

Menstrual blood-derived stromal stem cells from women with and without endometriosis reveal different phenotypic and functional characteristics

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ABSTRACT: Retrograde flow of menstrual blood cells during menstruation is considered as the dominant theory for the development of endometriosis. Moreover, current evidence suggests that endometrial-derived stem cells are key players in the pathogenesis of endometriosis. In particular, endometrial stromal stem cells have been suggested to be involved in the pathogenesis of this disease. Here, we aimed to use menstrual blood, as a novel source of endometrial stem cells, to investigate whether stromal stem cells from endometriosis (E-MenSCs) and non-endometriosis (NE-MenSCs) women differed regarding their morphology, CD marker expression pattern, proliferation, invasion and adhesion capacities and their ability to express certain immunomodulatory molecules. E-MenSCs were morphologically different from NE-MenSCs and showed higher expression of CD9, CD10 and CD29. Furthermore, E-MenSCs had higher proliferation and invasion potentials compared with NE-MenSCs. The amount of indoleamine 2,3-dioxygenase-1 (IDO1) and cyclooxygenase-2 (COX-2) in E-MenSCs co-cultured with allogenic peripheral blood mononuclear cells (PBMCs) was shown to be higher both at the gene and protein levels, and higher IDO1 activity was detected in the endometriosis group. However, NE-MenSCs revealed increased concentrations of forkhead transcription factor-3 (FOXP3) when compared with E-MenSCs. Nonetheless, interferon (IFN)- γ , Interleukin (IL)-10 and monocyte chemoattractant protein-1 (MCP-1) levels were higher in the supernatant of E-MenSCs-PBMC co-cultures. Here, we showed that there are inherent differences between E-MenSCs and NE-MenSCs. These findings propose the key role MenSCs could play in the pathogenesis of endometriosis and further support the retrograde and stem cell theories of endometriosis. Hence, considering its renewable and easily available nature, menstrual blood could be viewed as a reliable and inexpensive material for studies addressing the cellular and molecular aspects of endometriosis.

Key words: endometriosis / IDO1 / menstrual blood / stromal stem cells / surface marker expression

Introduction

Endometriosis, a benign gynecological disease with a prevalence of 6–10%, is generally described as the existence of endometrial tissues outside the uterine cavity. Common symptoms of endometriosis include chronic pelvic pain, dyspareunia, dysmenorrhea and infertility (Giudice, 2010). The exact etiology of endometriosis is still uncertain.

However, a dominant theory put forth by Sampson suggests that during the menstruation phase, endometrial tissue fragments find their way back through the fallopian tubes into the peritoneal cavity, where they implant and establish the lesions (Sampson, 1927). It should be noted, however, that retrograde flow of endometrial fragments occurs in the majority of women as a physiological phenomenon, whereas endometriosis occurs in 10% of these women (Halme et al., 1984); this

suggests the involvement of additional factors in the development of endometriosis.

Given the potential relationships between retrograde menstruation and the pathogenesis of endometriosis, the cellular content of the menstrual blood is probably the major player in the development of endometriosis. In fact, menstrual blood contains a heterogeneous population of cells, including endometrial stem cells, with a regenerative capacity (Meng et al., 2007; Murphy et al., 2008). These endometrial regenerative stem cells share several phenotypic and functional features with mesenchymal stem cells (MSCs) (Dominici et al., 2006) as they express CD29, CD44, CD73, CD90 and CD105, are negative for hematopoietic and endothelial markers, and are able to differentiate into different cellular lineages (Meng et al., 2007; Kazemnejad et al., 2013; Khanjani et al., 2013, 2014).

Even though certain immunological imbalances have been reported to promote the development of endometriosis (Laschke and Menger, 2007), the inherent nature of menstrual blood stromal stem cells (MenSCs) could also play a key role in the occurrence of this disease. Due to their immunoregulatory functions, indoleamine 2,3-dioxygenase-1 (IDO1), cyclooxygenase-2 (COX-2) and forkhead transcription factor-3 (FOXP3) could be regarded as major candidates serving the needs of MenSCs entering the peritoneal cavity.

IDO1 is an intracellular enzyme that is up-regulated during inflammation and suppresses T-cell-mediated immune responses (Munn et al., 2005). Interestingly, IDO1 is expressed at the feto-maternal interface (Jeddi-Tehrani et al., 2009) and was recently shown to play a pivotal role in proliferation, adhesion and invasion of endometrial stromal cells (ESCs) (Mei et al., 2012). On the other hand, COX-2, which is induced under inflammatory conditions (Vane et al., 1994; Ebert et al., 2005), is shown to be overexpressed in the eutopic and ectopic endometrium of endometriosis patients (Ota et al., 2001; Matsuzaki et al., 2004). FOXP3 is a transcription factor involved in development and function of regulatory T-cells (Tregs), which *per se* maintain immune system homeostasis (Yamagami et al., 2011). One study has reported that the recruitment of Tregs to both eutopic and ectopic endometrium is disrupted in women suffering from endometriosis (Berbic et al., 2010), suggesting a crucial role for FOXP3.

Several theories have been proposed to explain the development of endometriosis: Sampson's theory of retrograde menstruation, the coelomic metaplasia theory (Meyer's theory), the vascular and lymphatic metastasis theory (Halban's theory), the embryonic rest theory and the stem/progenitor cells theory (Sampson, 1927; Fallas, 1956; Sasson and Taylor, 2008). According to the latter, endometrial stem cells are involved in the pathogenesis of endometriosis. There is evidence confirming the existence of stem/progenitor cells in eutopic endometrial tissues and ectopic endometriosis lesions (Sasson and Taylor, 2008; Gargett and Masuda, 2010; Gargett et al., 2014). Considering the retrograde menstruation and stem cell theories, we speculated that menstrual blood as a key source of stromal stem cells might be the link between the endometrium and endometriosis lesions.

Accordingly, we hypothesized that MenSCs in women with endometriosis (E-MenSCs) differ inherently from their non-endometriosis counterparts (NE-MenSCs). With this in mind, we compared E-MenSCs and NE-MenSCs with respect to their morphological, phenotypic and functional features. We found for the first time that E-MenSCs differed from NE-MenSCs in terms of expression of certain surface markers, morphology, proliferation rate and expression of IDO1, COX-2 and

FOXP3 as well as induction of interferon (IFN)- γ , Interleukin (IL)-10 and monocyte chemoattractant protein-1 (MCP-1) secretion.

Materials and Methods

Antibodies

FITC-conjugated monoclonal antibodies against CD9, CD34, CD38 and CD133 as well as PE-conjugated monoclonal antibodies for CD10, CD29, CD44, CD45, CD73 and SSEA-4 were purchased from BD Biosciences (San Jose, CA, USA). PE-conjugated anti-CD105 and unconjugated mouse anti-human STRO-1 monoclonal antibodies were from R&D Systems (Minneapolis, MN, USA). Polyclonal FITC-conjugated sheep anti-mouse antibody was obtained from Avicenna Research Institute (Tehran, Iran). Polyclonal rabbit anti-human Oct-4 and FITC-conjugated goat anti-rabbit antibodies were from Abcam (Cambridge, MA, USA) and Sigma (St Louis, MO, USA), respectively. The entire corresponding isotype-matched control antibodies were obtained from the same companies as their test antibodies. All the aforementioned antibodies were used in flow cytometry experiments at the concentrations recommended by the manufacturers.

The following antibodies were used in chondrogenic differentiation experiments: a mouse anti-human Collagen type II antibody and an FITC-conjugated goat anti-mouse IgG (both from Abcam). The antibodies used in western blot assays were: polyclonal rabbit anti-human IDO1 antibody (Sigma), polyclonal rabbit anti-human COX-2 (Thermo Scientific Pierce Products, IL, USA), and goat anti-human FOXP3 antibodies (Abcam). Polyclonal horseradish peroxidase-linked secondary antibodies against either rabbit or goat IgG were prepared by Avicenna Research Institute (Iran).

