Angiogenic properties of endometrial mesenchymal stromal cells in endothelial co-culture: an *in vitro* model of endometriosis

Running Title: *In vitro* endothelial differentiation of endometrial MSCs

Canosa S[1†], Moggio A[2†], Brossa A[2], Pittatore G[1], Marchino GL[1], Leoncini S[1], Benedetto C[1], Revelli A[1] and Bussolati B[2].

[1]Gynecology and Obstetrics I, Department of Surgical Sciences, Physiopathology of Reproduction and IVF Unit, S. Anna Hospital, Torino, Italy; [2]Department of Molecular Biotechnology and Health Sciences, University of Torino, Italy

[†=] These authors should be regarded as joint First Authors

Correspondence to: Benedetta Bussolati, M.D., Ph.D., Department of Molecular Biotechnology and Health Sciences, via Nizza 52, 10126, Torino, Italy. E-mail: benedetta.bussolati@unito.it.

Abstract

Study question: Can endometrial mesenchymal stromal cells (E-MSCs) differentiate into endothelial cells in an *in vitro* co-culture system with HUVECs?

Summary answer: E-MSCs can acquire endothelial markers and function in a direct co-culture system with HUVECs.

What is known already: E-MSCs have been identified in the human endometrium as well as in endometriotic lesions. E-MSCs appear to be involved in the formation of the endometrial stromal vascular tissue and the support of tissue growth and vascularization. The use of anti-angiogenic drugs appears as a possible therapeutic strategy against endometriosis.

Study design, size, duration: This is an *in vitro* study comprising patients receiving surgical treatment of ovarian endometriosis (n=9).

Participants/materials, setting, methods: E-MSCs were isolated from eutopic and ectopic endometrial tissue and were characterized for the expression of mesenchymal and endothelial markers by FACS analysis and Real-Time PCR. CD31 acquisition was evaluated by FACS analysis and immunofluorescence after a 48h-direct co-culture with GFP^+-HUVECs. A tube-forming assay was set up in order to analyze the functional potential of their interaction. Finally co-cultures were treated with the anti-angiogenic agent Cabergoline.

Main results and the role of chance: A subpopulation of E-MSCs acquired CD31 expression and integrated into tube-like structures when directly in contact with HUVECs, as observed by both FACS analysis and immunofluorescence. The isolation of $CD31^+$ E-MSCs revealed significant increase of CD31, VEGFR2, Tie2 and Ve-Cadherin gene expression. On the other hand, the expression of mesenchymal genes such as c-Myc, Vimentin, N-Cadherin and SUSD2 remained unchanged. Cabergoline treatment induced a significant reduction of the E-MSC angiogenic potential.

Limitations, reasons for caution: Further studies are necessary to investigate the cellular and molecular mechanisms underlying the endothelial differentiation.

Wider implications of the findings: E-MSCs may undergo endothelial differentiation, and be potentially involved during the development of endometriotic implants. Cell culture systems that more closely mimic the cellular complexity typical of in vivo endometriotic tissues are required to develop novel strategies for treatment.

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Large scale data: Not applicable

Key Words: Endometriosis, mesenchymal stem cells, endothelial differentiation, cabergoline, anti-angiogenic therapy

Introduction

Endometriosis is a chronic and oestrogen-dependent disease characterized by the presence of ectopic endometrial tissue, composed by glands and stroma, outside the uterine cavity (Giudice, 2010; Bulun, 2009). A number of studies supported the presence of rare clonogenic epithelial and stromal cells with stem cell characteristics in the human endometrium (Chan *et al.,* 2004; Chan and Gargett, 2006; Gargett, 2006; Gargett *et al.,* 2009), thought to be physiologically involved in the cyclic endometrial regeneration after menstruation (Schwab *et al.,* 2005; Masuda *et al.,* 2010; Gargett *et al.,* 2016). Endometrial mesenchymal stromal cells (E-MSCs), in particular, are clonogenic mesenchymal like cells (Gargett *et al.,* 2009) expressing pericyte markers (Berger *et al.,* 2005; Spitzer *et al.,* 2012), and localized in the perivascular space of endometrial small vessels (Schwab and Gargett, 2007).

