Influence of the ectopic location on the antigen expression and functional characteristics of endometrioma stromal cells

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ABSTRACT

Research question

Endometriosis has been defined as a progesterone (P4)-resistance disease. Comparative studies of the ectopic and eutopic endometrium of patients have revealed differentially expressed genes that may be involved in the pathogenesis of endometriosis; are the alterations observed in the endometriotic cells already present in the eutopic endometrium or are acquired in the ectopic location?

Design

The response to decidualization with P4 and cAMP for up to 28 days was compared in different endometrial stromal cell (EnSC) lines established from samples of endometriomas (eEnSC), eutopic endometrium from women with endometriosis (eBEnSC), endometrial tissue from healthy women (BEnSC), and menstrual blood from healthy donors (mEnSC).

Results

Usual features of decidualized cells, such as the change in cell morphology and the expression of prolactin, were similarly observed in the three types of eutopic EnSCs studied, but not in the ectopic cells upon decidualization. Among the phenotypic markers analyzed, CD105 was down-regulated under decidualization in all cell types (mEnSC: $P = 0.005$, BEnSC: $P = 0.029$, eBEnSC: $P = 0.022$), except in eEnSCs. mEnSCs and BEnSCs underwent apoptosis during decidualization, whereas eBEnSCs and eEnSCs were resistant to the induction of cell death. Lastly, migration studies revealed that mEnSCs secreted undetermined factors during decidualization that inhibited cell motility, whereas eEnSCs showed a significantly lower ability to produce those migration regulating factors ($P < 0.0001$, $P < 0.001$ and $P = 0.0013$ for migration of mEnSC at 24, 48 and 72 h, respectively; $P < 0.0001$ for migration of eEnSC at all times studied).

Conclusions

This study provides novel insights into the differences between endometriotic and eutopic endometrial cells and reinforce the idea that the microenvironment in the ectopic location plays additional roles in the acquisition of the alterations that characterize the cells of the endometriotic foci.

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KEYWORDS: endometriosis, endometrial stromal cells, decidualization.

INTRODUCTION

Endometriosis is a common gynecologic disease characterized by the presence of endometrial glands and stroma outside the uterine cavity. It can affect the ovaries, pelvic organs, peritoneal cavity and, less frequently, distant organs such as lungs or pleura (Zondervan et al., 2018). Although endometriosis is still an enigmatic disease, several theories have been proposed to explain its etiology. The most widely accepted is the theory of retrograde menstruation, which states that menstruation ascends through the fallopian tubes and flows into the peritoneal cavity, favoring adhesion, invasion and growth of the endometrial fragments deposited there (Zondervan et al., 2018). However, the cases in which endometrial tissue appears in distant locations would not be attributable to this retrograde menstruation hypothesis. In addition, retrograde menstruation is frequent in healthy women in which endometriosis does not appear. Recently, it has been proposed the stem cell theory which could explain peritoneal as well as extraperitoneal endometriosis lesions (Taylor, 2020). This theory asserts that circulating stem cells (mesenchymal stem/stromal cells -MSC-) from bone marrow and MSC-related precursors from the endometrium basal layer could undergo a "erroneous homing" to different organs, even in remote locations, where they would differentiate into endometrial tissue (Maruyama and Yoshimura, 2012; Taylor, 2020).
During the secretory phase of the menstrual cycle and, especially, if pregnancy occurs, the endometrial stromal cells (EnSCs) located around the spiral arteries in the human endometrium undergo a process of differentiation, called decidualization, in response to progesterone (P4) and other ovarian hormones. In this reaction, EnSCs increase in size, change their fibroblastic shape to a rounder morphology and produce distinctive factors such as prolactin (PRL), insulin-like growth factor binding protein-1 (IGFBP-1) and IL-15 (Bergeron, 2000; Dunn et al., 2003; Richards et al., 1995). Interestingly, endometriosis has been defined as an estrogen-dependent and P4-resistant process, so that stromal cells in endometriosis foci (eEnSCs) show an antigen phenotype equivalent to that of EnSCs but do not completely decidualize (Bulun et al., 2006)
There is opposing literature regarding whether P4 resistance in endometriosis originates in the eutopic or the ectopic tissue (McKinnon et al., 2018). The eutopic endometrium of women with endometriosis have demonstrated an altered response to P4 (Osteen et al., 2005) and numerous