Patient selection

The patient group comprised endometriosis women ($n = 6$) (stages III–IV) undergoing laparoscopy for infertility or pain and the control group consisted of non-endometriosis subjects ($n = 6$) undergoing laparoscopy for tubal ligation. All donors were of reproductive age (20–45 years old; age mean \pm SD of 30 ± 4 and 29 ± 6 for endometriosis and non-endometriosis groups, respectively), with regular menstrual cycles. Subjects had not received hormones or GnRH agonist therapy for at least 3 months before and after surgery and had no history of malignancies or autoimmune diseases. Endometriosis scoring was carried out by an expert surgeon based on visual inspection during laparoscopy in accordance with the Revised American Fertility Society system (1997). Moreover, histological confirmation of endometriosis was performed for all tissue specimens based on the presence of endometrial glands and stroma. Participant enrollment took place between 2010 and 2012 at Tehran Clinic Hospital (Tehran, Iran), and all participants signed an informed consent form before enrolling in the study. The study was approved by the Institutional Review Board and the Ethics Committee for Medical Research of Avicenna Research Institute.

Isolation, morphological study, immunophenotyping and multi-lineage differentiation of MenSCs

MenSCs were isolated from menstrual blood specimens and characterized as per protocols described elsewhere (Nikoo et al., 2012; Kazemnejad et al., 2013). Briefly, menstrual blood samples were collected by the donors themselves using Diva Cup (Lunette, Stockholm, Sweden) on the second day of menstruation and were immediately transferred to the laboratory. Mononuclear cells were separated using Ficoll-Paque (GE Healthcare, Stockholm, Sweden), cultured and after 2 weeks used as MenSCs. The isolated MenSCs were confirmed to be positive for vimentin (a stromal cell cytoskeletal

marker), and negative for cytokeratin (an epithelial marker) (data not shown). MenSCs used in all experiments were from passages 2–4.

The shape index (SI) of E-MenSCs and NE-MenSCs was determined and compared. To this end, phase contrast micrographs taken from 2D culture of NE-MenSCs ($n = 6$) and E-MenSCs ($n = 6$) were investigated; in each micrograph, two frames were assessed by two observers (concordance $> 90\%$) for cell circularity and analyzed using ImageJ software (version 1.42I) (Abramoff et al., 2004). SI was calculated as per the following established formula: $SI = 4\pi \times \text{Area}/\text{Perimeter}^2$ (Cornhill et al., 1980; Schutte and Taylor, 2012). Accordingly, the SI for a circle and a line would be 1 and 0, respectively. Hence, the more circular a cell is, the closer its SI would be to 1, while in the case of spindle-like and more stretched cells, the SI would be closer to 0. Average SI for each NE-MenSC and E-MenSC was calculated; the means were then compared using the non-parametric Mann–Whitney test.

The multi-lineage differentiation capacity of both MenSCs types was determined as per a method published elsewhere (Darzi et al., 2012; Khanmoham-madi et al., 2012; Kazemnejad et al., 2012, 2013, 2014; Khanjani et al., 2013, 2014; Rahimi et al., 2014). In brief, with respect to osteogenic differentiation, MenSCs were treated with dexamethasone, β -glycerophosphate and ascorbate-phosphate (all from Sigma) and proper cell differentiation was confirmed through calcium-specific staining using an Alizarin red staining kit (Sigma). For induction of chondrogenic differentiation, cells were cultured in the presence of transforming growth factor $\beta 3$, bone morphogenetic protein 6, dexamethasone, ITS+ I (ITS+ bovine serum albumin and linoleic acid) and ascorbic acid (all from Sigma). Chondrogenic differentiation was investigated by immunofluorescent staining through treatment of cells with primary monoclonal mouse anti-human Collagen type II antibody, followed by staining with FITC-conjugated goat anti-mouse IgG. Nuclear staining was performed using DAPI (Sigma). To induce adipogenic differentiation, the cells were treated with dexamethasone, recombinant human insulin, 3-isobutyl-1-methyl-xanthine (all from Sigma) and rosiglitazone (Osvah Pharmaceutical Company, Tehran, Iran) for 6 days. Following a 3-day culture in DMEM-F12 supplemented with fetal bovine serum (FBS) (Gibco, Paisley, UK), the cells were transferred to a DMEM-F12 medium containing FBS, dexamethasone, recombinant human insulin and indomethacin for up to 12 days. Differentiated cells were stained for the existence of fat vacuoles using Oil red O staining (Sigma). For the control groups, undifferentiated cells were stained according to the aforementioned methods.

MenSCs were further assessed for the expression of certain surface and intracellular markers using flow cytometry. Staining procedures were performed according to our previous publications (Nikoo et al., 2012; Kazemnejad et al., 2013) using fluorochrome-labeled antibodies. In all experiments, the corresponding isotype-matched monoclonal antibodies were used as negative controls. Measurements were carried out by a Partec flow cytometer (Münster, Germany) and the results were analyzed and processed using Flowjo software (version 7.6.1).

Proliferation, invasion and adhesion assays

For proliferation experiments, NE-MenSCs and E-MenSCs that had undergone overnight serum starvation were seeded into 96-well plates (1×10^4 cells/well) in 100 μ l of phenol red-free DMEM (Gibco) supplemented with L-glutamine (2 mM) (Sigma), penicillin–streptomycin solution (50 IU/ml) (Sigma) and FBS (10%) (Gibco). Plates were cultured in a humidified incubator (5% CO_2) for 3 days. ^3H -thymidine (1 μCi) (GE Healthcare) was then added to each well and the incubation was continued for another 18 h. Cultures were harvested onto glass fiber filter papers (Titertek, Paris, France) and counted using a liquid scintillation counter (Wallac 1410; Pharmacia, Stockholm, Sweden). Results were expressed as mean count per minute (CPM) of triplicate cultures. Mitomycin-treated MenSCs were considered as the negative controls.

The invasiveness of NE-MenSCs and E-MenSCs in matrigel-coated inserts (8 μm pores) was evaluated using BD BioCoat Invasion Chambers (24-well plates) (BD) (Delbandi et al., 2013). MenSCs were cultured on the upper surface of the inserts (5×10^4 cells/insert) in an FBS-free DMEM-F12 medium. The lower part of the chambers contained the same medium with 5% FBS as the chemoattractant. After a 48 h incubation period, the cells that had passed through the pores and adhered to the lower surface of the inserts were counted in 50 randomly selected fields under an upright light microscope and averaged. The invasive ovarian cancer cell line, SKOV3, served as the positive invasion control.

The attachment ability of NE-MenSCs and E-MenSCs was investigated using fibronectin-coated 96-well attachment plates (BD) (Delbandi et al., 2013). A predetermined optimal cell number of 2.5×10^4 MenSCs/well was seeded into the wells of attachment plates in DMEM-F12, 5% FBS. After a 2 h incubation period, the non-adherent cells were removed and the adhered cells were fixed with ethanol (96%), and stained with crystal violet. For extraction of crystal violet, the cells were lysed with 10% (v/v) acetic acid. The extent of attachment was evaluated through measuring the optical density of wells using an Anthos 2020 microplate reader (Biochrom, Cambridge, UK) at 570 nm.

Transwell cultures

A transwell culture system (0.4 μm pores) (Corning, NY, USA) was used to prevent cell–cell contact. First, 2×10^5 NE-MenSCs or E-MenSCs were separately seeded into the lower chambers of transwells in RPMI-1640, 10% FBS. Next, peripheral blood mononuclear cells (PBMCs) obtained from two normal individuals were loaded into the upper chambers (2×10^5 from each individual/well) to set an allogenic mixed lymphocyte reaction (MLR); these cultures were called ‘MenSC/PBMC co-cultures’. In some wells, allogenic PBMCs were cultured in the absence of MenSCs (named ‘MLR cultures’). After a 72 h culture, both NE-MenSCs and E-MenSCs were harvested and used in the following experiments. In addition, the supernatants of co-cultures were collected and tested for cytokine concentrations. To achieve consistent results, PBMCs for all experiments were prepared from the same individuals.