Clonogenic E-MSCs have been also identified in cultures derived from ovarian endometriotic lesions (Chan *et al.,* 2011; Kao *et al.,* 2011). Ectopic E-MSCs showed a higher proliferation, migration and angiogenic ability than eutopic E-MSCs (Kao *et al.,* 2011). It is possible that E-MSCs abnormally shed during menstruation migrate into the peritoneal cavity and consequently proliferate, invade and generate endometriotic implants (Starzinski-Powitz *et al.,* 2001; Leyendecker *et al.*, 2002; Sasson and Taylor, 2008). In this context, the role of E-MSCs mainly appears to be the formation of the endometrial stromal vascular tissue and the support of tissue growth and vascularization through secretion of pro-angiogenic and growth supporting factors (Gargett *et al.,* 2014). In addition, clonally purified $SUSD2^{+}$ E-MSCs were shown to acquire endothelial marker expression and to integrate into renal blood vessels after xenograft under the kidney capsule, underlying an endothelial differentiative ability (Masuda *et al.,* 2012). In addition, circulating endothelial progenitor cells may contribute to the *de novo* vessel formation in endometriosis (Du and Taylor, 2007; Laschke *et al.,* 2011). However, the endometrial angiogenic process is mainly driven by recruitment of endothelial cells by surrounding tissues (Nisolle *et al.,*

1993; Maas *et al*., 2001; Laschke and Menger, 2007). The interaction of stromal and endothelial cells within endometrial angiogenesis has not been investigated yet.

Vascular endothelial growth factor (VEGF) appears to be the main regulator of endometrial angiogenesis. Indeed, VEGF polymorphisms seem to be associated to the risk of endometriosis (Li *et al*., 2013). In addition, increased levels of VEGF were found in the peritoneal fluid of women with endometriosis and in ectopic endometrial tissue, suggesting the relevance of a pro-angiogenic microenvironment in the development of the endometriotic implant (Donnez *et al.,* 1998; McLaren, 2000; Bourlev *et al*., 2006). The use of anti-angiogenic drugs therefore appears as a possible therapeutic strategy against endometriosis (Hull *et al*., 2003; Taylor *et al*., 2009; Pittatore *et al*., 2014). VEGF targeting using VEGF neutralizing antibodies or tyrosine kinase inhibitors effectively reduced growth of endometriotic implants, microvessel density and VEGF expression in models of endometriosis in mice, rats and monkeys (Park *et al.*, 2004; Van Langendonckt *et al.,* 2008; Ozer *et al*., 2013). Similarly, interfering with VEGF-VEGFR-2 signalling using a dopamine agonist displayed an anti-angiogenic effects in experimental endometriosis. Furthermore, we previously demonstrated that the tyrosine kinase inhibitor Sorafenib affected the angiogenic potential of ectopic E-MSCs *in vitro* and reverted their increased VEGF release (Moggio *et al*., 2012).

In the present study, we aimed to investigate the angiogenic process and to set up an *in vitro* model of endometriosis using stromal mesenchymal cells isolated from ovarian endometrial tissue. We found that E-MSCs acquired endothelial markers and contributed to *in vitro* tubulogenesis during co-culture with HUVEC cells. Finally, we evaluated the effect of the dopamine antagonist Cabergoline, also reported to affect VEGF signaling (Novella-Maestre *et al.,* 2009, 2012) in this model.

Materials and methods

Patients

The cell lines were obtained from nine patients receiving surgery for treatment of ovarian endometriosis in the Department of Surgical Sciences, University of Torino, between November 2013 and April 2015 after approval by the Ethics Review Board. Preoperative informed consent was obtained from each patient.

E-MSC isolation and culture

Two samples were collected from the same patient with endometriosis, one of eutopic tissue by gently scraping the endometrium and one of ectopic implant by surgical biopsy of the inner wall of the ovarian endometrial tissue. The tissues were immediately placed in a sterile tissue culture dish and dissected into small fragments using a scalpel blade in a sterile laminar flow. The obtained fragments were then enzimatically processed with 0.1% type I Collagenase (Sigma-Aldrich) for 30 minutes in a 37°C incubator. Later, cell aggregates were filtered through 60-mm and 120-mm meshes. Cells were seeded at a density of $1x10^4/cm^2$ viable cells (80% viable cells determined by trypan blue) in EBM: medium plus supplement kit without serum addition (Lonza) previously described for E-MSC isolation (Moggio et al., 2012). Dead cells were poured off 72 hours later and, after 5-7 days, cell clones were typically observed. Confluence was achieved 10-14 days after plating. Cells were passaged at confluence and after 2–3 days in the subsequent passages. The E-MSCs obtained (eutopic E-MSCs, n=9; ectopic E-MSCs, n=9) were cultured for 12 passages as maximum to test the proliferative capacity typical of MSCs. All the experiments were performed between passages 3 and 8. Eutopic and ectopic E-MSCs were used at the same cell passage.

