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Different Effect of Dienogest on Endometrium Mesenchymal Stem Cells Derived from Healthy and Endometriosis Tissues

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Background: Endometriosis (EM) is an inflammatory condition in which the endometrium is observed to develop outside the uterine cavity. Endometrium has conventionally been recognized as a rich source of endometrial mesenchymal stem cells (E-MSCs). The influence of dienogest, a medication frequently prescribed for EM, on E-MSCs has not been extensively investigated.

Aims: To explore effects of dienogest on the E-MSCs derived from healthy (E-MSCs-control) and diseased (E-MSCs-endometriosis) endometrial tissue samples in vitro.

Study Design: In vitro study.

Methods: We collected samples from healthy and diseased endometrial tissues. E-MSCs were derived from both healthy and EM tissues. The effect of dienogest (VISANNE) on E-MSCs was assessed by examining cell proliferation, telomerase activity, cell migration, and estrogen secretion levels after the isolation and characterization of E-MSCs.

Results: We discovered that cellular proliferation rate was higher in the E-MSCs derived from EM tissues compared to those derived from healthy tissue. The proliferation rate and telomerase activity were both suppressed by dienogest treatment, particularly in E-MSCs-endometriosis. The drug treatment also resulted in a decrease in the migration capacity of E-MSCs-endometriosis, from 60.4% to 59.2%. The expression of CXCL12, Ki67, and beta-catenin was analyzed in both E-MSCs-endometriosis and E-MSCs-control. The CXCL12 and Ki67 expressions were quite elevated in the E-MSCs-endometriosis without drug treatment compared to the E-MSCs-control. Following the treatment, these levels declined drastically to the levels close to E-MSCs-control. Similarly, this decrease in gene expression was accompanied by a decrease in estrogen secretion into the medium.

Conclusion: This research demonstrates that dienogest exerts a substantial impact on both stromal and stem cells, as it effectively controls the disease by reversing EM markers, despite the absence of progesterone receptors on endometrial stem cells.

INTRODUCTION

Endometriosis (EM) is prevalent in approximately 15% of the population, with a particularly high incidence among women of reproductive age.[1,2] Frequently resulting in chronic inflammation, this condition is characterized by the growth of functional endometrial tissue outside the uterine cavity and shares similarities with cancer, including hyperplasia, invasion, and dissemination.[3] Although EM is generally recognized as a hormone-dependent disease, the precise mechanism of its development remains unclear.

Endometrial mesenchymal stem cells (E-MSCs) are believed to contribute to the development of EM.[4] Certain studies indicate that EM may be the result of a stem cell-related disease.[5-7] In addition to their function in endometrial tissue regeneration, the involvement of stem cells in the onset of EM has also been implicated in

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the mechanisms of epithelial-to-mesenchymal transition and mesenchymal-to-epithelial transition mechanisms.[8] It is imperative to elucidate the relationship between EM and E-MSCs. Stem cell-based dysfunction is suspected to play a significant role in initial endometrial lesion formation.[2] Retrograde menstruation has been proposed as a mechanism by which a greater number of E-MSCs are released into the peritoneal cavity in women with EM.[9-11]

While it is anticipated that E-MSCs from EM patients will exhibit an aggressive phenotype with high proliferation and migration capabilities, stromal progenitor cells derived from these patients demonstrate low mitotic activity.[6,12,13] Current treatments for menstrual cycles and lesion formation concentrate on the limitation of ovarian function and estrogen (E_2) levels. Stem cells, which are essential for survival and growth, are present in menstrual debris and may contribute to endometrial pathology.

Progestin is an effective progesterone receptor agonist for treating EM. Dienogest, a fourth-class oral progestin, possesses the pharmacological properties of both progestins and 19-norprogestins,[14] including anti-estrogenic activities. It is an effective treatment for EM due to its highly specific progestogenic effect and lack of significant androgenic or glucocorticoid activities.[15] Dienogest has been demonstrated to effectively treat EM and suppress pathological disease processes, including endometrial proinflammatory and proliferative activities.[14,16,17] Dienogest was first approved for treatment of EM in Europe in 2009.[18] Despite its effectiveness in EM treatment, the mechanism of action of dienogest remains unclear. This study involved extracting stromal (stem) cells from endometrial tissue samples from EM patients and assessing the impact of dienogest in vitro. The study aimed to examine the influence of dienogest on E-MSCs derived from control (healthy) and EM (diseased) endometrial tissues in vitro.