Quantitative real-time PCR analysis of *IDO1*, *COX-2* and *FOXP3* genes

In the case of *IDO1*, prior to performing quantitative real-time PCR (qRT-PCR), NE-MenSCs were treated with IFN- γ (a typical *IDO1* inducer) and their ability to express the *IDO1* gene was confirmed using conventional reverse transcriptase–PCR (data not shown).

NE-MenSCs and E-MenSCs recovered from MenSC/PBMC co-cultures were tested for *IDO1*, *COX-2* and *FOXP3* expression using qRT-PCR. RNA extraction and cDNA synthesis were performed as per the method described in our previous publications (Jeddi-Tehrani et al., 2009; Zarnani et al., 2010). The cDNA obtained from each sample was then used as a template for qRT-PCR. The sequences of sense and antisense primers as well as those of the TaqMan fluorogenic probes are shown in Table I. Reaction conditions for qRT-PCR were as follows: 20 μ l of PCR mixture that included 1 μ l cDNA, 10 μ l of $2 \times$ PCR buffer (Takara, Shiga, Japan), 0.4 μ l of 10 μM sense and antisense primers (Operon, Sweden), 0.8 μ l of TaqMan probe (Operon, Sweden), 0.4 μ l of Dye II 50 \times (Takara) and 7 μ l of autoclaved distilled water. The reaction mixtures were incubated at 95°C for 10 s, followed by either 40 amplification cycles for *IDO1* or 50 cycles for *COX-2* or *FOXP3*, at 95°C for 5 s and 60°C for 34 s. The threshold cycle (Ct) was described as the fractional cycle number where the fluorescence passed the fixed threshold. Quantitative normalization of cDNA in each sample was carried out in reference to the expression of human housekeeping gene β -ACTIN. qRT-PCR amplifications were performed in triplicate using an ABI Prism 7500 (Applied Biosystems,

Table 1 Sequences of primers and probes.

IDO1	^a S: 5'-GCTGCTTCTGCAATCAAAGTAATT-3' ^b AS: 5'-CTTTTGGGTTTACATGATCGTGGA-3' Probe: [6-FAM]-5'-CACTTTGCTAAAGGCGCTGTTGGAAATAGC-3'-[TAMRA-6-FAM]
COX-2	S: 5'-GCAAATTGCTGGCAGGGTTGCT-3' AS: 5'-AGACATTTCTTTTCTCCTGTAAG-3' Probe: [6-FAM]-5'-CAGATGAAATACCACTCTTTTAATGAGTACC-3'-[TAMRA-6-FAM]
FOXP3	S: 5'-GTCTCTGGAGCAGCAGCTGGTG-3' AS: 5'-CCTTGTCGGATGATGCCACAGA-3' Probe: [6-FAM]-5'-AAGGAGAAGCTGAGTGCCATGCAGGCCC-3'-[TAMRA-6-FAM]
β-ACTIN	S: 5'-AGCCTCGCCTTTGCCGA-3' AS: 5'-CTGGTGCCTGGGGCG-3' Probe: [6-FAM]-5'-CCGCCGCCGTCACACCCGCC-3'-[TAMRA-6-FAM]

^aSense primer sequence.

^bAnti-sense primer sequence.

USA). The $\Delta\Delta C_t$ values were calculated and analyzed with REST-RG software (version 3).

SDS–PAGE and western blotting for IDO1, COX-2 and FOXP3 proteins

NE-MenSCs and E-MenSCs recovered from MenSC/PBMC co-cultures were homogenized using a lysis buffer as per the method described elsewhere (Jeddi-Tehrani et al., 2009). The samples were then placed on ice for 15 min (vortexed every 5 min). Next, they were centrifuged for 15 min at 12 000g. The supernatants were then collected and their protein concentration was determined by a BCA protein assay kit (Pierce, IL, USA). The proteins in samples were separated by SDS–PAGE (10%) (sample buffer and SDS–PAGE reagents from sigma), and transferred to a PVDF membrane (pore size: 0.45 μm) (Millipore, MA, USA) followed by incubation in a blocking buffer, composed of 5% non-fat dry milk powder (Sigma) in 0.05% PBS-Tween 20, for 2 h at room temperature. Upon washing, an overnight incubation at 4°C with anti-IDO1 (9.2 μg/ml), anti-COX-2 (2 μg/ml), or anti-FOXP3 antibodies (1 μg/ml) was performed. The membrane was then washed and probed with a horseradish peroxidase-linked secondary antibody (1:8000) for 1 h at room temperature. After four washing steps with PBS-Tween 20, the resulting signals were visualized using ECL Detection kit (GE Healthcare) according to the manufacturer’s instruction. In negative control lanes, the primary antibody was replaced by pre-immune serum from either rabbit or goat.

Determination of IDO1 enzyme activity

To determine IDO1 enzyme activity, kynurenine levels in supernatants of MenSC/PBMC co-cultures were measured using a spectrophotometric assay (Braun et al., 2005). To do so, the supernatants were mixed with 30% trichloroacetic acid (2:1), vortexed and centrifuged at 8000g for 5 min. Thereafter, 75 μl of the supernatant was added to an equal volume of Ehrlich reagent (100 mg of *p*-dimethylbenzaldehyde, 5 ml glacial acetic acid; Sigma) in a 96-well plate. Triplicate samples were run against a standard curve of defined kynurenine concentrations (0–100 μg/ml; Sigma). Optical density of the wells was measured using an Anthos 2020 microplate reader (Biochrom) at 492 nm. Supernatants of allogenic PBMCs cultured in the absence of MenSCs (MLR cultures) served as the negative control.

Cytokine assays

Supernatants of the MenSC/PBMC co-cultures were collected after 72 h and explored for the concentrations of IFN-γ and IL-10 by sandwich ELISA using

commercially available ELISA sets (BD Biosciences). Moreover, the levels of MCP-1 in the supernatants were measured using a sandwich ELISA kit (R&D, USA). Optical density of the wells was measured using an Anthos 2020 microplate reader (Biochrom) at 450 nm.

Statistical analyses

With respect to qRT-PCR, the comparisons were performed using REST-RG software (version 3), which uses Pair Wise Fixed Reallocation Randomization Test, the output of which is presented as mean ± SD. As for the other experiments, statistical differences were determined by the non-parametric Mann–Whitney test using Graphpad Prism software (version 5.04.) and data were shown as median with range. Differences were considered to be significant when $P \leq 0.05$. Six samples were allocated to either endometriosis or non-endometriosis group, unless otherwise mentioned.

Results

Morphological differences between NE-MenSCs and E-MenSCs

Microscopic observations showed that, in 2D cultures, NE-MenSCs had fibroblast-like spindle-shaped morphology (Fig. 1Aa) whereas E-MenSCs were less stretched and elongated (Fig. 1Ab). Consistently, the SI of E-MenSCs was significantly higher when compared with NE-MenSCs, confirming higher circularity of E-MenSCs (Fig. 1B). Furthermore, when cultured in matrigel (3D cultures), E-MenSCs formed small colonies (Fig. 1Ad), which were absent in NE-MenSCs cultures (Fig. 1Ac).

Differentiation of MenSCs into osteoblasts, chondrocytes and adipocytes

Mineralization was pronounced in both NE-MenSCs and E-MenSCs differentiated into osteoblasts. Moreover, cells differentiated into chondrocytes showed strong staining with anti-collagen type-II antibody. Finally, Oil red O staining confirmed formation of oil droplets in cells differentiated into adipocytes. Undifferentiated MenSCs were negative as related to all three specific staining protocols (Fig. 2).