Flow cytometric analysis

Cytometric analysis was performed using FACScan (Becton Dickinson) as previously described (Bruno *et al*., 2009). The cells suspensions were incubated with antibodies for 25 minutes at 4°C in 100 µl of phosphate-buffered saline with addition of 0.1% bovine serum albumin (Sigma). The following monoclonal antibodies, all fluorescein isothiocyanate or phycoerythrin conjugated, were used at 1:50 dilution: anti-CD29, -CD73, -CD90, -CD133, -CD140, -CD146 (Becton Dickinson), -SSEA-4, vascular endothelial growth factor receptor (VEGFR) 1, 2 and 3, Tie2, Ve-Cadherin (R&D Systems), -CD44, EPCAM (BioLegend), -CD31, -CD105, SUSD2 (Miltenyi Biotec), -CD45 (AbD Serotec). Fluorescein isothiocyanate or phycoerythrin mouse nonimmune isotypic IgG (R&D Systems) are used as control at the same dilution. At each experimental point, 10.000 cells were analyzed on a FACScan (Becton Dickinson).

Real-Time PCR analysis

Gene expression was performed by quantitative real time reverse transcription-polymerase chain reaction (RT-PCR) with Applied Biosystems StepOne, as previously described (Bussolati *et al.*, 2012). Total RNA was extracted using the mirVana RNA isolation kit (Ambion) according to the manufacturer's protocol. RNA was then quantified with Nanodrop 2000 (Thermo Scientific). Gene expression analysis and quantitative real-time PCR (qRT-PCR) were performed as follows: first-strand cDNA was produced from 200 ng of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time PCR experiments were performed in 20 µl reaction mixture containing 5 ng of cDNA template, the sequence-specific oligonucleotide primers purchased from MWG-Biotech, and the Power SYBR Green PCR Master Mix (Applied Biosystems). Relative quantization of the products was performed using the 48-well StepOne Real-Time PCR System (Applied Biosystems). Thermal cycling conditions were as follows: activation of AmpliTaq Gold DNA Polymerase LD at 95°C for 10 minutes, followed by 45 cycles of amplification at 95°C for 15 seconds and 60°C for 1 minute, and a final incubation at

95°C for 15 seconds. TATA binding protein (TBP) messenger RNA was used to normalize RNA inputs. Sequence-specific oligonucleotide primers are listed in Supplementary Table I. Fold change was calculated respect to control for all samples using the comparative ΔΔCT method, following the formula provided by the software (StepOne 2.3, Applied Biosystems):

$$RQ = 2^{-[(\Delta Ctsample)-(\Delta Ctreference)]}, \text{ where : } \Delta Ct = Ct_{specific-primer} - Ct_{TBP}.$$

HUVEC culture and generation of GFP-positive cells

HUVECs isolated from umbilical vein vascular wall were seeded on fibronectin-coated plates and cultured in endothelial cell basal medium with an EGM-MV kit (Lonza; containing epidermal growth factor, hydrocortisone, bovine brain extract) and 10% fetal calf serum (FCS) in a incubator (37°C, 5% CO2 atmosphere). Cell confluence was monitored by phase-contrast microscopy. For GFP insertion a pGIPZ lentiviral vector (Open Biosystems) was used. The 293T cell line was transfected with the construct using the ViraPower Packaging Mix (Life Technologies) for lentiviruses production. After titering the lentiviral stock, HUVECs were transduced with lentiviral particles following the manufacturer's instructions. Cells were selected by Puromycin (Gibco) (1000 ng/ml) and antibiotic-resistant cells were expanded. Cell infection was evaluated by $GFP^+ >$ 98%, as assessed by FACS analysis.

Co-culture systems

Co-culture system was established in direct contact or by using transwells (1 μm pore, Falcon, Becton Dickinson) in T75 flasks or 6-well plates (Corning Incorporated, NY, USA) respectively. HUVECs and E-MSCs were seeded into the two compartments of the culture wells at a ratio of 1:1 ($75x10^3$/cell line). For direct co-culture, a mix of HUVEC-E-MSC suspension at a ratio of 1:1 (3×10^5 cells/line) was seeded in T75 flask and in EBM in a humidified incubator (5% CO2, 37°C) for 48 hours. HUVECs and E-E-s were also cultured alone and used as control.

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189 **Tubulogenic assay**

190 In vitro formation of capillary-like structures was studied on growth factor-reduced Matrigel (BD
191 Biosciences, Franklin Lakes, NJ, USA) in 24-well plates. Eutopic and ectopic E-MSCs were stained
192 with Dil (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate) (Life Technologies)
193 according to manufacturer's instructions, and plated 1:1 with HUVEC-GFP on growth factor–
194 reduced Matrigel (BD Biosciences) for i*n vitro* formation of capillary-like structures. DIL staining
195 was assessed by flow cytometry at 0, 24 and 48h (not shown). Cells (6×10^4 cells per well) were
196 mixed and seeded onto Matrigel-coated wells in endothelial cell basal medium plus VEGF 10
197 ng/ml. Cell organization onto Matrigel was imaged after 24-48 hours with a Nikon Eclipse Ti
198 inverted microscope using a Nikon Plan 10X/0.10 objective and cells were kept on incubator at
199 37°C and 5% CO2 during the experiment (OKOLab, Italy).

