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Original Article

Modulation of tumor stem cell characteristics by 17 β -estradiol in human mesenchymal stem cells derived from ovarian endometriomaTa-Chin Lin ^{a, b}, Kai-Hung Wang ^{a, b, c, *}, Kuo-Hsiang Chuang ^d, An-Pei Kao ^e,
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ABSTRACT

Objective: Ovarian endometrioma is a cyst composed of endometrial tissue and is present in 20%–40% of patients with endometriosis. Endometriosis is an estrogen-dependent benign and chronic gynecological disease that affects women of reproductive age. Studies have reported that tumor stem cells can be isolated from numerous tumor types. Emerging evidence has indicated that tumor stem cells may be responsible for the development of endometriosis and endometrial tumors. The present study investigated the effects of 17 β -estradiol on levels of expression of stem cell markers and cell growth of human mesenchymal stem cells derived from ovarian endometrioma (hOVEN-MSCs).

Materials and methods: hOVEN-MSCs were isolated from human ovarian endometrioma. The proliferation potential of hOVEN-MSCs was measured by the cumulative population doubling and colony-formation efficiency. The gene expression of the hOVEN-MSCs was examined by the reverse transcription-polymerase chain reaction analysis. Protein expression assays were performed using flow cytometry and western blot analysis.

Results: This study demonstrated that hOVEN-MSCs can be isolated from ovarian endometrioma and that 17 β -estradiol was capable of increasing colony-forming efficiency and cell proliferation of these cells. In addition, we found that 17 β -estradiol not only increased the expression of the stem cell marker OCT-4, but also increased the expression of endometrial tumor stem cell markers CD133 and ALDH1 in hOVEN-MSCs.

Conclusion: The above results indicate an important role of 17 β -estradiol in cell growth of hOVEN-MSCs concomitant with enhanced expression of stem cell markers. This effect of 17 β -estradiol related to stem cell marker expression, if confirmed by further *in vitro*, *in vivo* studies, may be useful for developing new strategies for prevention and treatment of endometriosis and endometrioma.

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Introduction

Endometriosis is an estrogen-dependent benign and chronic gynecological disorder that affects women of reproductive age. The pathogenesis of endometriosis is poorly understood. Implantation theory suggests that viable endometrial cells are shed from the endometrium into the pelvic peritoneum or ovaries, possibly

through retrograde menstruation, and subsequently attach to, invade, and injure other tissues [1]. Evidence supporting the prevailing theory of retrograde menstruation suggests that endometrial mesenchymal stem cells (MSCs) may play a critical role in the pathogenesis of endometriosis [2–4].

According to tumor stem cell theory, tumors are clonal origin and consist of heterogeneous cell populations including tumor stem cells. Tumor stem cells that initiate and sustain tumor development have been reported for many tumor types [5–7]. Many tumor stem cell markers have also been identified as markers of normal stem cells in different tissues [8], indicating that normal

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stem cells can be tumor-initiating target cells [9]. Emerging evidence has indicated that tumor stem cell populations may be involved in the development of endometriosis or endometrial tumors [10]. Endometrial tumor stem cell markers have been identified, including CD133 [11,12] and ALDH1 [13,14].

Ovarian endometrioma is a cyst composed of endometrial tissue and is present in 20%–40% of patients with endometriosis [1]. The pathogenesis of endometriosis is multifactorial, and estrogens may play a key role in its development and progression [15,16]. Previously, we reported that 17 β -estradiol (an endogenous estrogen) induced the emergence of a subpopulation of cells expressing CD44^{+/high}/CD24^{-/low} breast tumor stem cell markers in a stem cell-derived breast epithelial cell line [17] and promoted epithelial-mesenchymal transition and tumor growth in estrogen receptor-positive human breast epithelial stem cells with HER2 overexpression [18]. Additionally, bisphenol A (an exogenous estrogen known to be an endocrine disruptor) could enhance cell proliferation and colony-forming efficiency, induce COX-2 gene expression, promote the migration and invasion of human uterine myoma MSCs [19] and induced epithelial-mesenchymal transition mediated by COX-2 up-regulation in human endometrial carcinoma cells [20]. In this study, we examined the effect of 17 β -estradiol on the expression of tumor stem cell markers, colony-forming efficiency, and growth rate of human ovarian endometrioma-derived MSCs. The results might shed light on the origin and mechanism of growth of endometrioma.