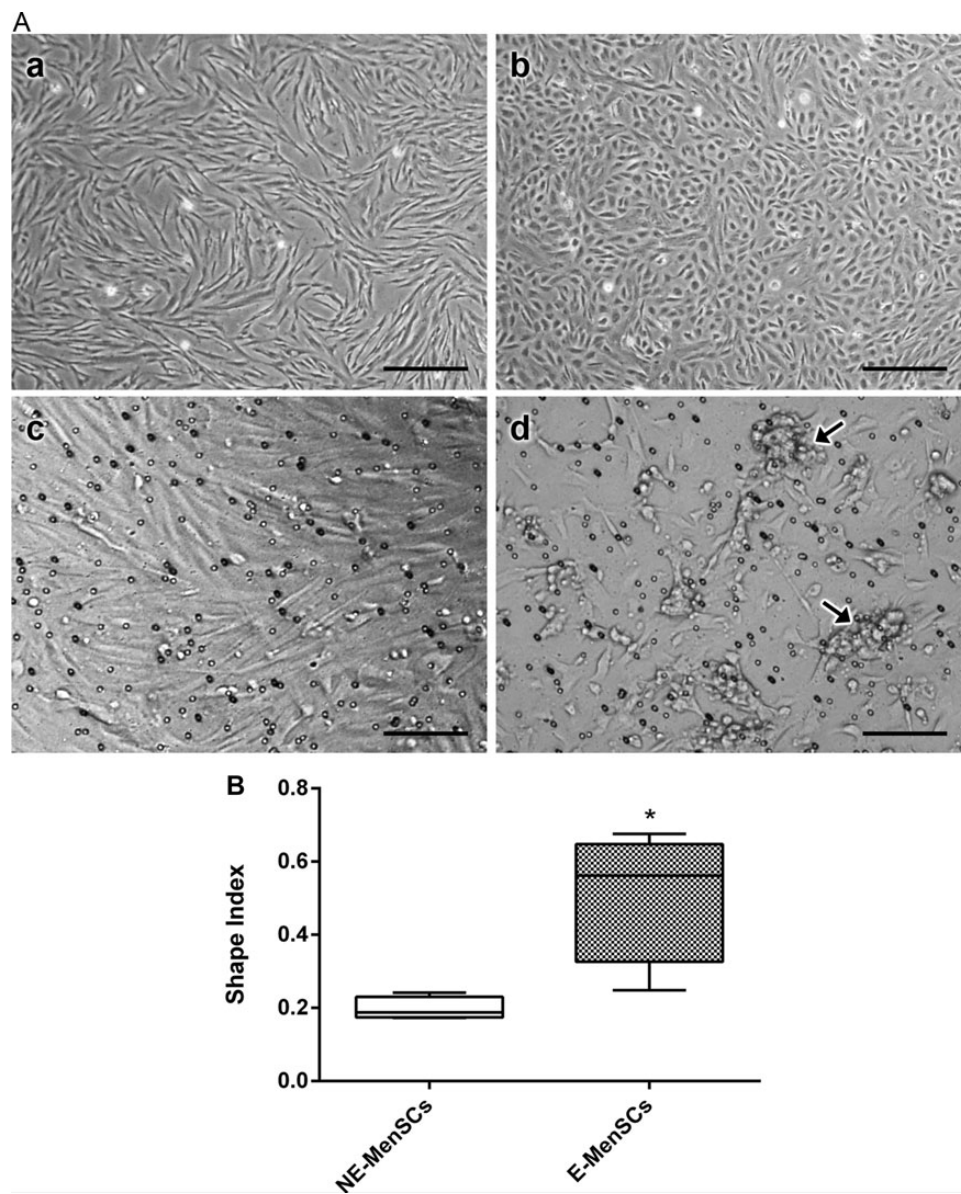


Figure 1 Morphological difference between NE-MenSCs and E-MenSCs. NE-MenSCs in 2D culture showed a fibroblast-like spindle-shaped morphology (Aa), while E-MenSCs were less stretched and more circular (Ab). In 3D culture, NE-MenSCs had fibroblast-like morphology without cell aggregates (Ac), whereas E-MenSCs formed cellular aggregates (black arrows) (Ad). The morphological differences in 2D cultures were quantified by calculating the shape indices of NE-MenSCs and E-MenSCs (B). * $P \leq 0.05$. Scale bar: 30 μm .

Differential expression of markers in NE-MenSCs and E-MenSCs

In the flow cytometry results, both MenSCs types were positive for CD9, CD10, CD29, CD73, CD105, Oct-4 and CD44, while negative for CD34, CD133, CD45, CD38, SSEA-4 and STRO-1 (Fig. 3A). To compare E-MenSCs and NE-MenSCs with respect to the expression/positivity level of CD9, CD10, CD29, CD73, CD105, Oct-4 and CD44, we used the mean fluorescence intensity (MFI); this is a measure directly proportional to the amount/number of a molecule on a cell population. In our results, the MFIs of CD9, CD10 and CD29

were significantly higher for E-MenSCs compared with that of NE-MenSCs ($P \leq 0.05$) (Fig. 3B).

Proliferation, invasion and adhesion potentials of NE-MenSCs and E-MenSCs

E-MenSCs showed a significantly higher proliferation capacity when compared with NE-MenSCs ($P \leq 0.05$) (Fig. 4A). The invasion ability of E-MenSCs was also significantly higher than that of the NE-MenSCs ($P \leq 0.05$) (Fig. 4B). Regarding adhesion capacity, no significant difference was observed between the two groups ($P \geq 0.05$) (Fig. 4C).

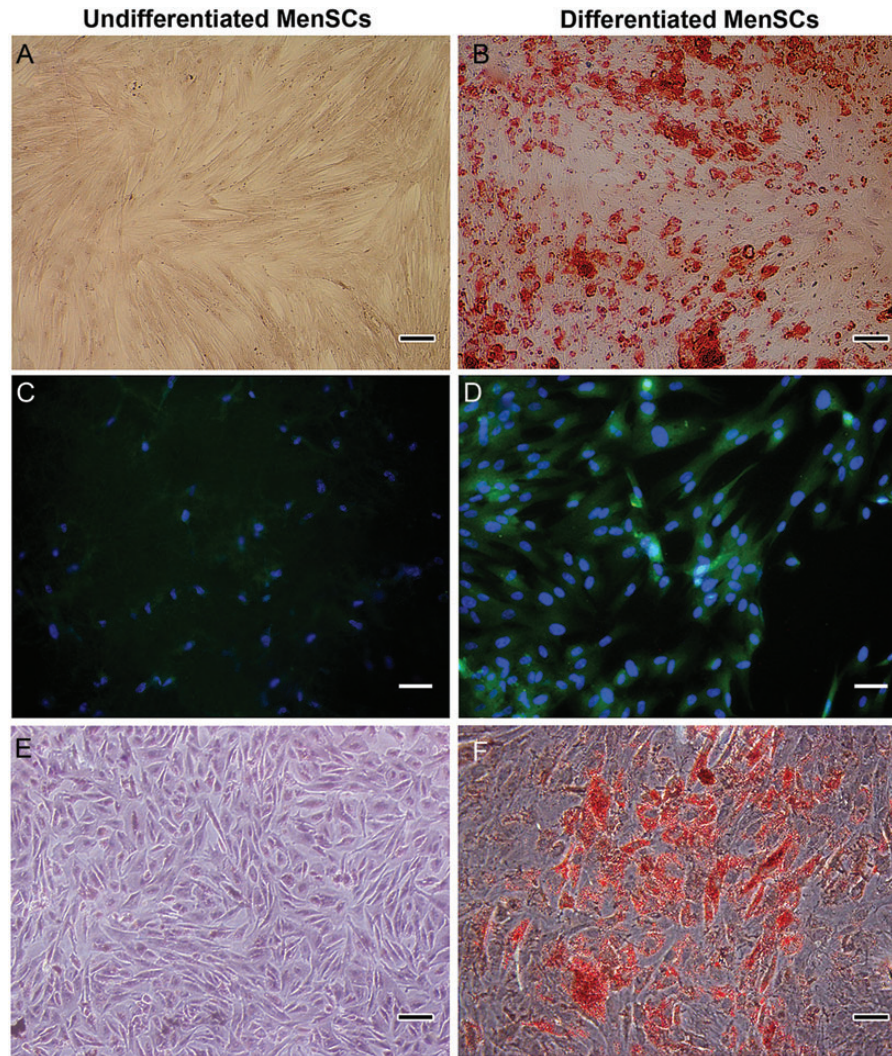


Figure 2 Multi-lineage differentiation potential of MenSCs. MenSC differentiation into osteoblasts (**B**), chondrocytes (**D**), and adipocytes (**F**) was assessed using Alizarin red staining, immunostaining of Collagen type II and Oil red O staining, respectively. The left panel shows staining of undifferentiated cells (**A**, **C**, **E**), as the control groups. Data of NE-MenSC are shown as the representative. Scale bar: 100 μ m.

