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

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

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

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24 **Abstract**

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

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

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

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

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

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

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

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

54 **Study funding and competing interest(s):** All authors declare that their participation in the study
55 did not involve actual or potential conflicts of interests.

56 **Large scale data:** Not applicable

57

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

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69 **Introduction**

70 Endometriosis is a chronic and oestrogen-dependent disease characterized by the presence of
71 ectopic endometrial tissue, composed by glands and stroma, outside the uterine cavity (Giudice,
72 2010; Bulun, 2009). A number of studies supported the presence of rare clonogenic epithelial and
73 stromal cells with stem cell characteristics in the human endometrium (Chan *et al.*, 2004; Chan and
74 Gargett, 2006; Gargett, 2006; Gargett *et al.*, 2009), thought to be physiologically involved in the
75 cyclic endometrial regeneration after menstruation (Schwab *et al.*, 2005; Masuda *et al.*, 2010;
76 Gargett *et al.*, 2016). Endometrial mesenchymal stromal cells (E-MSCs), in particular, are
77 clonogenic mesenchymal like cells (Gargett *et al.*, 2009) expressing pericyte markers (Berger *et al.*,
78 2005; Spitzer *et al.*, 2012), and localized in the perivascular space of endometrial small vessels
79 (Schwab and Gargett, 2007).

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

94 1993; Maas *et al.*, 2001; Laschke and Menger, 2007). The interaction of stromal and endothelial
95 cells within endometrial angiogenesis has not been investigated yet.

96 Vascular endothelial growth factor (VEGF) appears to be the main regulator of endometrial
97 angiogenesis. Indeed, VEGF polymorphisms seem to be associated to the risk of endometriosis (Li
98 *et al.*, 2013). In addition, increased levels of VEGF were found in the peritoneal fluid of women
99 with endometriosis and in ectopic endometrial tissue, suggesting the relevance of a pro-angiogenic
100 microenvironment in the development of the endometriotic implant (Donnez *et al.*, 1998; McLaren,
101 2000; Bourlev *et al.*, 2006). The use of anti-angiogenic drugs therefore appears as a possible
102 therapeutic strategy against endometriosis (Hull *et al.*, 2003; Taylor *et al.*, 2009; Pittatore *et al.*,
103 2014). VEGF targeting using VEGF neutralizing antibodies or tyrosine kinase inhibitors effectively
104 reduced growth of endometriotic implants, microvessel density and VEGF expression in models of
105 endometriosis in mice, rats and monkeys (Park *et al.*, 2004; Van Langendonck *et al.*, 2008; Ozer *et al.*,
106 2013). Similarly, interfering with VEGF-VEGFR-2 signalling using a dopamine agonist
107 displayed an anti-angiogenic effects in experimental endometriosis. Furthermore, we previously
108 demonstrated that the tyrosine kinase inhibitor Sorafenib affected the angiogenic potential of
109 ectopic E-MSCs *in vitro* and reverted their increased VEGF release (Moggio *et al.*, 2012).

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

116

117 **Materials and methods**

118 **Patients**

119 The cell lines were obtained from nine patients receiving surgery for treatment of ovarian
120 endometriosis in the Department of Surgical Sciences, University of Torino, between November
121 2013 and April 2015 after approval by the Ethics Review Board. Preoperative informed consent
122 was obtained from each patient.

123 **E-MSC isolation and culture**

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

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140 **Flow cytometric analysis**

141 Cytometric analysis was performed using FACScan (Becton Dickinson) as previously described
142 (Bruno *et al.*, 2009). The cells suspensions were incubated with antibodies for 25 minutes at 4°C in
143 100 µl of phosphate-buffered saline with addition of 0.1% bovine serum albumin (Sigma). The
144 following monoclonal antibodies, all fluorescein isothiocyanate or phycoerythrin conjugated, were
145 used at 1:50 dilution: anti-CD29, -CD73, -CD90, -CD133, -CD140, -CD146 (Becton Dickinson), -
146 SSEA-4, vascular endothelial growth factor receptor (VEGFR) 1, 2 and 3, Tie2, Ve-Cadherin (R&D
147 Systems), -CD44, EPCAM (BioLegend), -CD31, -CD105, SUSD2 (Miltenyi Biotec), -CD45 (AbD
148 Serotec). Fluorescein isothiocyanate or phycoerythrin mouse nonimmune isotypic IgG (R&D
149 Systems) are used as control at the same dilution. At each experimental point, 10.000 cells were
150 analyzed on a FACScan (Becton Dickinson).