genes known to be targets of P4 have been found dysregulated in the secretory endometrium from women with disease (Burney et al., 2007). However, several studies have also identified a large number of differentially expressed genes and microRNAs (miRNAs) between the ectopic and eutopic endometrium of women with endometriosis, which may regulate molecular pathways influencing the pathogenesis of endometriosis (Eyster et al., 2007; Ohlsson Teague et al., 2009; Teague et al., 2010).

Therefore, it is currently unknown whether the alterations detected in the eEnSCs are already present in the eutopic endometrium or are acquired in the ectopic location. Here, we compared the antigenic phenotype and functional characteristics in response to decidualization of EnSCs from ectopic (eEnSCs) and eutopic (eBEnSCs) endometrial tissue of patients with endometriosis. In addition, eEnSCs were compared with EnSC of healthy women obtained from two different sources: menstrual blood (mEnSC), as it is the best suited source of normal endometrial cells to compare to the chocolate-cyst (endometrioma), and endometrial biopsy (BEnSC) as the counterpart of the eutopic tissue of patients. The change in cell morphology, the production of prolactin, the induction of apoptosis and the antigen profile were analyzed in decidualization conditions. Moreover, the effect of decidualization on the migration abilities of eEnSCs and mEnSCs was investigated.

MATERIALS AND METHODS

Samples

Samples from patients with endometriosis were obtained by laparoscopic surgery at the Hospital Quirón Ruber Juan Bravo and Hospital La Zarzuela in Madrid. Endometrial stromal cells were isolated from 15 samples of eutopic and 30 samples of ectopic endometrium of patients of childbearing age. The endometriotic cells were isolated from the chocolate content of the endometriotic cysts. In all cases, the diagnosis of endometriosis was made intraoperatively and later confirmed by histological study of the sample. In accordance with the revised American

Society for Reproductive Medicine classification, all patients had moderate to severe endometriosis (stage III-IV disease).

Endometrial samples from 12 non-endometriosis patients were obtained using a hysteroscopic approach in women under the age of 40 who underwent the intervention for different reasons, excluding those in which there was suspicion of a malignant neoplastic process. All samples were obtained at the Hospital Quirón Ruber Juan Bravo and Hospital La Zarzuela in Madrid. mEnSC lines were obtained from 30 menstrual blood samples collected with menstrual cups or tampons and donated by healthy women aged 20 to 35 years. Those who were using any medication or with infectious, autoimmune, or other systemic or local disease were excluded.

All donors provided a written informed consent. The Research and Ethics Committee of University of Granada approved the study (reference number 186/CEIH/2016, approval date 4 July 2016).

Isolation and culture of EnSC lines

For eEnSC and mEnSC lines, the procedure started with the dilution and washing in phosphate buffer saline (PBS) of chocolate content and menstrual blood, respectively, followed by centrifugation on a Ficoll-Paque (Sigma-Aldrich, St. Louis, MO, USA) density gradient at 600 *g* for 20 min. Cells were collected from the interface, washed in PBS, and incubated in culture flasks for 24 h at 37ºC with 5% CO_2 in OptiMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 3% (v/v) fetal calf serum (FCS) (Thermo Fisher Scientific), 100 IU/mL penicillin, 100 µg/mL streptomycin and 0,25 µg/mL amphotericin (Sigma-Aldrich). After overnight incubation to allow adherent cells to attach to the flask, non-adherent cells in the supernatant were discarded. The medium was then replaced and changed thereafter every 3-4 days. After 1-3 weeks, adherent cells were morphologically uniform and covered the whole surface of the 25-cm^2 culture flask.