MATERIALS AND METHODS

Cell culture and characterization

This study was approved by the Kocaeli University Institutional Ethics Committee (approval number: KÜ GOKAEK 2020/4.01, project number: 2020/55, date: 12.03.2020), and written informed consent was obtained from each donor. In the control group, healthy endometrial tissue samples were collected from fertile women without a diagnosis of EM, adenomyosis, or hyperplastic endometrial disease and who did not receive hormonal or immunomodulatory therapy. The EM samples (n = 3) were obtained from patients diagnosed with EM in the proliferative phase through laparoscopic and pathological examination and who had not received hormonal or immunomodulatory treatment in the last three months.

The tissue samples were cut into small pieces for the purpose of stem cell isolation. The tissue fragments were mixed with 5 ml of 0.075% type 1 collagenase (Gibco, Thermo Scientific, Paisley, UK) in HBSS (Gibco) and incubated in a water bath at 37 ºC for 60 minutes. After centrifugation at 300 g for 5 minutes, the pellet was resuspended in 5 ml of HBSS, passed through a 100 µm-cell strainer, and centrifuged once more. The pellets were resuspended in α-MEM with 10% FBS (Gibco) and 1% penicilin-streptomycin (Gibco) and subsequently transferred to a culture flask. The plates were incubated at 37 ºC under 5% CO_2. The medium was replaced twice a week.

The cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Gibco), 1% Pen-Strep (Gibco), and 1% GlutaMAX (Gibco) at 37 °C in 5% CO_2. The cells were then detached using 0.05% Trypsin-EDTA (Gibco) and passaged by seeding again in RPMI 1640 medium. The cell morphology was evaluated under an inverted light microscope (CKX41, Olympus, Tokyo, Japan).

Following the isolation of the cells, a flow cytometer (FACSCalibur, BD Biosciences, SanJose, CA) was employed to identify MSC-positive markers (CD73, CD90, CD105) and -negative markers (CD34, CD45).[19] All antibodies were procured from BD biosciences.

Subsequently, differentiation analysis was conducted on the cells to determine their differentiation into chondrocytes, osteocytes, and adipocytes. The cells were initially cultured at a density of 3000/cm^2. For adipogenic differentiation, the cells were induced to differentiate by StemPro™ Adipogenesis Differentiation medium (Gibco) for three weeks. The intracellular lipid droplet formation was verified through 0.5% Oil Red O staining (Sigma-Aldrich). The osteogenic differentiation capacity was assessed by inducing cells with the StemPro Osteogenesis Differentiation Kit for four weeks. The calcium deposition was evaluated by Alizarin red S staining (2%, pH 4.1-4.3; Fluka, Buchs, Switzerland) to evaluate osteogenic differentiation. The chondrogenic differentiation was assessed by Alcian Blue staining after the differentiation was induced with the StemPro Chondrogenesis Differentiation Kit (Gibco).

Dienogest (Visanne, Bayer Weimer, Weimer, Germany) was dissolved in 20% DMSO in phosphate-buffered saline (PBS) at a pH of 7.4. The final concentration of dienogest was adjusted to 10 nM during drug supplementation assays to achieve the EC_{50} dose required to activate the progesterone receptor.[16]

Cell proliferation assay

The number of viable cells was determined by diluting CCK8 solution (Elabscience Biotechnology, Wuhan, China) to 10% in RPMI 1640 mediums in accordance with the manufacturer's instructions. Absorbance at 450 nm was assessed using a microplate reader (VersaMax Plus, Molecular Devices). The number of viable cells was determined on days 1, 3, and 5. On day 1, the results were expressed as a percentage of absorbance observed in control cells without dienogest (E-MSCs-control V-), normalized to 100%.

Migration assay

The wound healing assay was used to estimate cell migration in medium with or without dienogest supplementation. To achieve 90-100% confluence, the cells were plated on uncoated 6-well culture dishes at a density of 500,000 cells per well and incubated. The cell monolayers were scraped with a p200 pipette tip. The cells were then washed with PBS (Gibco) and cultured under standard conditions for 16 hours, in accordance with procedures defined previously. Photomicrographs were acquired employing an inverted light microscope (CKX41, Olympus, Tokyo, Japan) for wound closure

analysis. The in-built software of the microscope was used to estimate gap closure (distance).