200 **Immunofluorescence**

201 Immunofluorescence was performed on chamber slides (Sigma) on which cells were fixed in 4%
202 paraformaldehyde containing 2% sucrose for 15 minutes at 4°C, permeabilized with 0.1% Triton X-
203 100 (Sigma) for 8 minutes at 4°C, and then incubated overnight at 4°C with the appropriate
204 antibodies. Anti-CD31 antibody (Biomeda, 1:200) was used. Primary antibody was detected using
205 anti-mouse secondary antibody conjugated with Texas Red (Molecular Probes, 1:5000). DAPI dye
206 (Sigma) was added for nuclear staining, and imaging was performed using anLSM5 Pascal confocal
207 microscope (Carl Zeiss International). Substitution with an unrelated rabbit serum or mouse IgG
208 served as negative control.

209 **Cell sorting**

210 Cells were stained with anti-CD31 antibody (Miltenyi Biotec) and sorted using a BD FACSAria III,
211 equipped with the BD FACSDiva software v. 7.0. At least 10.000 events per sample were acquired,

obtaining three populations from each co-colture (GFP^+ HUVECs, eutopic/ectopic $GFP^{neg}/CD31^+$ E-MSCs and eutopic/ectopic $GFP^{neg}/CD31^{neg}$ E-MSCs) and analyzed separately by Real-Time PCR.

Drugs and Reagents

Sunitinib malate (Sigma-Aldrich, St Louis, MO, USA), was resuspended in DMSO to a final concentration of 10 mM and stored at 4°C. Sorafenib (Bayer Pharmaceuticals, Leverkusen, Germany) was resuspended in DMSO to a stock concentration of 10 mM and stored at -20°C. Bevacizumab, 25 mg/ml (Genentech) was stored at 4°C. Cabergoline powder 10 mg (Sigma) was dissolved in 885 µl of DMSO to a stock concentration of 25 mM and stored at -20°C. Sunitinib and Sorafenib were diluted 1:100.000 in the culture medium (final concentration 0.1 µM), Bevacizumab was diluted 1:1000 (final concentration 25 µg/ml) and Cabergoline was diluted 1:1000 (final concentration 25 µM). All the drugs were administered for 24 hours during cell cultures.

Statistical analysis

Results were expressed as means ± SD and analysed with GraphPad Prism V5. Differences in gene expression among groups were investigated by analysis of variance using non-parametric analysis by Kruskal-Wallis test with Dunn's post test. Eutopic and ectopic cell lines of the same patient were compared using a Wilcoxon test where indicated. Significance was set at $p < 0.05$.

Results

Eutopic and ectopic E-MSC characterization

In order to study the angiogenic potential of the mesenchymal like population present in eutopic and ectopic endometrial tissue, we cultured stromal cells from eutopic and ectopic tissues derived from patients affected by ovarian endometriosis (n=9). The clinical characteristics of the patient population enrolled for this study are listed in Table I. The obtained cell lines were characterized for their fibroblastic phenotype, adherence to plastic and expression of mesenchymal markers (Table II and Figure 1). As shown by Figure 1, E-MSCs expressed CD44, CD73, CD105, CD29 and CD90. Eutopic and ectopic E-MSCs showed a similar expression of mesenchymal markers. PDGFRb and SUSD2, considered more specific markers for endometriotic mesenchymal stem cell (Gargett et al., 2016) were expressed by a large fraction of cells. These characteristics suggest that, as reported (Bianco *et al.,* 2013; Gargett et al., 2016), E-MSCs represent a heterogenic population of mesenchymal stem cells and stromal fibroblast, sharing a number of markers and functions. CD146, a marker of both mesenchymal and endothelial cells (Wang and Yan, 2013) resulted significantly higher in the ectopic cell line. As shown in Table II, both eutopic and ectopic E-MSCs did not express markers of endothelial/hemopoietic cells such as CD34 and CD45, CD31, VEGFR2, Tie2 and Ve-Cadherin. The presence of epithelial cell contamination was excluded by lack of the epithelial marker EPCAM in both cell lines. These data indicate that Ectopic E-MSCs isolated from ovarian endometrial tissue have a mesenchymal phenotype similar to that isolated from peritoneal endometriosis (Moggio *et al.,* 2012).

Endothelial potential of eutopic and ectopic E-MSCs

We subsequently evaluated the endothelial angiogenic ability of eutopic and ectopic E-MSCs by an endothelial *in vitro* differentiation. We first characterized E-MSCs for the expression of endothelial markers. At the basal level, the cells expressed minimal levels of CD31 (Figure 2 and Table II).

