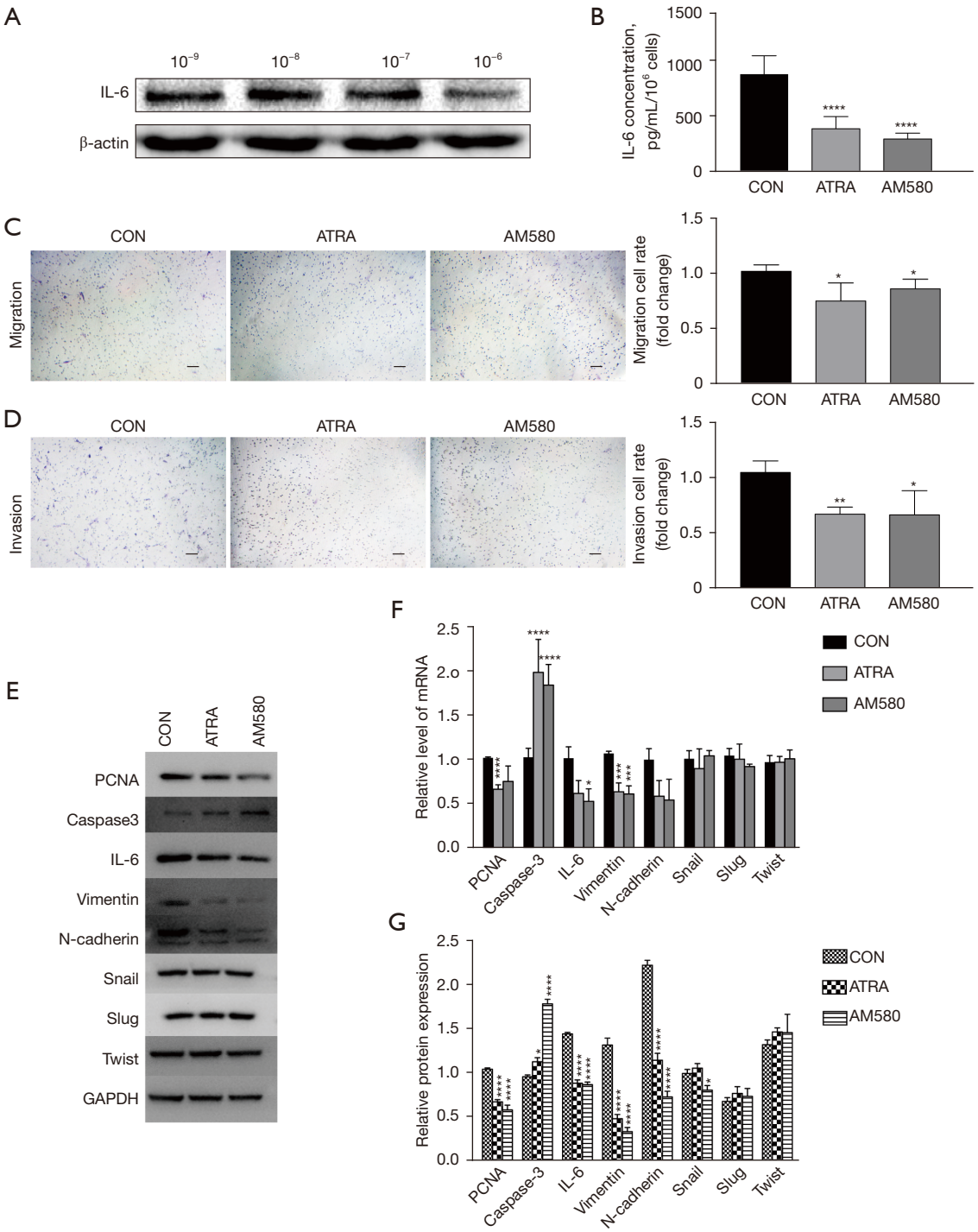

Figure 3 ATRA inhibits IL-6 and EMT in the large endometriotic stromal CFUs. (A) The large endometriotic stromal CFUs were treated with 10^{-6}, 10^{-7}, 10^{-8}, or 10^{-9} M ATRA for 48 h, and the expression of IL-6 was measured by western blot. Then, the large endometriotic stromal CFUs were treated with ATRA and AM580 for 48 h, and the level of IL-6 was measured by ELISA (B). The cell migration (C) and invasion (D) abilities were measured by the