IDO1, COX-2 and FOXP3 gene expression in NE-MenSCs and E-MenSCs

Quantitative RT-PCR analysis of *IDO1*, *COX-2* and *FOXP3* genes was performed in both NE-MenSCs and E-MenSCs collected from transwell MenSC/PBMC co-cultures. As shown in Fig. 5, the expression of *IDO1* in E-MenSCs was 10-fold higher than that in NE-MenSCs ($P \leq 0.001$). *COX-2* gene expression was also significantly up-regulated in E-MenSCs by a factor of 10 ($P \leq 0.01$) (Fig. 5). In the case of *FOXP3* gene, an 11-fold down-regulation was observed in E-MenSCs in comparison with NE-MenSCs ($P \leq 0.01$) (Fig. 5).

Protein level of IDO1, COX-2 and FOXP3 in E-MenSCs and NE-MenSCs

The concentrations of IDO1, COX-2 and FOXP3 proteins were determined in E-MenSCs and NE-MenSCs obtained from transwell MenSC/

PBMC co-cultures. With respect to IDO1 and COX-2, E-MenSCs produced significantly higher protein levels when compared with NE-MenSCs ($P \leq 0.05$) (Fig. 6A and B). As for FOXP3, NE-MenSCs showed significantly higher protein levels compared with E-MenSCs ($P \leq 0.01$) (Fig. 6A and B).

The activity of IDO1 produced in the supernatant of MenSC/PBMC co-culture

To investigate the functionality of IDO1, kynurenine concentrations were measured in the supernatant of MenSCs-PBMC co-cultures with reference to that of allogenic PBMCs cultured in the absence of MenSCs (MLR cultures). According to our results, IDO1 activity in the E-MenSCs was significantly higher than in the NE-MenSCs ($P \leq 0.05$) (Fig. 6C).

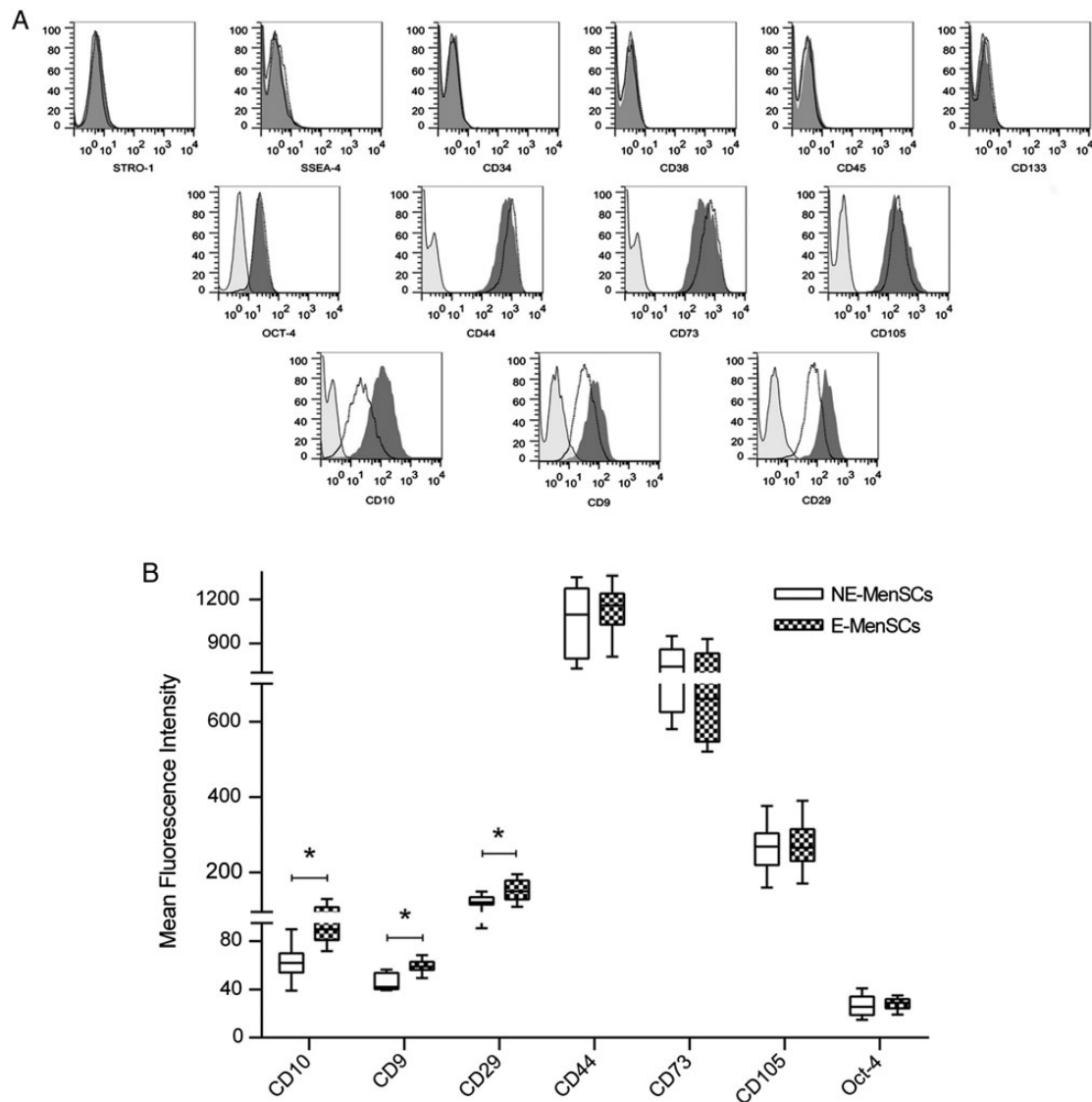


Figure 3 Flow cytometric comparison of NE-MenSCs and E-MenSCs. NE-MenSCs expressed typical MenSC CD markers, so that they were positive for CD9, CD10, CD44, CD29, CD73, CD105 and Oct-4, while negative for CD34, CD133, CD45, CD38, SSEA-4 and STRO-1. Light gray, open dashed and dark gray histograms correspond to isotype control, NE-MenSCs and E-MenSCs, respectively (A). MFI was used to compare NE-MenSCs with E-MenSCs with respect to the expression level of positive markers (B). * $P \leq 0.05$.

Differential cytokine production in MenSC/PBMC co-cultures of non-endometriosis and endometriosis patients

The effects of both MenSC types on the levels of IFN- γ , IL-10 and MCP-1 in MenSC/PBMC co-cultures were investigated and compared. The concentrations of IFN- γ ($P \leq 0.01$), IL-10 ($P \leq 0.05$) and MCP-1 ($P \leq 0.05$) were significantly augmented in the supernatant of E-MenSC/PBMC co-cultures compared with that of the NE-MenSC/PBMC co-cultures. Moreover, the two groups showed no statistical differences regarding the IFN- γ /IL-10 ratio (Fig. 7).

Discussion

In the present study, we demonstrated for the first time that E-MenSCs are different from NE-MenSCs in terms of morphology, expression level of some surface markers, proliferation capacity and expression of IDO1, COX-2 and FOXP3, together with the potential to establish a micro-environment containing IFN- γ , IL-10 and MCP-1.

E-MenSCs revealed a higher expression level of CD9, CD10 and CD29 when compared with NE-MenSCs. The accurate interpretation on this observation is still unclear to us, but it could partly be explained by considering the similarities between endometriosis and cancer.

