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ORIGINAL ARTICLE

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The extracellular signal-regulated kinase 1/2 triggers angiogenesis in human ectopic endometrial implants by inducing angioblast differentiation and proliferation

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Problem: The role of extracellular signal-regulated kinase (ERK)1/2-mediated angiogenesis during endometriotic nidation is unknown. We posit that ERK1/2-induced angioblast differentiation and proliferation promotes ectopic endometrial angiogenesis.

Methods of study: Human eutopic and ectopic endometria were immunostained for total- (T-) or phosphorylated- (P-) ERK1/2 or double-immunostained for P-ERK1/2-CD34 and PCNA-CD34. Estradiol (E_2), cytokines, normal peritoneal fluid (NPF) or endometriotic peritoneal fluid (EPF) ±PD98059, an ERK1/2 inhibitor, treaded primary human endometrial endothelial cells (HEECs) were evaluated by T-/P-ERK1/2 immunoblotting, MTT viability and tube formation assays.

Results: HEECs exhibited higher endothelial P-ERK1/2 immunoreactivity in ectopic vs eutopic endometria. Double-immunostained ectopic endometria displayed abundant CD34-positive angioblasts exhibiting strong P-ERK1/2 and PCNA immunoreactivity. EPF and vascular growth factor (VEGF)-A significantly increased HEEC proliferation and P-ERK1/2 levels. PD98059 reduced basal, EPF, and VEGF-induced HEEC proliferation and promoted vascular stabilization following tube formation.

Conclusion: Enhanced ERK1/2 activity in angioblasts by such peritoneal factors as VEGF, E_2 induces proliferation to trigger ectopic endometrial angiogenesis.

KEYWORDS

angioblast, angiogenesis, endometriosis, ERK1/2 MAPK, peritoneal fluid, proliferation

1 | INTRODUCTION

Endometriosis is a chronic inflammatory and estrogen-dependent disease characterized by the presence of endometrial tissue outside of the endometrium, with the ovarian surface, pelvic cavity, and occasionally extra pelvic organs.[1,2] Endometriosis affects more than 10%-15% of reproductive age women.[3] Unlike normal endometrium, aberrant regulation of ectopic endometrial implants by ovarian steroids results in endometriotic tissue growth accompanied by severe dysmenorrhea, chronic pelvic pain, and infertility.[2] Several etiologies for this disorder have been postulated, including coelomic metaplasia of cells lining the visceral and abdominal peritoneum and/or stem cells that persist in the embryonic remnants of the Mullerian system.[4,5] However, the most accepted etiological theory is retrograde menstruation, which enables recruitment of viable endometrial tissues into ectopic sites such as the peritoneal cavity with subsequent cellular attachment to peritoneal surfaces, implantation, and neovascularization prompting endometriotic tissue growth.[3,4] Previous studies demonstrated elevated peritoneal levels of pro-inflammatory chemokines monocyte

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chemo-attractant protein (MCP)-1, Interleukin (IL)-8, fractalkine, and increased inflammatory cell recruitment, and/or elevated levels of IL-6 and prostaglandins associated with pelvic pain in women with endometriosis.[6-10]

Mitogen-activated protein kinases (MAPKs) are intracellular signaling proteins located at the intersection of several signaling cascades regulating pathophysiological mechanisms ranging from inflammation, cell survival, and oxidative stress to tumorigenesis.[11] The extracellular signal-regulated kinase (ERK)-1 and 2 are 42 kDa and 44 kDa MAPKs, respectively, that are activated via phosphorylation mediated by steroids, cytokines, and/or carcinogens. Increased ERK1/2 activity modulates cell differentiation, survival, motility, and cytokine expression.[12]