transwell assay and the cells were stained by crystal violet. The expression levels of PCNA, caspase-3, IL-6, vimentin, N-cadherin, snail, slug, and twist were measured by western blot (E,G) and RT-qPCR (F). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Bar =100 μm. CFUs, colony forming units; EMT, epithelial-to-mesenchymal transition; ATRA, all-trans retinoic acid; AM580, retinoic acid agonist.

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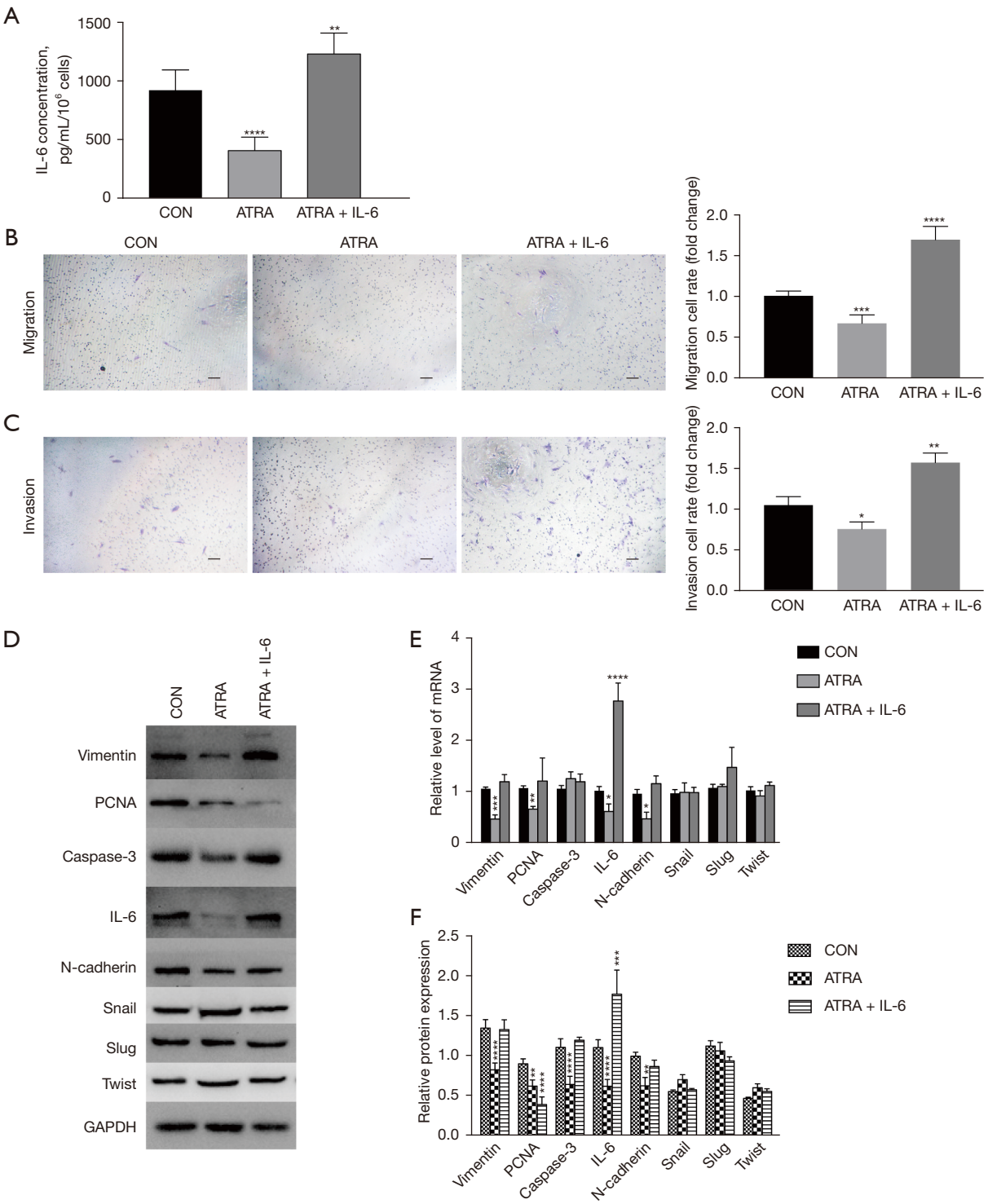