Telomerase activity assay

Telomerase activity was determined using the TRAP-TeloTAGGG PCR^{Plus} ELISA kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Briefly, 200,000 cells were washed with PBS (Gibco) and homogenized in ice-cold lysis buffer. The biotin-labeled primers, dNTPs, and Taq DNA polymerase were incubated with the cell extracts at 25 °C for 20 minutes. Subsequently, conventional PCR was conducted at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s; 50 °C for 30 s; 72 °C for 90 s; and a final extension at 72 °C for 10 min. The PCR products were denatured and labeled with digoxigenin (DIG) probe to detect telomeric repeats. The mixture was incubated in streptavidin-coated wells of 96 well-microplate (Roche). Finally, the microplate reader was used to measure the DIG-labeled hybrids using a peroxidase-conjugated anti-DIG antibody and a substrate (TMB). Relative telomerase activity is calculated as the ratio of the absorbance value of the sample to that of the control.

The bicinchoninic acid assay (BCA, Sigma-Aldrich) was employed to determine the total protein concentration. The samples were incubated at 60 °C for 30 minutes with 1% BCA working solution that was mixed with 0.4 M $CuSO_4$-solution (Sigma-Aldrich). The spectrophotometer (Versamax Plus) was used to measure the color change at 562 nm.

Gene expression analysis

The Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA) was used to isolate total RNA following cell culture. The cDNA synthesis kit (Thermo Scientific, Rockford, IL, USA) was used to synthesize cDNA in accordance with the manufacturer's instructions. The amplification of target genes [Ki67, CXCL12, beta-catenin, NR3C3, mitogen-activated protein kinase (MAPK)] was conducted in LightCycler 480-II (Roche) using gene-specific primers and the Power SYBR-Green Master Mix (Thermo, Applied Biosystems Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations. The Cp values were determined utilizing the LightCycler 480 software (release 1.5). Beta-actin was employed as a housekeeping gene during the normalization process. The ΔΔC p values were calculated in relation to E-MSCs-control V^-.

Estrogen ELISA

The cells were cultured in serum-free medium for 48 hours with dienogest addition (V^+) and without dienogest addition (V^-). The E_2 levels secreted into medium were assessed using ELISA in accordance with the manufacturer's instructions (CUSABIO, Wuhan Huamei Biotech, Wuhan, China). Briefly, a 50 µl sample was added to each well in duplicate. The plate was incubated at 37 °C for 1 hour following the addition of the antibody solution provided with the kit. The 100 µl substrate solutions were added to each well, and the plates were incubated for an additional 15 min at 37 °C. The reaction was stopped, and absorbance was measured at 450 nm.

Statistical analysis

The statistical significance was determined using ANOVA with a 95% confidence interval and Tukey's post-hoc test. The results are expressed as mean ± standard deviation, and values of $p < 0.05$ were considered statistically significant. All statistical analyses were performed using the SPSS 10.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Characterization of cells and proliferation

The cells isolated from EM patients and from healthy donors exhibited no significant differences (Figure 1). The cells were effectively cultured in the medium, preserving their mitogenic character for five days. The cellular clonogenic capacity of E-MSCs-EM was marginally higher than that of E-MSCs-control in the standard culture medium (V^-, without Visanne). Although the changes were not evident in the initial 24 hours, a significant increase in the number of cells in E-MSCs-EM V^- was observed after five days (Figure 1e) in comparison to E-MSCs-control V^- (Figure 1g). Following the addition of dienogest, the cell count in the culture was reduced in both the diseased and healthy groups.

The flow cytometry analysis demonstrated that both E-MSCs-EM and E-MSCs-control expressed the typical markers for mesenchymal stem cells, including CD29, CD73, and CD105. However, compared to E-MSCs-control, E-MSCs-EM displayed an elevated level of staining for endothelial and hematopoietic cell markers, CD34 and CD45. Although the detection level was below 5%, which indicates that the cells were negative for these surface markers, the E-MSCs-EM population exhibited 5.17% and 4.94% positive cells for CD34 and CD45 (Figure 2b1). In contrast, the E-MSCs-control population exhibited 2.01% and 1.28% positive cells for these markers (Figure 2a1). In E-MSCs-EM, the CD105 expression level decreased to 86.89%. The E-MSCs-control and E-MSCs-EM cells were induced chemically to differentiate into adipogenic, osteogenic, and chondrogenic cell lines. Adipocytes (Figure 2a2) and osteocytes (Figure 2a3) were successfully differentiated from E-MSCs-control cells. The differentiation into chondrocytes could be identified by staining glycosaminoglycans with Alcian blue stain (Figure 2a4). However, the staining intensity and the incidence of the stained cells were comparatively lower compared to the staining observed in the other two cell lines. This implies that the E-MSCs-control exhibit a lower propensity to differentiate into chondrocytes than adiopocytes and osteocytes. E-MSCs-EM differentiated into adipocytes with comparable efficiency to E-MSCs-control (Figure 2b2). Interestingly, the formation of calcium deposits, which were stained with Alizarin red S, was less pronounced than that observed in the E-MSCs-control (Figure 2b3). Compared to the E-MSCs-control, the staining intensity of glycosaminoglycans with Alcian blue was significantly higher (Figure 2b4). This suggests that E-MSCs-EM have a greater tendency to differentiate into chondrocytes than E-MSCs-control. Alterations in cell characteristics detected by flow cytometry may be associated with the shift in differentiation characteristics.