151 **Real-Time PCR analysis**

152 Gene expression was performed by quantitative real time reverse transcription-polymerase chain
153 reaction (RT-PCR) with Applied Biosystems StepOne, as previously described (Bussolati *et al.*,
154 2012). Total RNA was extracted using the mirVana RNA isolation kit (Ambion) according to the
155 manufacturer's protocol. RNA was then quantified with Nanodrop 2000 (Thermo Scientific). Gene
156 expression analysis and quantitative real-time PCR (qRT-PCR) were performed as follows: first-
157 strand cDNA was produced from 200 ng of total RNA using the High Capacity cDNA Reverse
158 Transcription Kit (Applied Biosystems, Foster City, CA). Real-time PCR experiments were
159 performed in 20 µl reaction mixture containing 5 ng of cDNA template, the sequence-specific
160 oligonucleotide primers purchased from MWG-Biotech, and the Power SYBR Green PCR Master
161 Mix (Applied Biosystems). Relative quantization of the products was performed using the 48-well
162 StepOne Real-Time PCR System (Applied Biosystems). Thermal cycling conditions were as
163 follows: activation of AmpliTaq Gold DNA Polymerase LD at 95°C for 10 minutes, followed by 45
164 cycles of amplification at 95°C for 15 seconds and 60°C for 1 minute, and a final incubation at

165 95°C for 15 seconds. TATA binding protein (TBP) messenger RNA was used to normalize RNA
 166 inputs. Sequence-specific oligonucleotide primers are listed in Supplementary Table I. Fold change
 167 was calculated respect to control for all samples using the comparative $\Delta\Delta\text{CT}$ method, following the
 168 formula provided by the software (StepOne 2.3, Applied Biosystems):

$$\text{RQ} = 2^{-[(\Delta\text{Ct}_{\text{sample}}) - (\Delta\text{Ct}_{\text{reference}})]}, \text{ where : } \Delta\text{Ct} \\ = \text{Ct}_{\text{specific-primer}} - \text{Ct}_{\text{TBP}}.$$

169

170 HUVEC culture and generation of GFP-positive cells

171 HUVECs isolated from umbilical vein vascular wall were seeded on fibronectin-coated plates and
 172 cultured in endothelial cell basal medium with an EGM-MV kit (Lonza; containing epidermal
 173 growth factor, hydrocortisone, bovine brain extract) and 10% fetal calf serum (FCS) in a incubator
 174 (37°C, 5% CO₂ atmosphere). Cell confluence was monitored by phase-contrast microscopy. For
 175 GFP insertion a pGIPZ lentiviral vector (Open Biosystems) was used. The 293T cell line was
 176 transfected with the construct using the ViraPower Packaging Mix (Life Technologies) for
 177 lentiviruses production. After titering the lentiviral stock, HUVECs were transduced with lentiviral
 178 particles following the manufacturer's instructions. Cells were selected by Puromycin (Gibco)
 179 (1000 ng/ml) and antibiotic-resistant cells were expanded. Cell infection was evaluated by GFP⁺ >
 180 98%, as assessed by FACS analysis.

181 Co-culture systems

182 Co-culture system was established in direct contact or by using transwells (1 μm pore, Falcon,
 183 Becton Dickinson) in T75 flasks or 6-well plates (Corning Incorporated, NY, USA) respectively.
 184 HUVECs and E-MSCs were seeded into the two compartments of the culture wells at a ratio of 1:1
 185 (75×10^3 /cell line). For direct co-culture, a mix of HUVEC-E-MSC suspension at a ratio of 1:1
 186 (3×10^5 cells/line) was seeded in T75 flask and in EBM in a humidified incubator (5% CO₂, 37°C)
 187 for 48 hours. HUVECs and E-E-s were also cultured alone and used as control.