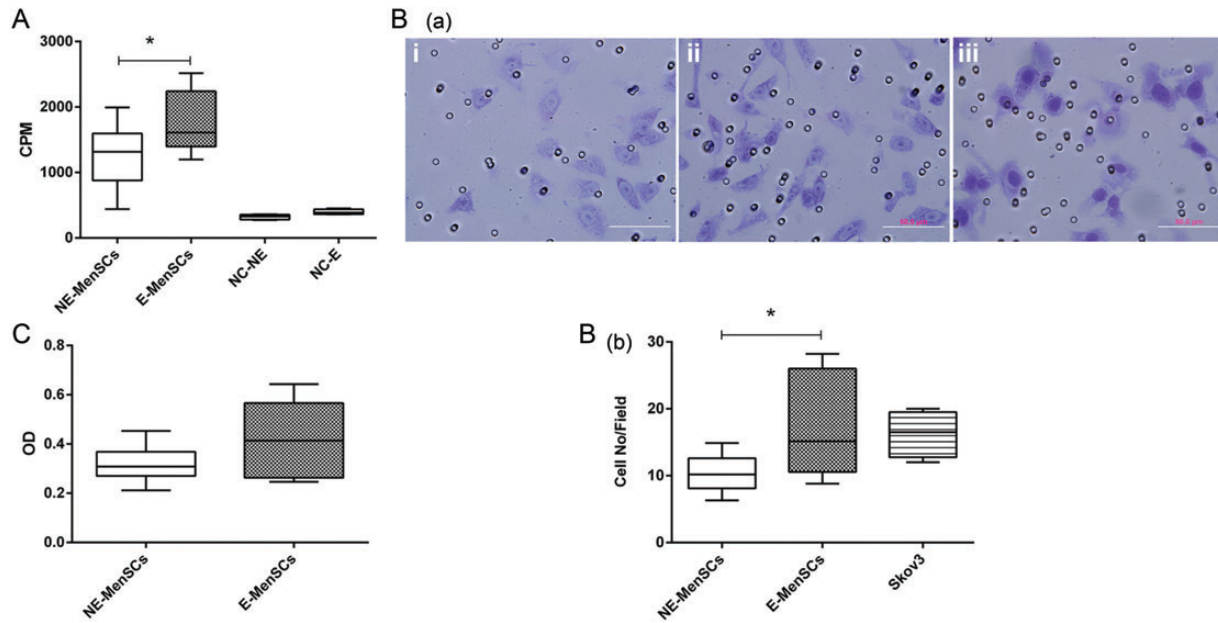


Figure 4 Proliferation, invasion and adhesion capacity of NE-MenSCs and E-MenSCs. The proliferation, invasion and adhesion potential of NE-MenSCs and E-MenSCs was measured using ^3H -thymidine incorporation, matrigel invasion assay and fibronectin adherence, respectively. **(A)** Mitomycin C-treated NE-MenSCs (NE-NC) and E-MenSCs (E-NC) served as negative control (NC) cells for cell proliferation assay. **(B)** A representative experiment displaying a microscopic view of invaded cells. (i) and (ii) correspond to NE-MenSCs and E-MenSCs while (iii) the Skov3 cell line served as the positive control. Scale bar: 50 μm . The number of NE-MenSCs and E-MenSCs that had digested through matrigel-coated membranes was counted (in 50 microscopic fields) and compared. **(C)** Adherence of NE-MenSCs and E-MenSCs to fibronectin-coated plates was measured by a colorimetric assay. Optical density (OD) was read at 570 nm. CPM, count per minute. * $P \leq 0.05$.

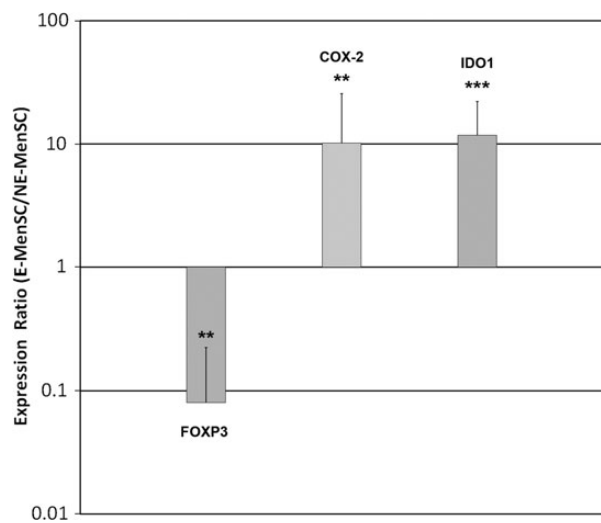


Figure 5 Quantitative assessment of *IDO1*, *COX-2* and *FOXP3* gene expression. NE-MenSCs and E-MenSCs were seeded into the lower sections of a transwell plate. In the upper chambers, allogenic MLR between two PBMC populations was set. Gene expression in both MenSC types was evaluated by qRT-PCR. *IDO1* and *COX-2* genes showed an up-regulation of more than 10-fold in E-MenSCs compared with that in NE-MenSCs, while *FOXP3* gene expression was 11 times lower in E-MenSCs. ** $P \leq 0.01$; *** $P \leq 0.001$.

In fact, endometriosis patients have been reported to be more prone to the development of ovarian cancer (Buis et al., 2013). In this regard, stromal cells existing in the milieu of several tumor types have been reported to differ from their normal counterparts with respect to expression of various CD markers (Belov et al., 2010; Cammarota et al., 2010; Meyer et al., 2011). CD9 mediates signal transduction, and has been shown to have a part in cellular events such as development, growth and motility. Hwang et al. (2012) reported the increased expression of CD9 in ovarian cancer tissues. Moreover, CD9 overexpression has been associated with the invasive, migratory and tumorigenic capacities of tumor cells in various cancers (Erovc et al., 2003; Nakamoto et al., 2009; Kohmo et al., 2010). Consistently, other studies addressing the relationship between cancers and CD10 expression have demonstrated CD10 up-regulation on tumor and stromal cells of different cancers such as breast and bladder (Abdou, 2007; Borgna et al., 2012). CD29 is generally known as a member of the integrin family (Van der Linden et al., 1995). This molecule has been shown to be involved in processes such as malignancy, metastasis, drug resistance and epithelial to mesenchymal transition (von Schlippe et al., 2000; Brakebusch and Fässler, 2005; Jensen et al., 2008). CD29 has been reported to be up-regulated on cancer stem cells providing them with a higher proliferation ability (Geng et al., 2013). There are currently no data available regarding the overexpression of CD29 in endometriosis stromal cells. However, the higher expression of CD29 on MenSCs might assist them in adhering to the peritoneal wall. Overall, higher expression of CD9, CD10 and CD29 on E-MenSCs, observed in the current study, may provide them with the same characteristics as tumor cells, leading to establishment

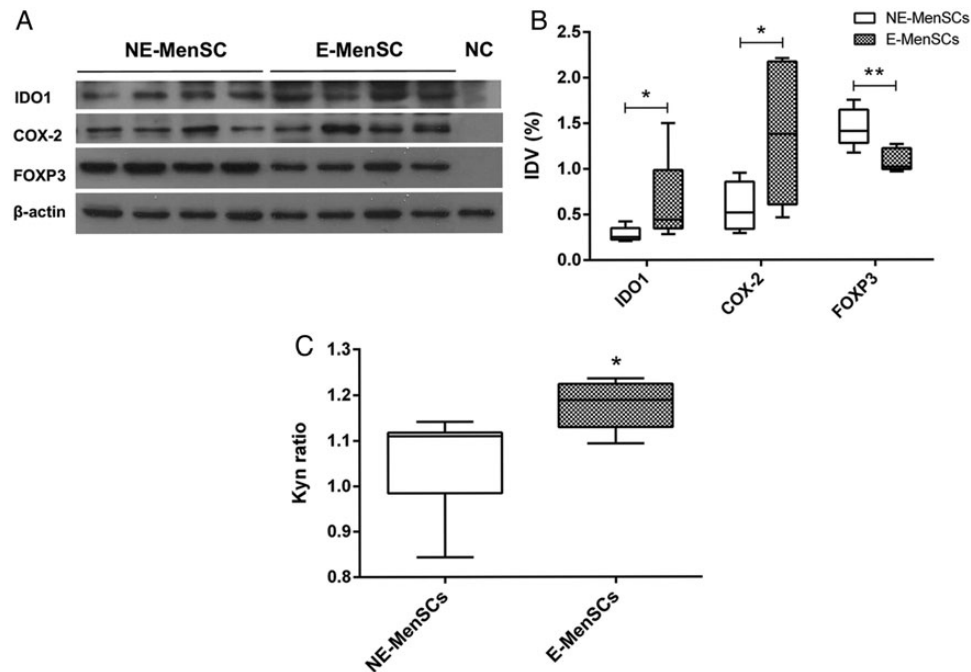


Figure 6 Determination of IDO1, COX-2 and FOXP3 protein concentration as well as IDO activity. IDO1, COX-2 and FOXP3 levels in both E-MenSCs ($n = 4$) and NE-MenSCs ($n = 4$) were assessed by western blot (A). Densities of all bands were determined using AlphaEaseFC software and normalized with that of β -actin bands (B). To assess IDO1 activity, the concentration of kynurenine in the supernatant of both E-MenSCs- and NE-MenSCs-PBMC co-cultures was determined and normalized to that of an MLR alone (C). NC, negative control; IDV, integrated density value; Kyn, kynurenine. $*P \leq 0.05$ and $**P \leq 0.01$.