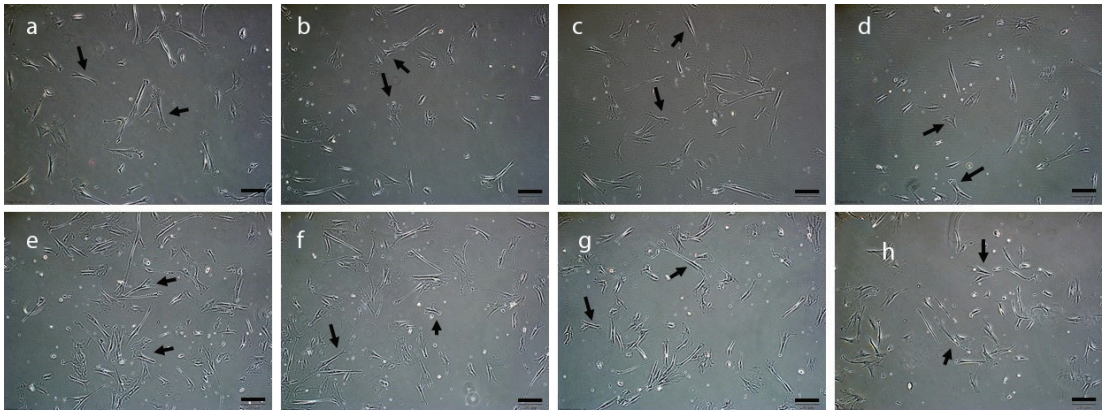

FIG. 1. Culture of E-MSCs-EM (a, b, e, f) and E-MSCs-control (c, d, g, h) in RPMI 1640 medium without (a, c, e, g) and with dienogest (b, d, f, h). Passage 3 cells derived from tissue exhibit spindle-like elongated cell morphology (black arrow) in cell culture. After culture for 24 hours (a-d), no significant difference was observed between the groups in cell number. After culture for 72 hours (e-h), E-MSCs-EM without drug supplementation (V^-) demonstrated a higher cell population (e) compared to the medium with drug supplementation (V^+) (f). In E-MSCs-control, no significant difference in cell population could be detected in groups without (g) and with (h) drug supplementation. Scale bar 200 µm.
E-MSCs-EM, endometrial mesenchymal stem cells-endometriosis.