BEnSC and eBEnSc lines were established from endometrial samples by explant culture. Briefly, samples were minced into fragments of about 1-3 mm^3, washed in PBS and explants were placed in culture flasks with OptiMEM medium, supplemented as indicated above, to allow for cell migration and proliferation. The medium was changed every 3 days without disturbing the

explants. Cells migrate out of the explant within 5-10 days. When the explants were surrounded by cells, they were trypsinized and subcultured.

In the low serum-containing medium used for cell culture, cell lines showed a stable antigen phenotype and functional activities for 8–12 weeks (up to ten passages) (Garcia-Pacheco et al., 2001; Muñoz-Fernandez et al., 2006). For all experiments in this study, cell lines were used at early passages (P2-P4, between 3 and 4 weeks after collection).

Decidualization

To induce decidualization, cell lines were cultured to 70% confluence (1.5 x 10^6 cells in a 75-cm^2 culture flask or 2 x 10^5 cells/well in 6-well plates, depending on the type of analysis) and treated with 300 nM P4 and 500 μM 8-bromo-cAMP (Sigma-Aldrich) for up to 28 days. The decidualization medium was changed every 4 days. Conditioned media (CM) were collected from cultures of undifferentiated and decidualized eEnSC and mEnSC, after 12 days of incubation without or with P4 and cAMP, and kept frozen until use.

Morphological analysis

Images of representative areas of undifferentiated and decidualized cell cultures were acquired using a Leica DMi8 optical microscope equipped with digital camera and processed using the free Image J software. Fibroblast-like cells and cells with rounded morphology were counted and the results were expressed as the percentage of rounded cells.

Detection of apoptotic cells

During decidualization, cells were collected every 7 days to determine the percentage of apoptotic cells. Hypodiploid apoptotic cells were detected by flow cytometry according to the published procedures (Gong et al., 1994). Briefly, cells were washed with PBS, fixed in cold 70% (v/v) ethanol, and later stained with propidium iodide while treated with RNase (Sigma-Aldrich). Sub-G1 apoptotic cells were quantified in a FACSCalibur cytometer with the Cell Quest software (BD Biosciences, San Jose, CA, USA).

Flow cytometry analysis

Analysis of antigen expression was assessed on the FACSCalibur flow cytometer as previously described (Ruiz-Magana et al., 2021). Briefly, cells were detached from the culture flask,

suspended in PBS and incubated with the appropriate monoclonal antibody for 30 min at 4 ºC in the dark. After incubation, cells were washed, suspended in PBS and analyzed in the flow cytometer. For intracytoplasmic labeling, cells were fixed with 4% paraformaldehyde (PFA) (Merck, Darmstadt, Germany) for 20 min at 4 ºC and permeabilized with cold 0.05 % PBS saponin (Merck) before the antibody was added. For indirect labeling, a labeled goat anti-mouse Ig was added after the first monoclonal antibody. The percentage of antibody-positive cells was calculated by comparison with the appropriate isotype control.

The monoclonal antibodies used for flow cytometry were against CD10-phycoerythrin (PE) (#312204), CD29- allophycocyanin (APC) (#303007), CD31-fluorescein isothiocyanate (FITC) (#303103), CD44-FITC (#338803), CD73-PE (#344003), CD105 (#323202), CD140b-PE (#323605), CD146-APC (#361015), cytokeratin (CK) (#628602), podoplanin (PDPN)-AlexaFluor 647 (#337008), SUSD2 (W5C5)-APC (#327408), (Biolegend, San Diego, CA, USA), α-smooth muscle actin (α-SM actin)-FITC or cyanine 3 (Cy3) (#F3777, #C6198, Sigma-Aldrich). The isotype controls used were immunoglobulin IgM, IgG1-FITC, IgG1-PE, IgG1-APC, IgG2A-AlexaFluor 488 and IgG2-PE (Biolegend). The secondary antibodies were FITC-labeled goat anti-mouse IgM and PE-labeled goat anti-mouse IgG (Thermo Fisher Scientific).