Materials and Methods

Isolation and culture of human stromal cells and MSCs derived from ovarian endometrioma

The human stromal cells and mesenchymal stem cells (hOVEN-MSCs) were developed and isolated from ovarian endometrioma. These tissues were derived from patients undergoing surgery for the treatment of endometriosis in the Department of Obstetrics and Gynecology of Kuo General Hospital, Tainan, Taiwan. The use of these tissues was approved by the hospital's Institutional Review Board (IRB No: A-13-K05 and B-18-K003). These tissues were minced by sterile scissors and then digested with Type II collagenase for 60–90 min in a 37 °C incubator. After digestion, the cells and cell aggregates were filtered serially through wire sieves with different pore sizes (100 μ m, 70 μ m and 40 μ m) to remove cell aggregates and epithelial cells. These stromal cells were collected and frozen for the following experiments. For isolation of hOVEN-MSCs, stromal cells were seeded in triplicate plates (10-cm-diameter) at cell density of 200 cells per dish. After incubation for 21 days, large colonies were isolated and trypsinized into single cells. These cells were diluted and seeded in 96-well plates at the density of about one cell per well. After culture for 14 days, proliferating colonies derived from single cells were trypsinized and cultured in a 10 cm diameter dish. The cells were allowed to grow in a modified DMEM/F-12/MCDB 153 (2/1, v/v) medium with 10% fetal bovine serum (FBS) until near confluence [2,21,22].

In vitro differentiation of hOVEN-MSCs

These hOVEN-MSCs were tested for differentiation potential, i.e. adipogenesis and osteogenesis. The cells were first subcultured and incubated in a DMEM medium with 10% FBS. The next day, differentiation induction was initiated by changing the medium with different supplementations. For adipogenesis, these hOVEN-MSCs were plated at seeding density of 5×10^4 cells/cm² in 35-mm-diameter dishes. The next day, cells were incubated in IDII medium for 2 days, then in I medium for 1 day. The cycle was repeated 4

times. IDII medium contains 3-isobutyl-1-methylxanthine (500 μ M), dexamethasone (1 μ M), indomethacin (1 μ M), and insulin (10 μ g/mL) in DMEM medium with 10% FBS. I medium is DMEM medium supplemented with 10% FBS and insulin (10 μ g/mL). Lipid vacuoles developed within adipocytes after induction were verified by Oil Red O staining (in red color). For osteogenesis, these hOVEN-MSCs were plated at the seeding density of 5×10^4 cells/cm² in 35-mm-diameter dish. The next day, cells were induced to differentiate in DAG medium for 2 weeks, with medium change once every 3 days. The DAG medium contains dexamethasone (0.1 mM), L-ascorbic acid-2-phosphate (50 μ M), and β -glycerophosphate disodium (10 mM) in DMEM medium with 10% FBS. The formation of calcified extracellular matrix by osteoblasts was visible and can be confirmed by von Kossa staining (in black color) [2,22].

Flow cytometry analysis of protein expression

The cells on plates were washed twice by ice-cold phosphate buffered saline (PBS) and removed by trypsinization. Single cells were obtained by filtration through a 40- μ m nylon mesh. Antibodies CD34, CD44, CD45, CD90, CD105, CD133, Notch1, Musashi-1, ALDH1, HLA-DR, and OCT-4 were used for surface and intracellular staining according to the manufacturer's recommendations. The labeled cells were then washed twice, resuspended in PBS, and analyzed using flow cytometry. Results are presented as mean \pm SD of the triplicate (N = 3).