Endothelial cells (HUVECs) were used as positive control (Table II). For the *in vitro* differentiation, the cells were seeded on attachment factor coated dishes and cultured in ENDO-GRO media plus VEGF (10 ng/ml), previously reported to induce endothelial differentiation of mesenchymal cells (Brossa et al., 2015). After 14 days of differentiation, we observed the acquirement of CD31 expression (Figure 2 A), as previously reported (Masuda *et al.,* 2012), confirming that E-MSCs may differentiate into endothelial cells.

To mimic the pericyte-endothelial interaction in endometriosis, we analyzed the endothelial differentiative ability of both eutopic and ectopic E-MSCs in a co-culture model with endothelial cells. Two different types of co-culture were prepared: an indirect stimulation, where HUVEC cells were plated on a trans-well, which does not allow a direct contact with E-MSCs (cell ratio of 1:1) or a direct co-plating of HUVECs and E-MSCs (cell ratio of 1:1). The HUVEC cells were marked by GFP expression obtained with a stable infection with lentiviral vector, (>98% expression in all experiments). In the indirect setting, the presence of HUVECs did not affect the expression of the endothelial marker CD31 in both eutopic and ectopic E-MSCs up to 7 days co-culture (Fig. 2 B). At variance, the direct co-culture of E-MSCs and HUVECs induced the presence of a population acquiring high CD31 expression by GFP negative E-MSCs, as observed by FACS analysis using a selective gating strategy and by immunofluorescence images (Figure 2 B and C and Figure 3 A). This effect was observed as early as 48 hours. No further increase was observed at longer co-culture times (4 and 7 days, not shown). Furthermore, in order to analyze the functional potential of E-MSC-HUVEC interaction, we set up a tube-forming assay onto Matrigel. As shown by Figure 3 B, E-MSCs could not organize in elongates tubular-like structures as HUVEC cells. When E-MSCs were plated together with HUVECs onto Matrigel, both cells contributed to the formation of tube-like structures (Figure 3 B). These data indicate that the direct contact between E-MSCs and HUVECs may influence the differentiating potential of E-MSCs into endothelial cells and their functional involvement.

Isolation and analysis of the CD31$^+$ E-MSC population

In order to analyze the nature of the E-MSC population expressing CD31 after HUVEC co-culture, we isolated GFP^{neg}/CD31$^+$ cells using a cell-sorter and we analyzed their gene expression compared to GFP$^+$ HUVECs (positive control), to the basal cells (not in co-culture) and to GFP^{neg}/CD31^{neg} E-MSCs after co-culture (Figure 4 A and B and Supplementary Fig. 1). Real Time PCR confirmed the increase in the expression of CD31 mRNA in the sorted CD31$^+$ E-MSCs compared to the basal and to CD31^{neg} E-MSCs after co-culture ($p<0.05$). In parallel, we observed a significant increase in VEGFR2 and Ve-Cadherin expression in both eutopic and ectopic CD31$^+$ E-MSC lines. Differently, Tie-2 expression was increased only in the ectopic CD31$^+$ E-MSCs in respect to basal and to CD31^{neg} cells. No significant differences were observed in the expression of VEGF in the different cell fractions (Figure 4 B). Moreover, the expression of the mesenchymal genes c-Myc and Vimentin, also expressed by HUVEC cells, was unchanged (Supplementary Figure 1). N-Cadherin and SUSD2, E-MSC markers, were expressed at higher levels in E-MSCs than in HUVECs, but they did not show significant variations comparing CD31$^+$ and CD31^{neg} cells (Supplementary Figure 1). Comparing the expression levels of endothelial markers in the GFP^{neg}/CD31$^+$ cells from eutopic and ectopic E-MSCs, whereas Tie2 was significantly increased in the ectopic cell line ($p<0.05$) whereas no difference was observed for CD31, VEGFR2 and Ve-Cadherin. Altogether these data reveal that a subpopulation of E-MSC may acquire an endothelial-like gene expression, implying a progressive endothelial differentiation.