Data were processed using FlowJo software (v.10, FlowJo LLC).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells with the TRIzol isolation method (Thermo Fisher Scientific). cDNA was synthesized from 0.5 μg of RNA using Access RT-PCR System kit (Promega Corporation, Madison, WI, USA) with Oligo-dT primers according to the manufacturer's protocol. For conventional PCR, the PCR Master Mix kit (Promega), along with the appropriate primers and cDNA samples, were mixed and placed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). After initial incubation for 3 min at 95ºC, each cycle consisted of 95ºC for 30 s, 58,5ºC for 45 s and 72ºC for 45 s, for a total of 35 cycles. The PCR products were size-separated on Gel Red-stained 1,5% (w/v) agarose gels (Biotium Inc, Fremont, CA, USA) and a 100-bp DNA ladder (Promega) was included in each run.

The following primers, obtained from the Instituto de Parasitología y Biomedicina, Granada, Spain, were used:

GAPDH: 5′- GCACCACCAACTGCTTAGC-3′ (forward) and 5′-GGCATGGACTGTGGTCATGAG-3′ (reverse).

Prolactin: 5′- AATCTGTTCCGCTGGTGACT -3′ (forward) and 5′-GAAGTGGGGCAGTCATTGAT -3′ (reverse).

Wound healing assay

Cells (1.5 x 10^4 cells/well) were seeded in 96-well plates until they reached 95% confluence, and a scratch was made in the monolayer using a sterile 200 μL micropipette tip. Culture medium with cell debris was then removed and replaced with the different culture media used in the experiment (OPTIMEM, CM from undifferentiated or CM from decidualized cells). Images were acquired at initial time (T0) and after 24, 48 and 72 h using 50x magnification on a Leica DMi8 optical microscope equipped with digital camera. The images were processed using the free Image J software. The cell-free area at each time point was subtracted from the T0 cell-free area and the results were expressed as percentage of covered area.

Statistical analysis

All experiments were repeated at least three times with different cell lines. The statistical analysis was performed using GraphPad Prism 6 software. Comparisons were carried out using the unpaired two-tailed t-test with Welch's correction. Values of $p < 0.05$ were consider significant.

RESULTS

Endometrial stromal cells from ectopic and eutopic endometrial tissue exhibit a similar antigen phenotype

The antigenic profile of EnSCs obtained from menstrual blood has been previously extensively analyzed (Ruiz Magana et al., 2020). Here, we compared the antigenic phenotype of eEnSC cell lines with that of mEnSCs and EnSCs obtained from endometrial biopsies of women with and without endometriosis. More than 90% of cells in all cell lines expressed the EnSC marker CD10 (Sumathi and McCluggage, 2002) as well as the MSC biomarkers CD29, CD44 and CD73, and

lacked CD31 and cytokeratin expression (Figure 1). They also exhibited similar levels of CD146 and α-SM actin. The expression of CD105 showed no significant variability, not only between different cell lines but also between samples of the same cell type. Moreover, CD140b, PDPN and SUSD2 presented a certain degree of variability, being their expression significantly higher in mEnSC, compared to the rest of cell lines (CD140b: $P = 0.0054$, $P < 0.001$ and $P = 0.0091$ for mEnSC versus BEnSC, eBEnSC and eEnSC, respectively; PDPN: $P = 0.018$ and $P = 0.0018$ for mEnSC versus BEnSC and eEnSC, respectively; SUSD2, $P < 0.001$ for mEnSC versus BEnSC, eBEnSC and eEnSC) (Figure 1).

Effects of decidualization on the phenotype and survival of EnSCs from women with endometriosis

In order to compare the decidualization ability of the different cell lines, they were treated with P4 and cAMP for 20 days. While mEnSCs, BEnSCs and eBEnSCs underwent a similar change from a fibroblastic-like appearance to a rounder shape characteristic of decidualized cells ($P = 0.014$, $P = 0.0003$ and $P = 0.0001$, respectively), eEnSCs did not significantly alter their morphology (Figure 2A and B). In addition, decidualization remarkably induced the mRNA expression of prolactin, as determined by RT-PCR, in all cell lines except for eEnSCs where a much lower increase of prolactin mRNA was observed in response to treatment with P4 and cAMP (Figure 2C).