Immunofluorescence staining

For immunofluorescence staining, the cells grown in 35-mm-diameter dish were washed with PBS and fixed by 4% paraformaldehyde in PBS for 20 min. After rinsing with PBS, the cells were permeabilized (0.5% triton x-100, 2% BSA and 0.05% NaN₃ in PBS) for 10 min. The cells were then incubated with the primary antibody (anti-Notch1, anti-Musashi-1, anti-OCT-4, anti-CD133, and anti-ALDH1) in PBS/triton/BSA at 25 °C overnight. The following day, the cells were incubated with a secondary antibody conjugated with PE in PBS/triton/BSA buffer for 1 h at 25 °C. 4,6-diamino-2-phenylindole (DAPI) was utilized to stain the nuclei under the condition of protection from light. Images were obtained on a fluorescence microscope after washing three times with PBS.

Colony-formation assay

To determine the effect of 17 β -estradiol on clonogenic ability, hOVEN-MSCs were plated at a density of 500 viable cells per 10-cm-diameter dish in medium with or without 1 μ M of 17 β -estradiol (N = 6). After incubation for 10 days, colonies were labeled when a clone had more than 40–60 cells. The labeled colonies were further cultured for 21 days until the cell colonies grew large enough to be visualized. These colonies on the culture dishes were stained with 0.5% crystal violet and counted for comparison of colony-forming efficiency.

Cell proliferation assay

The growth rate of the cell culture was calculated by dividing the cumulative population doubling level (CPDL) by days of culture (N = 6). The CPDL in a continual subculture and growth from a known number of cells (1×10^5 cells) were calculated using the equation $\ln(N_f/N_i)/\ln 2$, where N_i and N_f are initial and final cell numbers, respectively, and \ln is the natural logarithm.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells (5×10^5 cells) using 1 mL TRIzol reagent. RNA (1 μ g) was transcribed into cDNA using a reverse transcription system kit and cDNA was amplified with gene-specific primers in a polymerase chain reaction (PCR) machine. The primers were β -ACTIN (forward, 5'-ATGATATCGCCGCGCTCGTCGTC; reverse, 5'-CGCTCGGCCGTGGTGGTGAA), CD133 (forward, 5'-TCTTGACCGACTGAGACCAAC; reverse, 5'-ACTTGATGGATGCACCAAGCAC), ALDH1 (forward, 5'-TTGGAATTTCCCGTTGGTTA; reverse, 5'-CTGTAGGCCCA-TAACCAGGA), and OCT-4 (forward, 5'-GAGCAAAACCCGGAGGAGT; reverse, 5'-TTCTCTTCGGGCTGCAC). The PCR was performed in a PCR machine. The protocol was as follows, sequentially: 1 min denaturation at 95 °C; 30 reaction cycles each with 30 s of denaturation at 95 °C; 30 s of annealing at 52 °C; and extension at 72 °C for 1 min. The last polymerization step was performed at 72 °C for 10 min. The amplified products were separated on 2% agarose gel and stained with ethidium bromide. For the comparison of cell lines, β -ACTIN was used as a reference gene to normalize mRNA levels, and the same protocol and conditions were used. Results are presented as mean \pm SD of the triplicate ($N = 3$) and were statistically tested.

Western blotting

The proteins were extracted with 20% SDS lysis solution containing several protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 mM antipain, 0.1 mM aprotinin, 0.1 mM sodium orthovanadate, 5 mM sodium fluoride). Protein concentrations were measured using Biorad Protein Quantification kit. Same amounts of protein (15 μ g/lane) were separated by 12% SDS-PAGE and transferred from the gel to PVDF membranes. Immunoblotting was carried out using monoclonal antibody (anti-OCT-4, anti-CD133, anti-ALDH1, and anti- β -ACTIN). This was then followed by incubation with horseradish peroxidase-conjugated secondary antibody and detected with the ECL chemiluminescent detection reagent. The membranes were exposed to X-ray film for 15 s to 1 min. For the comparison of cell lines, β -ACTIN expression was used as a reference to standardize protein amount, and the same protocol and conditions were used. Results are presented as mean \pm SD of the triplicate ($N = 3$) and were statistically tested.