and propagation of endometriosis lesions. It is of note, however, that the observed differences in expression of these markers are obtained upon comparison of *in vitro* cultured cells; thus, we cannot certainly conclude that such differences would have been detected upon *in situ* assessments. Here, we tried to use MenSCs from lower passage numbers to obtain results closest to *in vivo* circumstances. Nevertheless, there is still a need to perform additional direct quantitative *in vivo* experiments to definitely verify this point.

We also showed that in 2D cultures, E-MenSCs were morphologically different from NE-MenSCs, the functional importance of which is not clear to us at present. However, based on theoretical and experimental evidence, cell shape can modulate signaling through inducing changes in plasma membrane curvature and hence affect cellular function (Lloyd, 2013). Additionally, here we showed that at least a small portion of E-MenSCs formed aggregates in 3D cultures, whereas NE-MenSCs did not grow into such structures. Rafii *et al.* (2008) indicated that the ascetic fluid of ovarian carcinoma patients generated stromal cell-derived aggregates, positive for markers such as CD9, CD10 and CD29. Collectively, bearing in mind that endometriosis has been described as a benign tumor-like disease (Ueda *et al.*, 2002), there is the possibility that at least a small number of cells with cancerous features exist among E-MenSCs; these cells might be indirectly involved in development of the rare cases of ovarian carcinoma observed in endometriosis patients.

The up-regulated expression of CD9, CD10 and CD29 could also be viewed as a means of non-invasive diagnosis of endometriosis. In fact, immunohistochemical detection of CD10 in endometriosis lesions has been suggested as a useful ancillary assay in diagnosis of endometriosis,

especially in women with minimal disease (Potlog-Nahari *et al.*, 2004). However, this is an invasive method, in that it demands obtaining tissue samples via laparoscopic operation. In our results, performing a flow cytometry test on more easily available menstrual blood specimens could be regarded as a less invasive diagnostic method. Nonetheless, to develop a practical screening technique, we would need to confirm the observed differences between NE-MenSCs and E-MenSCs with a larger population of donors.

Proliferation assays revealed that E-MenSCs had a significantly greater proliferation capacity when compared with NE-MenSCs. Similar observations have been reported by studies addressing the proliferation capacity of eutopic ESCs from women with and without endometriosis (Jones *et al.*, 1995; Nisolle *et al.*, 1997; Meresman *et al.*, 2002; Park *et al.*, 2009). In contrast to the current study, in our previous study, we indicated that ESCs from non-endometriosis women were superior to those from endometriosis patients in terms of proliferation rate (Delbandi *et al.*, 2013). This contradiction could be partly ascribed to the type of specimen (tissue biopsies versus menstrual blood samples), assays employed for cell proliferation quantification (XTT assay versus ^3H -thymidine incorporation), and more importantly, the menstrual phase of specimen collection (proliferative phase versus menstruation). Taken together, the higher proliferation rate could be considered as an essential survival matter for E-MenSCs entering the peritoneal cavity during menstruation.

To successfully implant in the ectopic sites (e.g. peritoneal wall), MenSCs need to have a substantial invasion ability. Our results revealed the increased invasiveness of E-MenSCs, which is in line with our previous

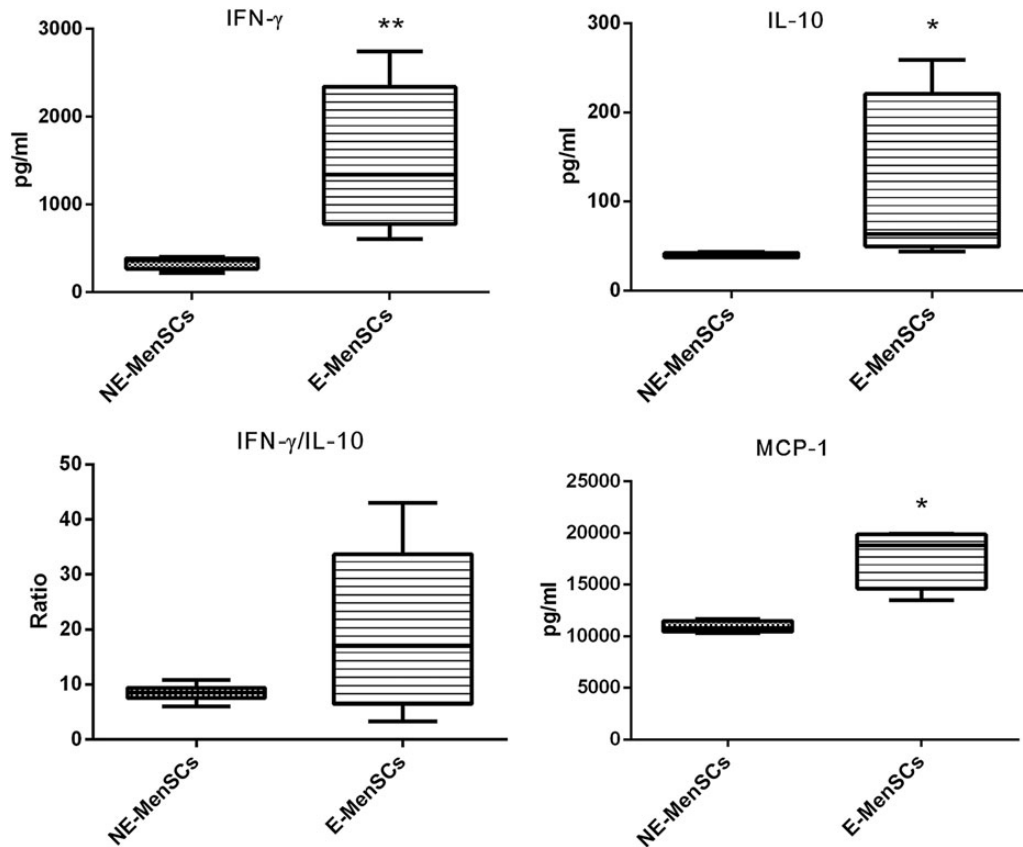


Figure 7 Differential secretion of cytokines in NE-MenSCs-PBMC and E-MenSCs-PBMC co-cultures. NE-MenSCs and E-MenSCs were seeded into the lower sections of a transwell plate. In the upper chambers, an allogenic MLR between two PBMC populations was set. The concentrations of IL-10, IFN- γ and MCP-1 in the supernatant of co-cultures were measured by ELISA. The two groups were also compared for the IFN- γ /IL-10 ratio. * $P \leq 0.05$; ** $P \leq 0.01$.

study (Delbandi et al., 2013) and other investigations on tissue-derived ESCs from endometriosis patients (Chung et al., 2002; Mulayim et al., 2004).

Our previous report (Delbandi et al., 2013) along with the data presented by others (Klemmt et al., 2007; Griffith et al., 2010; Adachi et al., 2011) have demonstrated the enhanced ability of endometriosis-derived ESCs to adhere to extracellular matrix. However, here, the higher attachment capacity of E-MenSCs compared with NE-MenSCs did not reach a significant level, which could partly be attributed to the difference between the specimen source in the present study (menstrual blood) and that of the previous works (endometriotic lesions). Moreover, we cannot ignore the possibility that significant results would have been observed with a larger sample size.