188

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 *in 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,

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

215 **Drugs and Reagents**

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

224 **Statistical analysis**

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

229

230 **Results**

231 **Eutopic and ectopic E-MSC characterization**

232 In order to study the angiogenic potential of the mesenchymal like population present in eutopic and
233 ectopic endometrial tissue, we cultured stromal cells from eutopic and ectopic tissues derived from
234 patients affected by ovarian endometriosis (n=9). The clinical characteristics of the patient
235 population enrolled for this study are listed in Table I. The obtained cell lines were characterized for
236 their fibroblastic phenotype, adherence to plastic and expression of mesenchymal markers (Table II
237 and Figure 1). As shown by Figure 1, E-MSCs expressed CD44, CD73, CD105, CD29 and CD90.
238 Eutopic and ectopic E-MSCs showed a similar expression of mesenchymal markers. PDGFRb and
239 SUSD2, considered more specific markers for endometriotic mesenchymal stem cell (Gargett et al.,
240 2016) were expressed by a large fraction of cells. These characteristics suggest that, as reported
241 (Bianco *et al.*, 2013; Gargett et al., 2016), E-MSCs represent a heterogenic population of
242 mesenchymal stem cells and stromal fibroblast, sharing a number of markers and functions. CD146,
243 a marker of both mesenchymal and endothelial cells (Wang and Yan, 2013) resulted significantly
244 higher in the ectopic cell line. As shown in Table II, both eutopic and ectopic E-MSCs did not
245 express markers of endothelial/hemopoietic cells such as CD34 and CD45, CD31, VEGFR2, Tie2
246 and Ve-Cadherin. The presence of epithelial cell contamination was excluded by lack of the
247 epithelial marker EPCAM in both cell lines. These data indicate that Ectopic E-MSCs isolated from
248 ovarian endometrial tissue have a mesenchymal phenotype similar to that isolated from peritoneal
249 endometriosis (Moggio *et al.*, 2012).

250 **Endothelial potential of eutopic and ectopic E-MSCs**

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

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

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

279 **Isolation and analysis of the CD31⁺ E-MSC population**

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

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

299 In order to evaluate the efficacy of anti-angiogenic drugs in limiting the endothelial differentiation
300 of E-MSCs during the direct co-culture with HUVECs we tested the effect of Sorafenib, Sunitinib,
301 Bevacizumab treatment, used at not toxic concentrations as previously described (Fiorio Pla et al.,
302 2014; Brossa et al., 2015). No significant reduction in the percentage of CD31 expressing E-MSCs
303 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 μ M 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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324 **Discussion**

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

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

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

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

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

369 The angiogenesis is a fundamental process for the physiological growth of the endometrium, as well
370 in the establishment of endometriosis (Donnez *et al.*, 1998; McLaren *et al.*, 2000; Laschke *et al.*,
371 2007; Du and Taylor, 2007). Indeed different studies suggest the therapeutic efficacy of anti-
372 angiogenic therapy targeting VEGF for endometriosis eradication (Hull *et al.*, 2003; Nap *et al.*,
373 2004; Taylor *et al.*, 2009; Pittatore *et al.*, 2014). However, anti-angiogenic drugs currently available

374 on the market are quite expensive and are mainly used in oncology, being able to cause relevant
375 undesired effects. Differently, the dopamine agonist Cabergoline, which has been used for several
376 years to block lactation or treat hyper-prolactinemia, appears as an easier and more practical
377 option. The mechanism responsible for the anti-angiogenic effect of Cabergoline is still unclear,
378 possibly relaying on its ability to block the VEGF-VEGFR2 interaction (Novella-Maestre *et al.*,
379 2009). In our study, Cabergoline but not other anti-VEGF drugs impaired, in part, endothelial
380 differentiation. However, it did not affect the ability of E-MSCs to organize into tubular structures.
381 These data support a prominent role for stromal-endothelial interactions rather than for VEGF-
382 mediated signals in the early endothelial differentiation of E-MSCs (at 48 hours) and suggest a
383 VEGF-independent effect of Cabergoline. At variance, VEGF could be important as a later signal,
384 as VEGF addition to the culture medium promoted endothelial differentiation after 14 days.
385 Therefore, strategies aimed to inhibit endometriosis should be considered in the context of the
386 different cell types present in the ectopic microenvironment.

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

391

392 **Authors' Roles**

393 The study was design by CS, MA and BB. CS, MA and BA performed the experiments and were
394 responsible for the acquisition and analysis of the data. PG and LS enrolled the patients and MGL
395 was involved in the surgical treatment. MA, CS, BB, BC and RA contributed to the final
396 interpretation of the data, the drafting of the manuscript and gave their final approval. § CS and MA
397 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 \pm 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 \pm 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 differentiation 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 \pm 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.

580

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.