Statistical analysis

Results shown were obtained from at least three separate experiments. Statistical analyses were performed using Student's *t*-test for comparison of two different treatments. Data represented the mean \pm standard deviation. *P* values < 0.05 were considered statistically significant.

Results

Isolation and characterization of human mesenchymal stem cells derived from ovarian endometrioma (hOVEN-MSCs)

Human stromal cells and hOVEN-MSCs were developed and isolated from human ovarian endometrioma as described in the Materials and Methods section and depicted in Fig. 1A. The stromal cell morphology was large and flat, whereas the hOVEN-MSCs morphologically resembled fibroblasts (Fig. 1A). The ability to differentiate into multiple mesenchymal lineages is a qualifying criterion for cells to be considered as mesenchymal stem cells (MSCs). After induction with specific medium supplementations, as

described in the Materials and Methods section, the hOVEN-MSCs were found to differentiate into adipocytes and osteoblasts as revealed by positive staining with Oil Red O (for lipid droplets) and von Kossa (for calcified extracellular matrix) staining, respectively (Fig. 1B). Flow cytometry analysis revealed the expression of MSC markers CD44, CD90 and CD105, as well as the regulation of cell differentiation and self-renewal from stem cell marker Notch1 and endometriosis and endometrial carcinoma tissue marker Musashi-1 [23,24] in stromal cells and hOVEN-MSCs. The expression of stem cell markers and carcinoma tissue marker was higher in hOVEN-MSCs than in stromal cells. These hOVEN-MSCs did not show expression of cell surface markers, including CD34, CD45, and HLA-DR, which are specific markers for hematopoietic stem cells, and leukocytes (Fig. 2A). These hOVEN-MSCs clearly show characteristics of MSCs as defined by the International Society for Cellular Therapy [25], namely, 1) plastic-adherent; 2) expression of CD44, CD90 and CD105 and non-expression of CD34, CD45, and HLA-DR; and 3) the ability to differentiate into adipocytes and osteoblasts *in vitro* (Fig. 1 and 2A). By immunofluorescence staining, in addition to the expression of Notch1, Musashi-1 and OCT-4, hOVEN-MSCs were also found to express two major endometrial tumor stem cell markers CD133 and ALDH1 (Fig. 2B).

17 β -estradiol enhanced growth rate and clonogenic ability of hOVEN-MSCs

The 17 β -estradiol-treated hOVEN-MSCs demonstrated a faster growth rate than untreated hOVEN-MSCs (0.83 ± 0.06 and 0.66 ± 0.04 population doubling per day, respectively) (Fig. 3A). In addition to enhancing cell growth, 17 β -estradiol enhanced the clonogenic properties of the hOVEN-MSCs. After 21 days of single-cell plating, the 17 β -estradiol-treated hOVEN-MSCs exhibited 2.4-fold higher colony-forming efficiency compared to untreated hOVEN-MSCs ($32.7\% \pm 5\%$ and $13.5\% \pm 4\%$, respectively) (Fig. 3B). The effect of 17 β -estradiol on cell proliferation and colony-forming efficiencies were reversed by the estrogen antagonist ICI 182,780 (Fig. 3). These results clearly indicate that 17 β -estradiol (1 μ M) enhanced the colony-forming ability and growth rate of hOVEN-MSCs.