Effect of Cabergoline treatment on the endothelial potential of E-MSCs

In order to evaluate the efficacy of anti-angiogenic drugs in limiting the endothelial differentiation of E-MSCs during the direct co-culture with HUVECs we tested the effect of Sorafenib, Sunitinib, Bevacizumab treatment, used at not toxic concentrations as previously described (Fiorio Pla et al., 2014; Brossa et al., 2015). No significant reduction in the percentage of CD31 expressing E-MSCs as evaluated by FACS analysis after Sorafenib, Sunitinib or Bevacizumab treatment was obtained

304 (data not shown). We subsequently focused on Cabergoline, a dopamine receptor2 agonist also
305 shown to impact neo-angiogenesis and endometrial lesions (Novella-Maestre *et al*., 2009, 2010).
306 Cabergoline was administered after 24 hour co-culture. We observed that the increase in CD31
307 expression obtained after co-culture was significantly reduced by 24 hour treatment with
308 Cabergoline 25 μM in both eutopic and ectopic cell line (Figure 5 A and B). Differently,
309 Cabergoline did not affect the incorporation of E-MSCs in HUVEC tubular structures (Figure 5 C).
310 It could be speculated that VEGF signalling is only partly involved in the early endothelial
311 differentiation observed and that this is largely dependent on endothelial-stromal contact.

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Discussion

In the present study we focused our attention on the angiogenic potential of E-MSCs isolated from eutopic and ectopic endometrial tissue. We found that endothelial co-culture promoted the acquirement of endothelial markers and function, and that this required a direct cell-to-cell contact. In addition, Cabergoline treatment partly inhibited this process.

E-MSCs are mesenchymal like cells localized in the perivascular space of endometrial small vessels (Schwab and Gargett, 2007), and possibly involved in support of angiogenesis and endothelial integrity. We also previously found that stromal cells isolated from ectopic endometrial lesions expressed higher levels of VEGF and HIF in respect to those isolated from eutopic endometrial tissue which may in turn promote angiogenesis (Moggio *et al.,* 2012). This result was confirmed in the present study using ectopic E-MSCs from ovarian endometrial tissue. E-MSCs were previously shown to differentiate into endothelial cells when cultured within endothelial growth factors (Oswald et al., 2004; Masuda *et al*., 2012). We also found in the present study the acquisition of CD31 by a fraction of the cells after 14 days of culture with endothelial differentiating medium. Moreover, it was recently shown that E-MSCs are able to acquire endothelial markers (CD31, Ve-cadherin and KDR) when plated onto nanofibrous scaffold in the presence of angiogenic factors, suggesting their potential angiogenic property in selected culture conditions (Shamosi *et al.,* 2016).

To understand if the interaction with endothelium could cause an activation of the differentiative program of E-MSCs, we co-cultured them with HUVECs. As proved by both FACS and immunofluorescence images, we demonstrated that E-MSCs could acquire an endothelial phenotype as soon as after 48 hours and participate into vessel organization *in vitro* together with endothelial cells. Eutopic and ectopic E-MSCs showed a similar behaviour and acquisition of endothelial markers. However, the ectopic E-MSCs specifically increased Tie2, the angiopoietin-2 receptor that could possibly represent a specific ectopic E-MSC marker. The identification of a subpopulation, around 10% of cells, acquiring high levels of CD31 and expressing endothelial

markers could be dependent on the heterogeneity of E-MSCs, possibly comprising both mesenchymal stem cells and stromal fibroblasts. The basal characterization and the expression of SUSD2 indicated a fraction of mesenchymal stem cell population larger than 10% in our culture. Indeed, these populations represent a continuum, as the mesenchymal stem population may spontaneously differentiate into fibroblasts, and they share several functions (Barragan *et al*., 2016). The absence of a pure mesenchymal stem population could limit the entity of the endothelial differentiation.

Of interest, this rapid acquirement of the endothelial phenotype appears to be due to cell-to-cell interactions, possibly involving adhesion related mechanisms, rather than on growth factor release, as no differentiation was observed with an indirect co-culture. In addition, the use of a VEGF specific inhibitor was unable to reduce the acquisition of endothelial markers. These data suggest that E-MSCs might be involved in the vessels formation, by an interaction with endothelial cells. Further studies are required to identify the contact-activated pathways involved. These evidences confirm the direct functional contribution of E-MSCs to the endothelial microenvironment and suggest that these cells may play a role in the pathogenesis of gynaecological diseases, such as endometriosis and adenomyosis, due to inappropriate shedding of stem cells or alterations in the stem cell niche (Gargett, 2006). It is also conceivable that a fraction of E-MSCs could contribute to angiogenesis and vessel formation in their pericyte/perivascular role in ectopic vessels in addition to their ability to differentiate into endothelial cells. However, more than 80% of blood vessels observed in endometriosis implants are pericyte-free (Hull *et al.,* 2003).