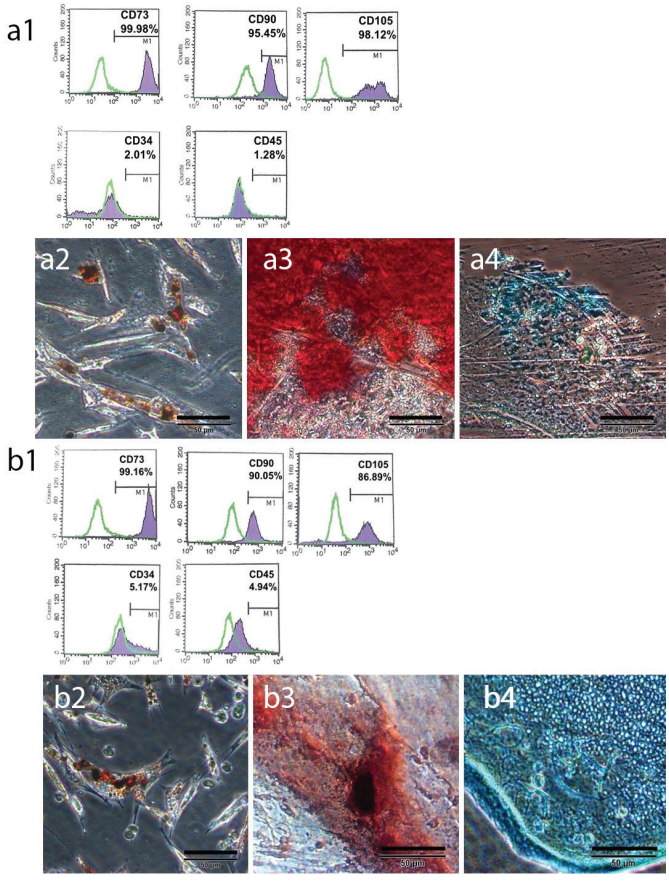

FIG. 2. Characterization of stem cells. Cells derived from the endometrial tissues were characterized both by flow cytometer for E-MSCs-control (a1) and E-MSCs-EM (b1), and by trilineage cell differentiation into adipocytes (a2, b2), osteocytes (a3, b3), and chondrocytes (a4, b4) for E-MSCs-control and E-MSCs-EM, respectively. Scale bar 50 µm.
E-MSCs-EM, endometrial mesenchymal stem cells-endometriosis.

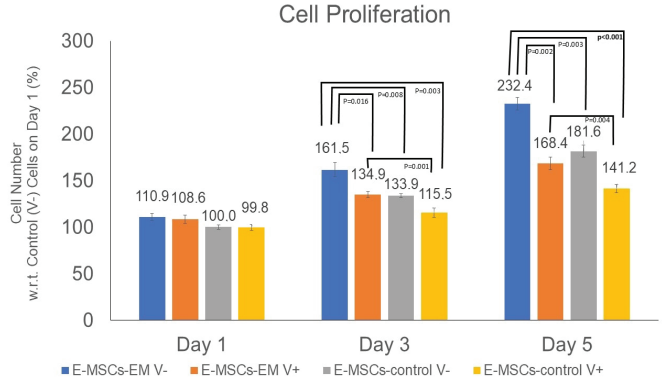

FIG. 3. Cell proliferation analysis results over the five days. The numbers are represented in percentage with respect to control culture on day 1, taking its level to be 100%.
E-MSCs-EM, endometrial mesenchymal stem cells-endometriosis.

The cell proliferation assay was conducted for five days, and the results were estimated by CCK8 (Figure 3). Although the cell counts on the first day of culture did not reveal any significant difference, E-MSCs-EM in V^- medium on day 5 exhibited a significant proliferation of 232.4% ± 6.9 in comparison to E-MSCs-control on day 1 (100%). The drug supplementation in medium (V^+) suppressed growth of E-MSCs-EM by 168.4% ± 6.6 (Figure 3). This level was close to the proliferation rate of E-MSCs-control V- on day 5, which was determined to be 181.6% ± 6.5. In E-MSCs-control V^+ on day 5, proliferation was decreased to 141.2% ± 4.3 compared to the non-drug supplemented counterpart (181.6% ± 6.5). At a 10 nM dose, the cell number did not decrease during the culture with dienogest. However, the inhibitory effect of dienogest on cell proliferation was statistically significant on days 3 and 5 in E-MSCs-EM.

Migration assay

Consistent with the cell proliferation results, compared to E-MSCs-control V^-, E-MSCs-EM exhibited a higher migration rate in the medium without dienogest. The migration capacity was marginally reduced by the addition of dienogest, as the percentage of filled gap area by cells decreased from 60.4% to 57.7% in comparison to E-MSCs-EM V^- (Figure 4a) and E-MSCs-EM V^+ (Figure 4b). Compared to cells from a healthy donor, E-MSCs-EM still possessed greater migration capacity. Dienogest also influenced cell migration in healthy cells (E-MSCs-control), resulting in a decrease from 57.7% to 56%. Although the drug's impact on migration characteristics was detectable, the extent of the change was relatively minor (Figure 4c).

Telomerase activity assay

In addition, the telomerase activity of the cells was estimated to further investigate the impact of the drug on E-MSC lines. The telomerase activity was found to be significantly high in E-MSCs-EM V^-. The high proliferation rate may be attributed to the high telomerase activity. The level was determined to be 8.22% ± 2.83 relative to those of T293-cells (Figure 5). Dienogest decreased telomerase activity to 0.25%, while no significant activity was observed in healthy tissue cells.