17 β -estradiol increased expression tumor stem cell markers in hOVEN-MSCs

We examined the expression of OCT-4 and two major endometrial tumor stem cell markers, CD133 and ALDH1, in hOVEN-MSCs with or without 17 β -estradiol-treatment using RT-PCR analysis. The results revealed that the expressions of OCT-4, CD133, and ALDH1 were significantly higher in 17 β -estradiol-treated hOVEN-MSCs than the control without 17 β -estradiol treatment (2.25-, 2.38- and 1.55-fold increases, respectively, for OCT-4, CD133, and ALDH1; Fig. 4A). We then conducted experiments using flow cytometry analysis to determine if 17 β -estradiol increased the expression of tumor stem cell markers in hOVEN-MSCs. The analysis indicated that expressions of OCT-4, CD133, and ALDH1 in 17 β -estradiol-treated hOVEN-MSCs were 1.17-, 1.25-, and 1.48-fold higher than in untreated hOVEN-MSCs, respectively (Fig. 4B). To confirm these results from RT-PCR and flow cytometry analysis, hOVEN-MSCs and 17 β -estradiol-treated hOVEN-MSCs were tested for the protein expression of OCT-4, CD133, and ALDH1 by western blot analysis. The results, indeed, showed that the protein expression of OCT-4, CD133, and ALDH1 were statistically significantly higher in 17 β -estradiol-treated hOVEN-MSCs than in hOVEN-MSCs without 17 β -estradiol treatment (1.83-, 2.93- and 1.32-fold increases, respectively, for OCT-4, CD133, and ALDH1; Fig. 4C). The above results consistently demonstrate that 17 β -estradiol

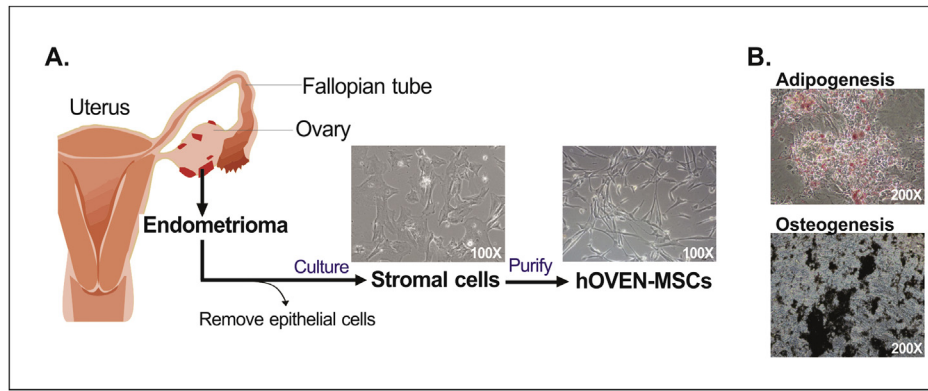


Fig. 1. Isolation and identification of human MSCs derived from ovarian endometrioma (hOVEN-MSCs). (A) Illustration of the stepwise isolation procedure of stromal cells and MSCs from ovarian endometrioma. Stromal cell morphology was large and flat, whereas the morphology of hOVEN-MSCs resembled fibroblasts. (B) hOVEN-MSCs were induced to differentiate into adipocytes (stained red by using Oil Red O staining of lipid droplets) and osteoblasts (stained black by using von Kossa staining of calcified extracellular matrix).