The angiogenesis is a fundamental process for the physiological growth of the endometrium, as well in the establishment of endometriosis (Donnez *et al.,* 1998; McLaren *et al.,* 2000; Laschke *et al*., 2007; Du and Taylor, 2007). Indeed different studies suggest the therapeutic efficacy of anti-angiogenic therapy targeting VEGF for endometriosis eradication (Hull *et al.*, 2003; Nap *et al.*, 2004; Taylor *et al.*, 2009; Pittatore *et al.*, 2014). However, anti-angiogenic drugs currently available

on the market are quite expensive and are mainly used in oncology, being able to cause relevant undesired effects. Differently, the dopamine agonist Cabergoline, which has been used for several years to block lactation or treat hyper-prolactinemia, appears as an easier and more practical option. The mechanism responsible for the anti-angiogenic effect of Cabergoline is still unclear, possibly relaying on its ability to block the VEGF-VEGFR2 interaction (Novella-Maestre *et al*., 2009). In our study, Cabergoline but not other anti-VEGF drugs impaired, in part, endothelial differentiation. However, it did not affect the ability of E-MSCs to organize into tubular structures. These data support a prominent role for stromal-endothelial interactions rather than for VEGF-mediated signals in the early endothelial differentiation of E-MSCs (at 48 hours) and suggest a VEGF-independent effect of Cabergoline. At variance, VEGF could be important as a later signal, as VEGF addition to the culture medium promoted endothelial differentiation after 14 days. Therefore, strategies aimed to inhibit endometriosis should be considered in the context of the different cell types present in the ectopic microenvironment.

In conclusion, our data suggest that E-MSCs could be driven by the surrounding endothelial cells to differentiate and to take part to the formation of endothelium and new blood vessels. Moreover, we propose a simple cell culture system that closely mimics the cellular complexity typical of in vivo endometriotic tissues. This system might be useful to test novel strategies for treatment.

Authors' Roles

The study was design by CS, MA and BB. CS, MA and BA performed the experiments and were responsible for the acquisition and analysis of the data. PG and LS enrolled the patients and MGL was involved in the surgical treatment. MA, CS, BB, BC and RA contributed to the final interpretation of the data, the drafting of the manuscript and gave their final approval. [§] CS and MA equally contributed to the manuscript.

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534 **Figure legends**

535 **Figure 1. Expression of mesenchymal and hematopoietic markers by eutopic and ectopic E-**
536 **MSCs.** Representative FACS analysis of eutopic and ectopic E-MSCs are shown. The filled area
537 shows binding of the specific antibody, and the grey line shows the isotypic control. All ectopic and
538 eutopic E-MSC lines, obtained from each patient (n=9), were characterized and showed similar
539 marker expression.

540 **Figure 2. Characterization of CD31 acquisition after endothelial differentiating conditions.**
541 Eutopic and ectopic E-MSCs were cultured for 14 days in *in vitro* endothelial differentiation
542 medium (A), or were cultured with HUVEC cells in indirect culture setting (Indirect), or in direct
543 cell-to-cell contact (Co-culture) (B). **A**. Representative FACS analysis showing the acquirement of
544 CD31 expression (red filled area) by eutopic and ectopic E-MSCs after 14 days incubation in
545 endothelial differentiation medium. The black line is the isotypic control. **B**. Percentage of CD31
546 expressing cells by E-MSCs in indirect culture with HUVECs and of GFP^{neg} E-MSCs in co-culture
547 conditions with HUVECs. Data are the mean ± SD of four independent experiments. *=$p<0.05$;
548 **=$p<0.001$ vs Basal. **C.** Upper panels are the representative two-colour flow cytometry traces of
549 CD31 and GFP expression by ectopic E-MSCs, (red, 99.8% $GFP^{neg}/CD31^{neg}$) and HUVECs (green,
550 98.7% $GFP^{+}/CD31^{+}$). Lower panels show the gating strategy of cells in co-culture: gates were
551 performed on the GFP^{neg} cell population (red) and GFP^{+} population (green), and the expression of
552 CD31 was evaluated in the GFP^{neg} gate only to avoid contamination of the $GFP^{+/}CD31^{+}$ cell
553 population.

554 **Figure 3. Endothelial differentiation and tubular-like organization of E-MSCs after co-culture**
555 **with HUVECs.** A. Representative immunofluorescence images showing CD31 expression (red) by
556 GFP+ HUVEC cells and GFP negative E-MSCs 48 hour after direct co-culture. In merged pictures
557 $GFP^{neg}/CD31^{+}$cells are indicated by yellow arrows. B. Representative micrographs of the tubular-
558 like networks formed by ectopic E-MSCs (red), HUVEC cells (green) or co-cultured cells. When

559 co-cultured with HUVECs, E-MSCs functionally organized within the elongated structures formed
560 by HUVECs. Nuclei were stained with Hoechst dye 33258. Three experiments were performed with
561 similar results. Original magnification: A. x400, B: x200.