Since apoptosis has been reported to occur in parallel with decidualization (Leno-Duran et al., 2014), we compared the effect of decidualization on the viability of the different cell lines. As expected, a time-dependent increase in the induction of apoptosis of mEnSCs (day 7: $P = 0.022$, day 14: $P = 0.035$, day 21: $P < 0.001$, day 28: $P < 0.0001$) and BEnSCs (day 21: $P = 0.026$, day 28: $P = 0.033$) was observed after incubation with P4 and cAMP for up to 28 days (Figure 2D and E). However, there was no apoptosis in either eBEnSC or eEnSC (Figure 2F and G).

We also analyzed the antigen profile of the different cell lines under decidualization conditions. The expression of distinctive markers, such as CD10 and CD29, did not change in response to decidualization in any of the cell lines. Moreover, the proportion of CD146+ and SUSD2+ cells did not significantly vary with decidualization (Figure 3). In contrast, the expression of CD105

was significantly down-modulated in response to P4 and cAMP in all cell types except for eEnSCs (mEnSC: $P = 0.005$, BEnSC: $P = 0.029$, eBEnSC: $P = 0.022$). We further found that the levels of CD140b and α-SM actin only decreased significantly in decidualized mEnSCs ($P = 0.032$ and $P = 0.021$, respectively), whereas slight non-significant reduction of both antigens was also observed in BEnSCs upon decidualization (Figure 3).

Effects of decidualization on the migration ability of ectopic and eutopic EnSC

Under decidualization conditions, it has been reported a reduction in the motility of eutopic EnSCs while endometriotic cells seem to retain their ability to migrate (Lavogina et al., 2021; Sultana et al., 2017). To further characterize this different behaviour of eutopic and ectopic cells, we studied the migration of mEnSCs and eEnSCs upon incubation with the conditioned medium (CM) obtained from cultures of undifferentiated (U) and decidualized (D) mEnSCs and eEnSCs by the wound closure assay. Migration in OptiMEM medium was also analyzed as a control. Interestingly, similar profiles were observed in both cell types (Figure 4A). Migration of mEnSC and eEnSC similarly increased upon incubation with OptiMEM and CM from undifferentiated cells (CM -U) in a time-dependent manner while motility of both, mEnSCs and eEnSCs, was significantly inhibited when cultured with CM from decidualized cells (CM -D) at all-time points analyzed (mEnSC: $P < 0.0001$ for CM -U versus CM -D at all times and conditions; eEnSC: $P = 0.0017$ for CM eEnSC-U versus CM eEnSC-D at 24 h, $P < 0.0001$ for the rest of the comparisons of CM -U and CM -D). The most remarkable differences were found when we compared the migration of cells upon incubation with CM from decidualized mEnSCs and CM from decidualized eEnSCs (Figure 4A, black and white bars; Figure 4B, left and rights panels) so that inhibition of migration of both, mEnSCs and eEnSCs, was significantly higher in the presence of CM mEnSC-D (mEnSC: $P < 0.0001$, $P < 0.001$ and $P = 0.0013$ for 24, 48 and 72 h, respectively; eEnSC: $P < 0.0001$ for all times). In addition, a slight but significant difference was found in the motility of mEnSCs and eEnSCs only when cultured with CM eEnSC-D, being the capacity for migration of eEnSCs less impaired than that of mEnSCs in this case (Figure 4B, right lower and upper panels; Figure 4C). The same experiment was conducted with BEnSC and eBEnSC lines,

both showing a migration capacity similar to that of mEnSCs and lower than that of eEnSCs upon incubation with CM eEnSC-D (Figure 4C).

DISCUSSION

Nowadays, knowing the exact mechanism of the pathogenesis of endometriosis remains a challenge. Even though several theories have been proposed to explain the origin of endometriosis (Taylor, 2020; Zondervan et al., 2018) and a variety of factors – such as genetic, epigenetic, immunological, hormonal and environmental – have been involved in the onset of the disease (Bulun et al., 2019; Symons et al., 2018; Szukiewicz et al., 2021; Zondervan et al., 2018), it is not yet clear whether the alterations found in the endometriotic cells are intrinsic to them or induced by the ectopic location (McKinnon et al., 2018). Progesterone resistance is one of the main characteristics of the endometriotic tissue. In the present study, for the first time to our knowledge, we have compared the response to decidualization with P4 and cAMP in ectopic EnSCs obtained from endometriomas (eEnSCs) and eutopic EnSC from three different sources and we have shown that eEnSCs are more resistant to changes induced by decidualization than eutopic EnSC from patients with endometriosis.