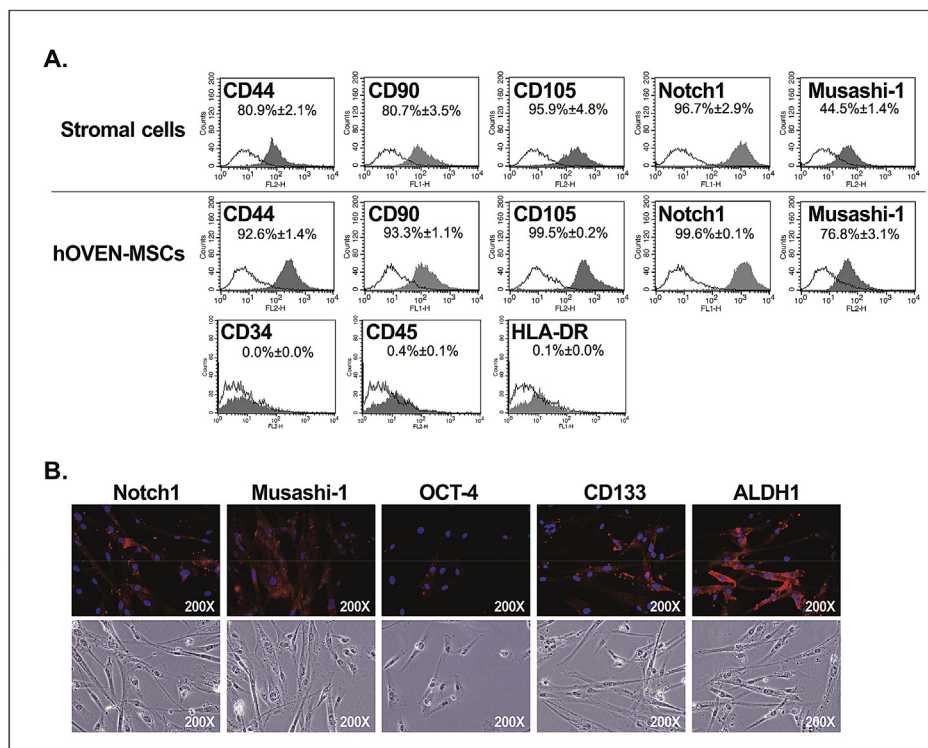


Fig. 2. Expression of MSC and tumor stem cell markers in hOVEN-MSCs. (A) Flow cytometry analyses revealed that the expressions of CD44, CD90, CD105, Notch1, and Musashi-1 were higher in hOVEN-MSCs than that in stromal cells. hOVEN-MSCs did not show the expression of CD34, CD45, and HLA-DR. The values are presented as mean \pm SD of the triplicate (N = 3) (B) hOVEN-MSCs expressed Notch1, Musashi-1, OCT-4, CD133 and ALDH1 (red) as detected by immunofluorescence staining. DAPI staining (blue) shows the locations of the nuclei. Upper panels, observed under a fluorescence microscope; lower panels, the corresponding phase images.

upregulated OCT-4, CD133, and ALDH1 expression in hOVEN-MSCs, the increases were especially significant for the former 2 genes (more than 2-fold increase by RT-PCR and western blot analysis).

Discussion

Endometriosis is a common chronic gynecological disorder defined as the presence of ectopic endometrial tissues, mainly in the pelvic peritoneum and ovary [1]. Endometriosis is a stem cell disease, and several theories have been proposed regarding its pathogenesis [2–4]. MSCs have been derived from various tissues and may be used for various clinical applications because of their high potential for differentiation into various cell types and ready

expansion of cell populations *in vitro*. In this study, we characterized hOVEN-MSCs with regard to cell proliferation and expression of tumor stem cell markers in response to 17 β -estradiol. The results indicate that 17 β -estradiol increased cells growth rate, colony-forming efficiency, and OCT-4, CD133, and ALDH1 expression in hOVEN-MSCs. Since these cells with stem cell characteristics were derived from endometrioma, they are qualified as tumor stem cells. Future *in vivo* tumorigenicity study of these cells should provide convincing supporting evidence.

Human endometrium is a dynamic tissue that regenerates, differentiates, and shed in more than 400 cycles during the reproductive phase of a woman's life. Therefore, endometrial stem cells exhibit regenerative capacity. The continual proliferative and