There is accumulating evidence suggesting that a set of immune response-related malfunctions is responsible for the development of endometriosis (Dmowski et al., 1993; Wu et al., 2000; Kyama et al., 2003). On the other hand, the cellular component of menstrual blood is also suggested to play a crucial part in this disease. Accordingly, we sought to determine whether MenSCs from endometriosis patients are inherently different from their normal counterparts. To this end, we studied the reaction of NE-MenSCs and E-MenSCs to inflammatory-like circumstances, known to influence the pathogenesis

of endometriosis. In this context, both MenSC types were separately seeded into MLR and compared for their ability to express IDO1, COX-2 and FOXP3, as molecules with central immunomodulatory roles. Moreover, the concentrations of IFN- γ , IL-10 and MCP-1 in the co-cultures were determined.

E-MenSCs produced higher amounts of IDO1 both at the gene and protein levels. Consistently, IDO1 activity in the supernatant of E-MenSCs-PBMC co-cultures was shown to be superior to that of the control. The only previous study investigating the role of IDO1 in endometriosis showed that endometriosis-derived eutopic and ectopic ESCs express higher levels of IDO1 compared with healthy eutopic ESCs. Moreover, IDO1 could up-regulate COX-2 and matrix metalloproteinase-9 expression in ESCs and support their proliferation, adhesion and invasion abilities (Mei et al., 2012). Hence, our findings on higher proliferation and invasion capacity of E-MenSCs could be in part related to the higher IDO1 level and activity.

Since IFN- γ is known as the major inducer of IDO1 expression, we further determined IFN- γ level in supernatants of MLRs that were established in the presence of either E-MenSCs or NE-MenSCs. Higher amounts of IFN- γ were detected in the endometriosis group. Similarly, we observed enhanced MCP-1 concentrations in E-MenSCs-PBMC co-cultures. IFN- γ and MCP-1 have been demonstrated to synergistically

increase monocyte activation (Ritter and Moll, 2000). Notably, elevated levels of MCP-1 in peritoneal fluid and endometrial glands of endometriosis patients have been reported (Jolicœur *et al.*, 1998; Boucher *et al.*, 2000). Thus, we postulate that the higher concentration of MCP-1 might account for higher IFN- γ production by monocytes and macrophages in E-MenSC/co-cultures. This might *per se* be the reason for the up-regulated IDO1 expression in E-MenSCs. We also observed that, in line with IFN- γ concentrations, IL-10 levels were higher in E-MenSCs co-cultures, which could be viewed as a counter-regulatory mechanism formed secondarily in reaction to IFN- γ enhancement. As a result, we observed no significant differences between the two groups regarding the IFN- γ /IL-10 ratio. Moreover, these results further imply that, as suggested by others (Tariverdian *et al.*, 2007), endometriosis is not a simple enhancement of inflammatory responses, but rather a complex disequilibrium in the cytokine network. Taken together, it could be suggested that, in comparison with their normal counterparts, E-MenSCs have a greater potential to direct the inflammatory responses in their own favor, thereby facilitating the development of endometriosis.

There are data suggesting that COX-2 is involved in the pathogenesis of endometriosis by promoting the generation of prostaglandins (Fagotti *et al.*, 2004). Furthermore, it has been reported that eutopic stromal cells from endometriosis patients express higher levels of COX-2 in comparison with those from normal women (Attar and Bulun, 2006). In addition, Kao *et al.* (2011), through developing an *ex vivo* model, demonstrated the role of COX-2 in increasing the invasive capacity of MSCs derived from ectopic endometrioma. In our results, E-MenSCs recovered from MenSC/PBMC co-cultures showed increased expression of COX-2, suggesting their enhanced capacity to secrete this molecule under inflammatory circumstances, which are supposed to favor the development of endometriosis.

We further assessed the expression of FOXP3 in both MenSC types. In a recent study, Sundin *et al.* (2011) demonstrated that MSCs expressing higher levels of FOXP3 were superior to those with lower FOXP3 expression in terms of MLR suppression. Furthermore, in a non-human primate model of endometriosis, Braundmeier *et al.* (2012) indicated that the expression of FOXP3 gene and the frequency of Tregs in eutopic endometrium were diminished compared with the normal control. The authors suggested that this could account for the infertility cases observed in endometriosis patients. Consistently, another investigation reported the decreased expression of FOXP3 mRNA in eutopic endometrium of women with primary unexplained infertility (Jasper *et al.*, 2006). In our results, compared with NE-MenSCs, E-MenSCs displayed decreased FOXP3 expression. Given the immunomodulatory roles of FOXP3, this phenomenon may be a causative factor compounding the inflammatory milieu in the peritoneal cavity, thereby deteriorating the disease status.

There is accumulating evidence suggesting the potential role of endometrial stem cells in the development of endometriosis (Gargett and Chan, 2006; Chan *et al.*, 2011; Figueira *et al.*, 2011; Gargett *et al.*, 2014). It has been shown that in endometriosis patients, several stemness-related markers are expressed by epithelial and/or stromal cells isolated from both eutopic and ectopic endometrium (Chan *et al.*, 2011; Silveira *et al.*, 2012). On the other hand, previous studies have reported the existence of progenitor cells with high proliferative and differentiation capacities in menstrual blood (Meng *et al.*, 2007; Patel *et al.*, 2008). Here, we observed that MenSCs of endometriosis patients are

different from their normal counterparts with respect to the generation of key molecules involved in the pathogenesis of endometriosis; collectively, these results further support the stem cell theory of this disease.

In this study, for the first time, we provided evidence indicating that MenSCs from endometriosis patients differed from those of non-endometriosis women as judged by their morphology, certain CD marker expression, invasion capacity and proliferation rate, as well as the expression of IDO1, COX-2 and FOXP3, and induction of certain cytokines. These results imply that MenSCs might play key roles in the initiation and development of endometriosis. Moreover, we suggest that regarding their morphological and immunophenotypic features, E-MenSCs play indirect roles in the rare cases of ovarian cancer observed in endometriosis patients; in fact, there is evidence suggesting that through pathways such as interaction with tumor cells and production of soluble factors (e.g. cytokines), stromal cells provide a microenvironment suitable for development and metastasis of epithelial ovarian cancers (Rafii *et al.*, 2008; Spaeth *et al.*, 2009).

A major clinical challenge has been to find a non-invasive and reliable assay for diagnosis of endometriosis. The abnormal immunophenotype of E-MenSCs shown in this study opens the door for development of novel flow cytometry-based assays to diagnose endometriosis. Furthermore, thus far, nearly all of the studies on the differences between endometriosis and non-endometriosis stromal cells have focused on endometrial tissue biopsies, while the present study was conducted on menstrual blood samples. Given that in most cases, the results of the current work are comparable to those of the previous studies on endometrial biopsies, for future studies, we propose menstrual blood as a renewable, inexpensive and easily available source with the potential to be replaced for endometrial biopsies which are obtained through invasive methods. Moreover, the results of this work delineate some new aspects of ESCs in the pathogenesis of endometriosis supporting the stem cell/progenitor theory of this disease. Nonetheless, we need to perform further investigations to unravel the precise mechanisms underlying the interaction between both MenSCs types and key immune cells, such as natural killer cells, macrophages, dendritic cells and T-cells.

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Authors' roles

S.N. performed all experiments and statistical analyses, and took the lead in the manuscript preparation. M.E. was in charge of the study design and critically reviewed the manuscript. M.J.-T. edited the manuscript and approved the final version to be published. A.S. was responsible for patient selection, sample preparation and gynecological consultation. M.B. assisted in performing proliferation and MLR experiments, and was involved in manuscript drafting and preparation of figures. S.V.

assisted in performing western blot assays and provided valuable technical input. S.K. commented on and managed the multi-lineage differentiation experiments. A.H.Z. was in charge of the study design, critically reviewed the paper and approved the final version of the manuscript.

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Conflict of interest

None declared.

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