We have recently described that EnSCs from menstrual blood (mEnSCs) from normal women underwent changes in cellular morphology and apoptosis and secreted PRL during decidualization, although to a lesser extent than decidual stromal cells (DSCs) (Ruiz Magana et al., 2020). Now, a similar response has been observed for EnSCs obtained from endometrial biopsy of healthy women (BEnSC), confirming the equivalence of both sources, menstrual blood and endometrial biopsy, of endometrial cells. In agreement with previous reports comparing the decidualization capacity of eEnSCs with that of EnSCs from healthy donors (Sultana et al., 2017; Yin et al., 2012), we also found that eEnSCs mostly retained their fibroblastic morphology and barely expressed PRL in response to treatment with P4 and cAMP. Moreover, we showed that eEnSCs did not undergo apoptosis, even after prolonged incubation with P4 and cAMP. Interestingly, no apoptosis was observed in EnSC obtained from eutopic endometrium of patients (eBEnSCs), even they

changed their morphology to a rounder shape and produced PRL upon decidualization, suggesting a partial response to this process. Klemmt *et al*. previously reported similar morphological changes, but a reduced PRL secretion, in cultures of eutopic endometrial stromal cells from women with endometriosis in comparison with cells from healthy women, after in vitro decidualization (Klemmt et al., 2006). Moreover, although they were not quantified, polygonal/rounded cells were found in cultures of decidualized endometriotic stromal cells, even their ability to secrete PRL was significantly lower than that of eutopic cells. Discrepancies with our results may be due to the different experimental conditions, as they decidualized with cAMP alone (Klemmt et al., 2006), while we used the standard protocol for decidualization with P4 and cAMP (Gellersen and Brosens, 2003). In agreement with our results and the role of P4 in the regulation of PRL production, similar levels of PRL have been reported to be produced by eutopic cells from women with and without endometriosis in response to P4, but not to cAMP (Aghajanova et al., 2009). Regarding the resistance to apoptosis, it has been considered a characteristic of endometriotic cells and different authors have demonstrated that spontaneous apoptosis in endometriotic lesions is lower than that in the eutopic endometrium of patients and this, in turn, lower than that of endometrial tissue from control women (Gebel et al., 1998; Imai et al., 2000; Meresman et al., 2000). Here, we found that eBEnSCs were as resistant to the induction of apoptosis during decidualization as eEnSCs, which is in accordance with the altered expression of antiapoptotic and proapoptotic genes reported in the eutopic endometrial cells from women with endometriosis (Ahn et al., 2016). In particular, the antiapoptotic genes Bcl-2 and Bcl-xL have been shown to be increased in the proliferative and early secretory endometrium from patients with endometriosis, compared with women without disease (Braun et al., 2007; Burney et al., 2007; Meresman et al., 2000), and reduced levels of the proapoptotic genes p53 and caspase-1 have been described in the eutopic endometrium of patients (Braun et al., 2007). Moreover, miRNAs families involved in the regulation of cell cycle and cell death are also down-regulated in the endometrium of women with endometriosis, such as the miR-9 family, one of whose targets is Bcl-2 (Burney et al., 2009). Altogether, these data provide a basis for the observed resistance of eBEnSCs and eEnSCs to decidualization-induced apoptosis.