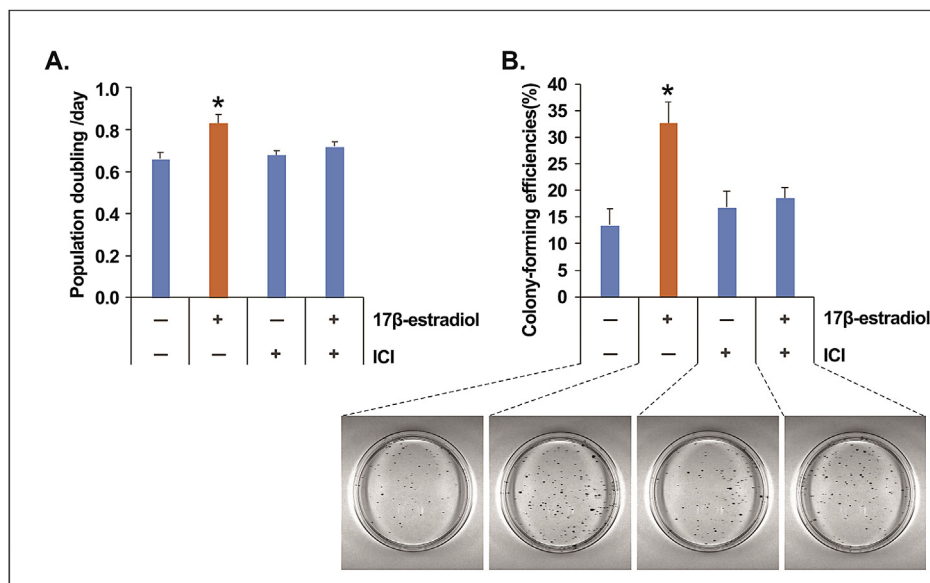


Fig. 3. 17 β -estradiol enhanced the growth rate and clonogenic ability of hOVEN-MSCs. (A) Proliferation of 17 β -estradiol-treated hOVEN-MSCs was significantly faster than the hOVEN-MSCs control without 17 β -estradiol (N = 6). (B) Colony-forming efficiency of 17 β -estradiol-treated hOVEN-MSCs was significantly higher than that of hOVEN-MSCs control without 17 β -estradiol (N = 6). The increases induced by 17 β -estradiol treatment may be reversed by treatment with estrogen antagonist ICI 182,780 (ICI). *P < 0.05.