Figure 4 Exogenous IL-6 reverses the inhibitory effect of ATRA in the large endometriotic stromal CFUs. The large endometriotic stromal CFUs were treated with ATRA, with or without exogenous IL-6 for 48 h, and the level of IL-6 was measured by ELISA (A). The cell migration (B) and invasion (C) abilities were measured by the transwell assay and the cells were stained by crystal violet. The expression levels of vimentin, PCNA, caspase-3, IL-6, N-cadherin, snail, slug, and twist were measured by western blot (D,F) and RT-qPCR (E). *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001. Bar =100 μm. ATRA, all-trans retinoic acid; CFUs, colony forming units.

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Overall, our findings suggested that IL-6 mediated the EMT of large endometriotic stromal CFUs to promote EMs progression, while ATRA could inhibit this process specifically.

Discussion

This study revealed a correlation between IL-6 and EMT in EMs, which demonstrated that elevated IL-6 stimulated the EMT process in endometriotic stem cell enrichment sets, whereas ATRA specifically inhibited this process.

In 2004, Chan *et al.* demonstrated that human endometrial epithelial and stromal cell suspensions could form CFUs (32). Nearly 15 years later, several different types of stem/progenitor cells have been gradually detected in the human endometrium. Stem/progenitor cells have also been identified in the ectopic endometria and have manifested a similar ability to form colonies (41). Incumbent data linked the observation of endometriotic stem cells to Sampson'MAOs theory (42). Once these cells from the menstrual debris reached the pelvic cavity or other ectopic sites, their stem-cell characteristics, which included high self-renewal ability, resistance to hormonal therapy, and regeneration, promoted the formation of endometriotic lesions. This is able to account for the disparity between the EMs morbidity and retrograde menstruation rate. Furthermore, researchers proposed that the severity of EMs depended on the "stemness" degree of the endometriotic cells. In addition, clonogenic epithelial cells and stromal cells from ectopic endometria in EMs patients had higher proliferative and invasive potential (8,43). Consequently, evidence supporting the endometrial stem cell theory is growing (44,45). In our study, the large stromal CFUs from the endometrium and ovarian endometrioma both possessed higher renewal and clonogenic capacity. Then, the large endometriotic stromal CFUs were confirmed to be representative of endometriotic stromal stem cells, which could differentiate into osteogenesis, chondrogenesis, and adipogenesis, and expressed the stem cell markers CD90 and CD146.

A prior study demonstrated an increased level of IL-6 in endometriotic cells, revealing that endometriotic cells with immune dysfunction played a part in the pathogenesis of EMs (46). The promotion of mammosphere formation, stem cell self-renewal, cancer cell stemness, and stem cell recruitment (47,48) by IL-6 has also been shown, supporting the view that IL-6 could directly act on stem cell function. IL-6 has been proven to be associated with tumor angiogenesis, proliferation, migration, metastasis, and chemotherapy resistance in many types of cancers (49-52). Subsequent publications suggested that IL-6 could promote the EMT process (48,53,54) by inhibiting the expression of E-cadherin and inducing vimentin, N-cadherin, snail, and twist expression in breast adenocarcinoma cells (53), in conjunction with epidermal growth factor (EGF) through activating JNK2/STAT3 in ovarian cancer (55), and inducing hypoxia associated with the EMT of small cell lung cancer (56), thereby improving the migration and invasion abilities of cells. Additionally, IL-6 has been shown to promote the migration and invasion capabilities of endometriotic cells (57,58). Notably, our data further demonstrated that an enrichment set of endometriotic stem cells manifested a stronger EMT potency, accompanied by higher IL-6 expression, providing powerful evidence that EMs originated from endometriotic stem cells which were stimulated by abnormal IL-6.