The comparative study of the antigen phenotype in the four types of cell lines revealed a similar profile with no substantial differences between cells derived from ectopic (eEnSCs) and eutopic endometrium, either from patients (eBEnSCs) or healthy women (BEnSCs). Our results reinforce previous data showing a similar expression of MSC markers in endometrial stem cells obtained from ectopic endometrial tissues compared with cells from the endometrium of either patients (Kao et al., 2011) or healthy donors (Koippallil Gopalakrishnan Nair et al., 2015; Liu et al., 2020). We only found significant differences in the phenotype of mEnSCs, showing a higher expression of CD140b, PDPN and SUSD-2. The overall greater expression of MSC markers in mEnSC (Bozorgmehr et al., 2020; Gargett et al., 2016) suggests that menstrual blood is more suitable than endometrial biopsy as a source for obtaining more undifferentiated endometrial MSC which, along with their easy accessibility, make it promising for therapeutic approaches (Bozorgmehr et al., 2020).

In response to decidualizing factors, DSCs have been recently reported to down-modulate the expression of several pericyte/MSC markers such as CD140b, CD146, α-SM actin and SUSD2 (Ruiz-Magana et al., 2021), a change probably related to the location and functional variations of these cells during decidualization. Likewise, decidualization of mEnSCs significantly reduced the expression of CD140b and α-SM actin and induced a slight, although no significant decrease, in the percentage of CD146+ and SUSD2+ cells. These differences between DSCs and mEnSCs may be due to the demonstrated different ability of both cell types to decidualize (Ruiz Magana et al., 2020). Interestingly, the expression of the aforementioned antigens did not significantly vary upon decidualization in the rest of cell lines, although they all showed a trend to decrease in BEnSCs. The different response of mEnSCs and BEnSCs could be explained on the basis of the more undifferentiated state of mEnSCs suggested above. The only MSC marker that was regulated in all cell lines, except those derived from ectopic tissue, was CD105. Endoglin or CD105 is a transmembrane glycoprotein which plays an essential role in angiogenesis (Duff et al., 2003). Its expression was reported to negatively correlate with the degree of differentiation of umbilical cord blood derived-MSCs (UCB-MSCs) so it was proposed as a marker of the differentiation status of these cells (Jin et al., 2009). Our results show that the four types of cell lines displayed a

heterogeneous CD105 expression profile in their undifferentiated state and, similar to UCB-MSCs, a decrease in the expression of CD105 was observed in mEnSCs, BEnSCs and eBEnSCs upon differentiation with P4+cAMP. In contrast, and according to their resistance to decidualization, the level of CD105 did not change in eEnSC in response to treatment with decidualizing factors.

The capacity of EnSC to migrate is one of the cellular functions altered during decidualization. Different authors have reported that decidualization of endometrial cells in vitro with P4 and cAMP induces an intense decrease in basal cell motility (Chen et al., 2020; Lavogina et al., 2021; Sultana et al., 2017). On the other hand, ectopic endometrial MSCs from women with endometriosis have shown a higher migration ability than eutopic MSCs, either from patients or healthy women (Kao et al., 2011; Liu et al., 2020), and retained this ability upon decidualization (Sultana et al., 2017). As decidualized cells acquire a secretory phenotype, we have addressed the study of the changes in motility in response to decidualization from a different perspective, trying to examine whether decidualized cells could secrete factors that reduce their own motility rather than lose their migration ability. Certainly, our results show that mEnSCS, as well as eEnSCs, reduced their motility when incubated with the CM from decidualized mEnSCs suggesting that, during decidualization, EnSCs may produce some factors capable of inhibiting migration. Given the impact of EnSC motility on endometrial tissue remodeling during endometrial regeneration and embryo implantation, it is not surprising that this process is highly regulated and that decidualized EnSCs themselves produce factors that contribute to its regulation. The specific products and the mechanism mediating this effect are yet to be determined. It has been recently published that secretion of decorin, a small leucine-rich proteoglycan that interacts with transforming growth factor (TGF)-β among many other molecules, is enhanced during decidualization and required for the acquisition of the decidual phenotype (Halari et al., 2020). In addition, decorin repress throphoblast migration and invasion (Halari et al., 2020). It should be interesting to study whether this protein may also restrain endometrial cell motility. In accordance with their defective response to decidualization, ectopic EnSCs showed a lower ability to produce those motility regulating factors as migration of mEnSCs and eEnSC was significantly higher after incubation with CM from decidualized eEnSCs than when incubated with CM from decidualized mEnSCs. Regarding

potential differences in the migration capacity of ectopic and eutopic cells, either from patients or healthy women, they were only exhibited in response to incubation with CM from decidualized eEnSCs. It is reasonable to speculate that the greater capacity of ectopic cells to migrate can be only appreciated in those conditions in which motility is limited, while in situations of strong inhibition (CM-mEnSC-D) or, on the contrary, optimal for migration (OptiMEM, CM-U), differences are not evident.