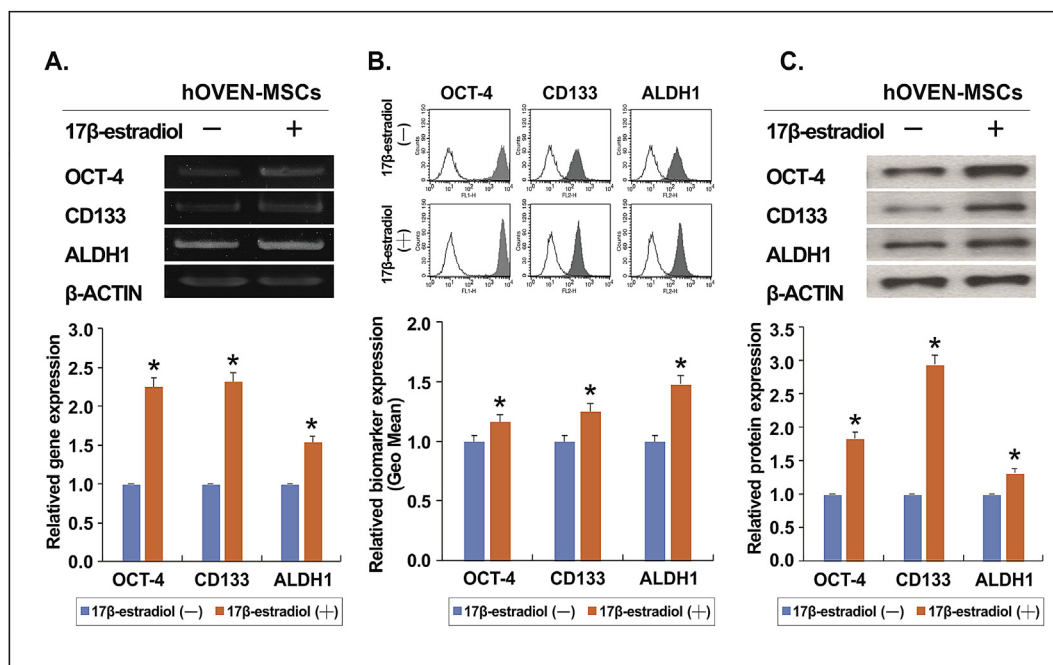


Fig. 4. 17 β -estradiol increased the expression of tumor stem cells markers in hOVEN-MSCs. Treatment of hOVEN-MSCs with 17 β -estradiol (1 μ M) increased the expression of OCT-4, CD133, and ALDH1 measured by (A) mRNA expression using RT-PCR (N = 3) and protein expression measured using (B) flow cytometry analysis (N = 3) and (C) western blot analysis (N = 3). (A) mRNA and (C) protein expression were determined by densitometry. β -ACTIN served as an equal loading control. The results are presented as the ratios of the values for 17 β -estradiol-treated (+) hOVEN-MSCs to those of untreated (–) hOVEN-MSCs. *P < 0.05.

regenerative traits of endometrial stem cells may contribute to endometriosis formation. The hOVEN-MSCs we isolated indeed possess these characteristics of MSCs as defined by the International Society for Cellular Therapy [25] and shown by our results, namely, 1) capacity to adhere to plastic, 2) expression of MSC markers (i.e., CD44, CD90 and CD105) (Fig. 2A) and stem cell markers (i.e., Notch1 and OCT-4) (Fig. 2B), and 3) the ability to differentiate into adipocytes and osteoblasts *in vitro* (Fig. 1). In

addition, these cells expressed endometriosis and endometrial carcinoma tissue marker, Musashi-1 (23, 24) (Fig. 2B).

Several *in vitro* studies have reported that estrogen act as proliferation and differentiation agents in endometrial cells [26] and accelerate the progression of endometriosis by upregulating β -catenin expression [16]. Tumor stem cells have been isolated from various human tumor tissues [6,7]. Endometrial tumor stem cell markers, including CD133 and ALDH1, have been identified in

previous research [11–14]. In addition to identification through specific markers, tumor stem cells may be identified by their capacities to self-renew, generate new tumors, and increase tumor migration and invasion [27]. The results of this study indicate that 17 β -estradiol enhanced the expression of endometrial tumor stem cell markers (Fig. 4), cell proliferation and colony-forming ability (Fig. 3) in hOVEN-MSCs.

OCT-4, a member of the POU domain transcription factor family, plays a key role in the regulation of self-renewal and pluripotency in embryonic stem cells, germ cells, and adult stem cells [28,29], whereas Nanog is a homeobox gene and plays a critical role in maintaining self-renewal and the undifferentiated state of pluripotent stem cells [30]. *In vitro* studies have revealed that the elevated expression of OCT-4 and Sox-2 in human endometrial cells contributed to reprogramming of these cells into induced pluripotent stem cells, indicating that OCT-4 and Sox-2 are stemness-related factors in the human endometrium [31]. In our previous study, the ectopic expression of OCT-4 in amniotic fluid MSCs simultaneously increased the expression of the three most crucial pluripotent genes: OCT-4, Nanog, and Sox-2 [32]. Several other studies have demonstrated that OCT-4 is expressed in normal human endometrium, ectopic endometrium, and endometrial cancer [33,34]. Our previous study also revealed OCT-4 expression in human ectopic endometrial MSCs [2]. In addition, aberrant OCT-4 expression promotes cell migration in endometriosis [34] and regulates epithelial-mesenchymal transition in colorectal [35] and ovarian cancer cells [36]. Moreover, Au et al. demonstrated that TGF- β 1 promotes cell migration through OCT-4 expression in endometriosis [37].

In summary, we have isolated human endometrial tumor stem cells (hOVEN-MSCs) that showed mesenchymal stem cell characteristics and expressed OCT-4, ALDH1, CD133 and Musashi-1. The expression of Musashi-1 indicated the endometrioma nature of these cells. 17 β -estradiol treatment was found to increase the expression of the former 3 genes and promote the growth rate and colony-forming efficiency, indicating a role of estrogen in the growth of these tumors. These results in conjunction with future *in vivo* studies might provide new strategies for prevention and treatment of endometriosis and endometrioma.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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