Gene expression analysis

The impact of dienogest on the expression of beta-catenin (Wnt-signaling), Ki67 (proliferation), CXCL12 (stem cell survival and proliferation), NR3C3 (progesterone receptor), and MAPK (Figure 6) was analyzed. These genes were expressed at the maximum levels in the E-MSCs-EM, which also exhibited the highest levels of proliferation and migration. Dienogest significantly decreased Ki67 and CXCL12 expression in the E-MSCs-EM by approximately sixfold and fivefold, respectively. Additionally, it decreased the level of beta-catenin and did not impact the expression of these genes in E-MSCs-control. Both groups of E-MSCs exhibited NR3C3 expression; however, the response of this expression to dienogest was distinct. Following the dienogest treatment, NR3C3 expression decreased by nearly 2.5-fold in E-MSCs-EM, while the decrease was estimated to be about 1.4-fold in E-MSCs-control (Figure 6). Similarly, in E-MSCs-EM, the medium lacking dienogest exhibited the highest MAPK expression. The dienogest treatment reduces the MAPK expression by approximately 50% of the preceding level. Notably, the MAPK expression in E-MSCs-control was not significantly affected by the dienogest effect.

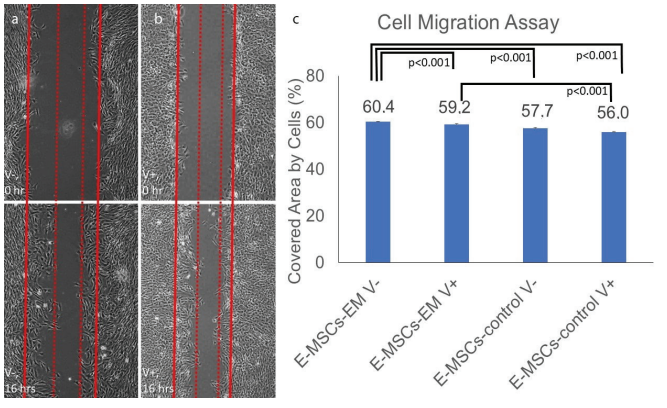

FIG. 4. Cell migration (scratching) analysis. On culture of E-MSCs-EM that had attained nearly full confluency, a cell-free region was created. The dienogest supplemented medium (V^+) (b) was compared with the culture medium not supplemented with the drug (V^-) (a). During 16 hours of culture (below), the capacity of cells to migrate to the free region and fill the gap was observed. Later, the filled regions were compared with the initial gap at time 0 hour (above). The assay was repeated for all cell groups, including the control group (c).
E-MSCs-EM, endometrial mesenchymal stem cells-endometriosis.

Estrogen ELISA

Dienogest treatment reduced E_2 expression, as expected (Figure 7). This reduction in E_2 levels may be associated with a decrease in CXCL12 levels, which also regulate E_2 production. The decrease from 94.6 pg/ml ± 22.8 to 45.0 pg/ml ± 3.5 was substantial; however, it remains elevated compared to the E-MSCs-control, in which E_2 was estimated to be 29.6 pg/ml ± 7.4 (V^-) and 17.6 pg/ml ± 0.7 (V^+).

DISCUSSION

Interpretation

Endometriosis is a stem cell-based disease, and it is crucial to examine both E-MSCs and endometrial cells in this context. In our study, mitotically active cells were extracted and cultured from ectopic endometrial tissue. Despite the absence of any morphological distinction between E-MSCs-control and E-MSCs-EM, their response to dienogest was significantly different. The investigation investigated the influence of dienogest on ectopic E-MSCs. Mesenchymal stem cell-specific markers were expressed by the isolated E-MSCs from both EM and control tissue samples. Although E-MSCs-EM cells expand at a higher rate, research has demonstrated a reduced proliferation rate and a decrease in telomerase expression. Ectopic E-MSCs-EM were observed to have