In summary, this study demonstrates that eutopic endometrial cells from women with endometriosis may exhibit alterations in some phenomena associated to decidualization, such as the induction of apoptosis. However, endometrioma cells must undergo further changes in the endometriotic foci, probably derived from their interaction with the microenvironment in the ectopic location, that contribute to the acquisition of their high resistance to decidualization. Further studies with samples from different ectopic sites are needed to determine whether different microenvironments may similarly influence the characteristics and decidualization resistance of eEnSCs.

DECLARATION OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

M.J.R-M. contributed to the study design, execution, data analysis and critical discussion. J.M.P. collected samples and participated in execution and data analysis. T.L., C.M.-M. and R.M.-A. participated in execution and data analysis. A.C.A.-M. contributed to the study design, data interpretation and critical discussion. E.G.O. and C.R.-R. were responsible for the conception and study design, financial support, data analysis and interpretation, and manuscript writing. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1. Antigen phenotype of ectopic and eutopic EnSCs obtained from different sources. mEnSCs, BEnSCs, eBEnSCs and eEnSCs were stained with antibodies for different antigens and analyzed by flow cytometry. The graphs represent the mean percentage of positive cells for the indicated markers. Error bars show the SEM from 10 (mEnSC), 7 (BEnSC and eBEnSC) and 15 (eEnSC) different cell lines.

Figure 2. Comparative analysis of cell morphology, prolactin production and induction apoptosis in response to decidualization. mEnSCs, BEnSCs, eBEnSCs and eEnSCs were incubated either for 20 days (A, B, C) or for up to 28 days (D, E, F, G) without (undifferentiated) or with P4 and cAMP. A) Morphological changes during decidualization. Images of a representative cell line of each cell type are shown. B) Mean percentage of rounded cells after quantification of representative areas (n=3 per sample). Error bars show SEM of three independent experiments with different cell lines. C) Detection of prolactin by RT-PCR in the four cell lines after decidualization. The expression of the reference gene GAPDH was determined as a control. D-G) Percentage of sub-G1 apoptotic cells analyzed every 7 days, during decidualization, by flow cytometry. Error bars show SEM from eight (mEnSC and eEnSC) and five (BEnSC and eBEnSC) independent experiments with different cell lines.

Figure 3. Comparative analysis of the antigen phenotype upon decidualization. Antigen expression was determined by flow cytometry in mEnSCs, BEnSCs, eBEnSCs and eEnSCs after incubation for 20 days without (undifferentiated) or with P4 and cAMP. The bar charts represent the mean percentage of antigen-expressing cells. Error bars show the SEM from five (mEnSC and eEnSC) and three (BEnSC and eBEnSC) different cell lines.

Figure 4. Effect of CM from undifferentiated and decidualized mEnSC and eEnSC on the migration of ectopic and eutopic EnSCs. A) Motility of mEnSCs (upper panel) and eEnSCs (lower panel) was determined by the wound healing assay after incubation for 24, 48 and 72 h with control medium (OptiMEM), CM from undifferentiated cells (CM mEnSC-U, CM-eEnSC-U) or CM from decidualized cells (CM mEnSC-D, CM eENSC-D). Wound closure is represented as the percentage of covered area with respect to time zero (time of scratch). B) Representative pictures of the migration of mEnSC and eEnSC incubated for 72 h with CM from decidualized cells. C)

Comparison of the migration of mEnSCs, BEnSCs, eBEnSCs and eEnSCS at the indicated times upon incubation with CM from decidualized eEnSCs, as represented by the percentage of wound closure. In A) and C), error bars show the SEM from three independent experiments with different cell lines.