562 **Figure 4. Isolation and characterization of endothelial differentiated $CD31^+$ E-MSCs.** (A)
563 Representative dot plots showing flow cytometric analysis of direct co-culture experiments in which
564 three cell populations were sorted: $GFP^+/CD31^+$ HUVECs, $GFP^{neg}/CD31^+$E-MSCs and
565 $GFP^{neg}/CD31^{neg}$ E-MSCs. (B) Quantitative RT-PCR analysis showing the expression of endothelial
566 markers CD31, VEGFR2, Tie2, Ve-Cadherin and VEGF in E-MSCs (basal), and in sorted GFP
567 $^{neg}/CD31^+$ and GFP $^{neg}/CD31^{neg}$ E-MSCs after direct co-culture with HUVECs. HUVECs were used
568 as positive control. Data were normalized to TBP mRNA and to 1 for HUVECs and expressed as
569 relative quantification (RQ). Data are mean ± SD of four different cell lines. *$p<0.05$ Basal vs
570 $CD31^+$; § $p<0.05$ $CD31^+$ vs $CD31^-$.

571 **Figure 5. Effect of Cabergoline treatment on endothelial differention of E-MSCs in co-culture**
572 **with HUVECs.** Cabergoline was added to the 48 hour co-culture experiments of E-MSCs and
573 HUVECs. **A and B**: Representative dot plots and quantification of the $CD31^+$ cells analyzed by
574 cytofluorimetric analysis in the GFP^+ HUVECs and GFP^{neg} E-MSCs. Data are mean ± SD of 4
575 different cell lines. *$p<0.05$ Basal vs Ctr; § $p<0.05$ Ctr vs Cabergoline. C. Representative
576 micrographs of the tubular-like networks formed by co-cultured ectopic E-MSCs (red) and
577 HUVECs (green) in the absence or presence of Cabergoline. No difference was observed. Nuclei
578 were stained with Hoechst dye 33258. Three experiments were performed with similar results.
579 Original magnification: x200.

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581 **Table I. Patients' clinical characteristics.**

Patient #	Samples	Age (years)	Previous pregnancies	Average menstrual cycle length (days)	Other diseases
1	Eutopic/Ectopic	34	No	28	No
2	Eutopic/Ectopic	28	No	28-30	No
3	Eutopic/Ectopic	38	1 miscarriage at 8 weeks gestational age	27	No
4	Eutopic/Ectopic	40	1 live birth	28-30	No
5	Eutopic/Ectopic	25	No	25	No
6	Eutopic/Ectopic	32	1 miscarriage at 7 weeks gestational age	28-30	No
7	Eutopic/Ectopic	34	No	28	No
8	Eutopic/Ectopic	29	No	28-30	No
9	Eutopic/Ectopic	41	No	28	No

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584 **Table II.** Cytofluorimetric evaluation of surface marker expression by eutopic and ectopic E-

585 MSCs and HUVECs.

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	Eutopic E-MSC	Ectopic E- MSC	HUVEC
CD44	97.0 ± 1.9 %	97.4 ± 2.0 %	72.4± 2.3 %
CD73	96.8 ± 3.2 %	94.9 ± 4.3 %	97.0± 0.4 %
CD105	77.8 ± 20.5 %	87.3 ± 10.3 %	95.7± 0.7 %
CD146	2.6 ± 3.3 %	23.0 ± 6.3 %*	98.5± 1.3 %
CD29	99.0 ± 1.0 %	97.3 ± 2.0 %	99.7± 0.3 %
CD90	68.2 ± 21.0 %	86.7 ± 16.7 %	0.6 ± 0.1 %
CD34	1.1 ± 0.8 %	2.7 ± 1.0 %	0.7± 0.2 %
CD45	0.4 ± 0.3 %	0.5 ± 0.2 %	0.2± 0.1 %
CD31	0.2 ± 0.1 %	0.5 ± 0.3 %	98.6 ± 1.0 %
VEGFR2	1.7 ± 0.5 %	0.8 ± 0.5 %	80.6 ± 9.6 %
Tie2	0.9 ± 0.2 %	1.3 ± 0.3 %	9.8 ± 0.1 %
Ve-Cadherin	0.2± 0.2 %	1.0± 0.3 %	86.9± 2.3 %
SUSD2	56.1± 7.3 %	43.3±9.7 %	1.7± 0.2 %
PDGFRb	71.1± 6.9 %	52.8±13.0 %	1.6± 2.3 %
EPCAM	0.2± 0.1 %	0.5± 0.2 %	0.8± 0.2 %

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589 Quantitative expression of mesenchymal, hematopoietic and endothelial markers assessed by FACS
590 analysis. Values represent the percentage of positive cells and are expressed as mean ± SD of all
591 nine lines in study for eutopic and ectopic E-MSCs. Three cell lines of HUVECs were tested as
592 control. *=$p<0.001$ ectopic vs eutopic E-MSCs.