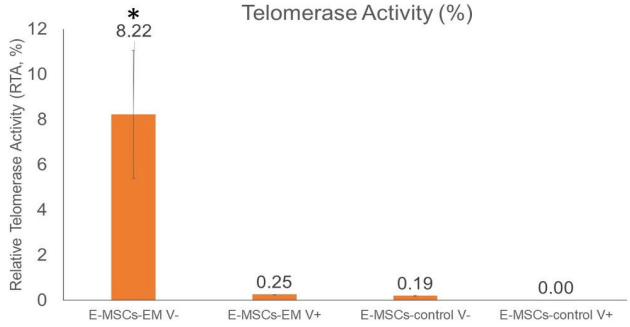

FIG. 5. Telomerase enzyme activity. Relative telomerase activity with respect to those observed in T293 cells was evaluated based on the protocol provided within the kit. Only E-MSCs-EM V^- exhibited a substantial amount of telomerase activity. The statistically significant results ($p < 0.05$) compared to E-MSCs-control V^- are indicated by an asterisk (*).
E-MSCs-EM, endometrial mesenchymal stem cells-endometriosis.

a lower proliferation rate on fibronectin-coated plates and to be less invasive than eutopic EM and non-endometrial derived-stromal cells. This was attributed to firm adhesion of ectopic EM-stem cells to the extracellular matrix. Nevertheless, recent reports have demonstrated an upregulation of TERT (telomerase enzyme activity) and Ki67, which may be attributed to the regulatory effects of nuclear factor-kappa B (NF-κB) on TERT expression. The MAPK expression was analyzed to assess the inflammatory signaling. The induced proliferation in E-MSCs-EM might be due to high Ki67 expression. Certain papers have suggested a potential link between the recurrence of EM and telomerase activity and Ki67,[20-22] as EM shares certain characteristics with tumorigenesis and metastasis.[23] In addition to hormonal stimulus, proliferation of ectopic E-MSCs-EM is regulated by other factors through Ki67.[24] Diminished NR3C3 expression in cells may also be related to cell proliferation. It is conceivable that proliferation is primarily suppressed in cells with high NR3C3 expression, as the cell response to dienogest is correlated with the progesterone receptor level. This can result in the selective culture of cells, resulting in the predominance of cells with reduced NR3C3 expression in the culture over time. Consequently, the NR3C3 expression was reduced in E-MSCs-EM in the presence of dienogest. The decrease was not substantial; however, the selective culture for an extended period may exacerbate the reduction in progesterone receptor expression in cells.

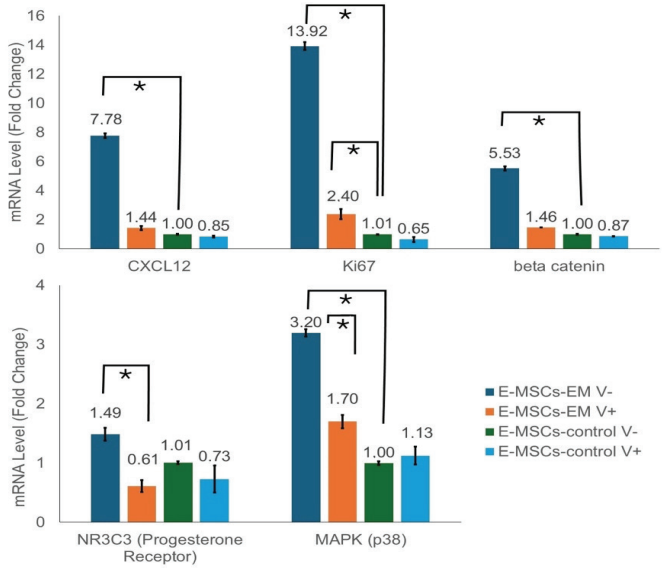

FIG. 6. Gene expression analysis. Beta-catenin, which is a crucial protein in the canonical Wnt-signaling pathway; cell proliferation marker Ki67; and CXCL12, which is known as stromal derived factor-1 and plays critical roles in stem cell survival and proliferation, were assessed. Furthermore, the cellular expression of progesterone receptor gene (*NR3C3*) and mitogen-activated protein kinase (p38) was analyzed. The results are expressed in terms of comparison to E-MSCs-control V^- (gray). The statistically significant results ($p < 0.05$) compared to E-MSCs-control V^- (gray) are indicated by an asterisk (*).
E-MSCs-EM, endometrial mesenchymal stem cells-endometriosis; MAPK, mitogen-activated protein kinase.