Consistently, it has been proven that targeting IL-6 could decrease the invasion and metastasis abilities of pancreatic cancer-associated fibroblast cells and lung cancer cells via repressing the EMT process (31,59). Next, our study elucidated the therapeutic effect of ATRA on EMs by revealing that ATRA inhibited EMT through decreasing IL-6 in endometriotic stem cells. In the EMs research field, many studies have found that retinol metabolism related to abnormal immunological changes in inflammatory processes could contribute to EMs development and/or progression (60,61). RA has been found to have potential in suppressing EMs via diverse pathways, such up-regulating gap junctional intercellular communication and connexin-43 (62) and suppressing IL-6 (63) in human endometrial stromal cells. Besides, a transcriptome analysis performed in ATRA-treated endometriotic stromal cells showed many influenced genes associated with the negative regulation in cellular proliferation were up-regulated, and the 17-beta-dehydrogenase 2 gene, which converts estradiol into estrone, was decreased (64). In an experimental mouse model, ATRA treatment reduced the endometriotic implant growth caused by decreased IL-6 and MCP-1 production, and enhanced peritoneal macrophage differentiation, showing an inhibitory effect on EMs development (65). In addition, fenretinide (a synthetic retinoid analogue) treatment induced endometriotic stromal cell apoptosis and increased STRA6 expression, thereby potentially reversing the retinoid metabolism dysfunction in EMs (66). Lu *et al.* further demonstrated that RA could up-regulate beclin1, a fundamental protein involved in autophagy, to

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suppress endometriotic stromal cell growth (67). As well, RA displayed reproductive protective capacity in a rat EMs model (68). In our *in-vitro* study, down-regulation of IL-6 by ATRA resulted in decreased endometriotic stem cell proliferation and EMT tendency. Nevertheless, the role of IL-6 in the microenvironment of EMs needs further in-depth study to fully evaluate the therapeutic potential.

Our present study provides a new strategy for the use of ATRA in the non-hormonal treatment of EMs, especially aimed at endometriotic stem cells. New combinatorial therapeutic approaches may explore the possibility of harnessing the anti-invasive properties of ATRA while blocking stemness-associated characteristics using a second drug. Nevertheless, due to the small clinical sample size, this study is limited. Studies with a larger sample size and *in vivo* studies are required to validate our results, and more investigations are also needed to probe into the exact molecular mechanisms involved.

Acknowledgments

Funding: The research was supported by grants from the Natural Science Foundation of China (No. 81401184), the scientific research of Wenzhou (No. 2018Y0759), the Natural Science Foundation of Zhejiang Province (No. LY20H040005), the Medical and Health Research Project of Zhejiang Province of China (No. 2017KY479).

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://dx.doi.org/10.21037/apm-21-2175

Data Sharing Statement: Available at https://dx.doi.org/10.21037/apm-21-2175

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/apm-21-2175). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). Written informed consent was obtained from each patient and the Second Affiliated Hospital of Wenzhou Medical University provided ethical approval (No. 2014-28).

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Cite this article as: Li L, Gao H, Pan L, Zhao Y, Liang Z, Zhang Q, Wang D. All-trans retinoic acid inhibits epithelial-to-mesenchymal transition (EMT) through the down-regulation of IL-6 in endometriosis. Ann Palliat Med 2021;10(11):11348-11361. doi: 10.21037/apm-21-2175

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