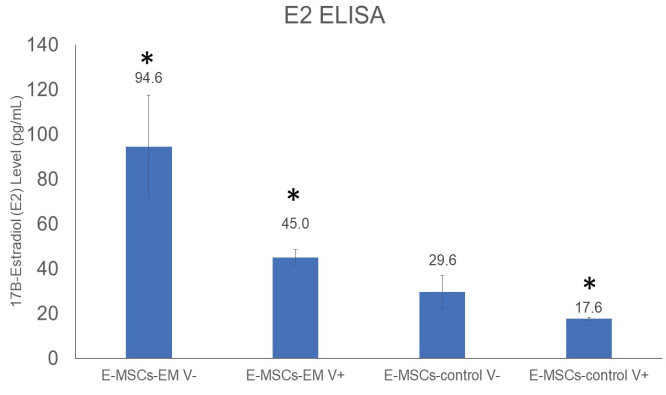

FIG. 7. Estimation of E_2 (17B-E_2) level secreted into medium. After 48 hours of culture, the E_2 level was measured and normalized to the total protein in the medium. The statistically significant results ($p < 0.05$) as compared to E-MSCs-control V^- are indicated by an asterisk (*).
E-MSCs-EM, endometrial mesenchymal stem cells-endometriosis.

The high migration and gap-filling capacity of E-MSCs-EM was evident compared to control cells, which contrasts with some reports.[25] Ectopic EM cells have also demonstrated higher migration and invasion rates. E-MSCs-EM cells were previously defined as E-cadherin negative cells that migrate to ectopic sites in vivo and are crucial in pathogenesis.[24] Ectopic endometrial cells, a cause of EM, create an inflammatory environment in peritoneal cavity. The precise mechanism is not yet understood, but it may involve NF-κB signaling, triggered by tumor necrosis factor-α and CXCL12, to regulate cell behavior. The expression of MAPK was also regulated by NF-κB signaling, and the suppression of MAPK expression might also control the chronic inflammation observed in EM.

Main findings

This study discovered that CXCL12 expression was elevated in human endometriotic lesions compared to healthy controls, consistent with findings from previous research.[26] The production of CXCL12 may be reduced by hormone-based therapies, which are frequently employed to treat EM. This implies that progestins may impart some of their effectiveness through this mechanism.[27] Dienogest, which acts as a progesterone receptor agonist, downregulates CXCL12 expression and beta-catenin, a key regulator of the canonical Wnt pathway. This pathway is known to be abnormally activated in EM.[3,28] Dienogest treatment suppressed both Wnt/β-catenin and CXCL12, potentially resulting in a reduction in stem cell recruitment.

Although the progesterone receptor was not detected in E-MSCs through immune staining,[29] other studies have revealed that these cells exhibit responsiveness to progesterone by reducing the decidualization markers prolactin and IGFBP-1.[30,31] We demonstrated that the *NR3C3* gene, which encodes the progesterone receptor, is expressed by the MSCs isolated from the ectopic endometrial tissue. Dienogest impacts the E-MSCs in terms of cell proliferation, migration, and telomerase activity. Previous research does not provide a definitive explanation regarding the origin of EM or the role of stem cells in its early onset. However, it has been revealed that

stem cells play a significant role in EM pathogenesis. Pharmaceutical agents that are employed to manage EM would evidently affect these stem cells, which would subsequently modulate surrounding cells and contribute to mitigating EM-related pathology. The study discovered that dienogest significantly reduced telomerase activity, proliferation, and migration of E-MSCs derived from ectopic EM tissue. This implies that dienogest may reduce the mitotic activity of E-MSCs in EM patients by inhibiting its pathogenesis through a reduction in CXCL12, Ki67, beta-catenin, and MAPK expression levels. Although stem cells have not been demonstrated to directly involve in EM, their tissue-supporting characteristics might have a detrimental impact on the tissue and result in chronic inflammation. These findings indicate that endometrial stem cells are involved in the pathogenesis of EM, either directly or indirectly.

Ethics Committee Approval: This study was approved by the Kocaeli University Institutional Ethics Committee (approval number: KÜ GOKAEK 2020/4.01, project number: 2020/55, date: 12.03.2020).

Informed Consent: Written informed consent was obtained from each donor.

Data Sharing Statement: The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Authorship Contributions: Concept- H.U.Ş., T.Ş.; Design- H.U.Ş., T.Ş., G.D.; Supervision- Y.Y.; Materials- H.U.Ş., T.Ş.; Data Collection or Processing- H.U.Ş., G.D.; Analysis or Interpretation- H.U.Ş., G.D., S.F.R.; Literature Review- H.U.Ş., T.Ş., S.F.R., Y.Y.; Writing- H.U.Ş., T.Ş., G.D., S.F.R.; Critical Review- T.Ş., Y.Y.

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