Angiogenesis plays an essential role in the growth and survival of endometriotic lesions.[8] Previously, we and others demonstrated that ERK1/2 signaling contributes to growth of endometriotic lesions by enhancing proliferation of endometriotic stromal cells.[11,12] Our prior in situ analysis revealed that phosphorylated ERK1/2 (P-ERK1/2) exhibits menstrual cycle-dependent changes in normal endometrial stromal and glandular cells and is activated at abnormally high levels in eutopic and ectopic endometrium of women with endometriosis.[11] Inhibition of ERK1/2 in primary cultures of human endometrial stromal cells reduced proliferation and increased apoptosis. Estradiol (E_2) treatment does not increase P-ERK1/2 level in human endometrial stromal cell cultures derived from eutopic endometrium of women without endometriosis. However, E_2 significantly enhances P-ERK1/2 levels in cultured human endometrial stromal cells isolated from eutopic endometrium of women with endometriosis, suggesting that aberrant estrogen-mediated activation of ERK1/2 signaling stimulates endometriotic cell survival.[11] Similarly, E_2 treatment enhances MCP-1 and IL-8 transcription and secretion in cultured human endometrial endothelial cells (HEECs) isolated from women with endometriosis, but not from women without endometriosis. Such elevated secretion of endothelial cell-derived MCP-1 and IL-8 is implicated in promoting endometriosis-related inflammation by recruiting monocytes and neutrophils.[13,14] However, the intracellular mechanism(s) of E_2-induced endothelial cell-mediated increases in MCP-1 and IL-8 levels in endometriosis is/are not known. Moreover, whether E_2-induced endothelial MCP-1 and IL-8 enhance ERK1/2 activity in HEECs in an autocrine manner requires investigation as MCP-1 elevates P-ERK1/2 levels in several cell types.[15,16]

In view of our previous observations of the role played by ERK1/2 in human endometrial stromal cell proliferation, we hypothesized that endometriotic pro-inflammatory milieu and/or local continuous E_2 production induce endothelial progenitor cell (angioblast) differentiation and survival by enhancing ERK1/2 activity, eliciting increased vascularization of ectopic endometrial implants. Thus, the current study compared ERK1/2 activity in endothelial cells in eutopic and ectopic endometrial sections and investigated both in vitro regulation and the role of ERK1/2 activity in HEEC differentiation, survival, angiogenesis, and expression of pro-angiogenic cytokines MCP-1, IL-8, and vascular growth factor (VEGF).[8,17,18]

2 | MATERIALS & METHODS

2.1 | Tissue collection

Serial paraffin sections of endometrial specimens were obtained from Yale Pathology Tissue Services under the protocol approved by the Yale Human Investigation Committee. For this study, five eutopic endometrial and 11 paired eutopic-ectopic endometrial specimens were obtained from women with endometriosis undergoing laparoscopy for infertility or pelvic pain and used for immunohistochemical analysis. The menstrual cycle day was established from each woman's menstrual history and confirmed by endometrial histology using the criteria of Noyes et al.[19] Ectopic endometrial implants in the sections were confirmed by histological examination. Endometrial samples were grouped according to menstrual cycle phase: proliferative eutopic (n = 7) or proliferative ectopic (n = 6) (days 1-14 of the cycle) or secretory eutopic (n = 9) or secretory ectopic (n = 5) (days 15-28). For in vitro studies, previously frozen HEECs (n = 6) from normal women undergoing laparoscopy for myomectomy were thawed and grown to confluence as described.[20]

2.2 | Immunohistochemistry

Deparaffinized 5-μm serial endometrial sections were boiled in 10 mmol/L citrate buffer (pH 6.0) for 15 minute for antigen retrieval, then immersed in 3% H_2O_2 (in 1/1 methanol/distilled water) for 10 minute to quench endogenous peroxidase activity. After washing with Tris-buffered saline (TBS; pH: 7.4) ×3 for 5 minute, the slides were incubated with 5% blocking goat serum (Vector Labs, Burlingame, CA, USA) in TBS for 30 minute at RT. Excess serum was drained, and the slides were incubated with either rabbit polyclonal antitotal (T)-ERK1/2 or rabbit monoclonal antiphosphorylated (P)-ERK1/2 (Thr202/Tyr204; Cell Signaling Technology, Danvers, MA, USA) in 1% normal goat serum overnight at 4°C. After several washes, biotinylated goat anti-rabbit IgG (Vector Labs) was added for 30 minute at RT. The antigen-antibody complex was detected using an avidin-biotin-peroxidase kit (Vector Labs) for 30 minute at RT and visualized using DAB (3, 3-diaminobenzidine tetrahydrochloride dihydrate; Vector Labs). Sections were counterstained with hematoxylin.

To identify endothelial progenitor cells and their proliferative capacity, a second set of serial sections was double-immunostained with antibodies either for P-ERK1/2 and CD34 (an endothelial cell progenitor marker; DAKO, Glostrup, Denmark) or for proliferating cell nuclear antigen (PCNA; Sigma-Aldrich, St. Louis, MO, USA) and CD34, respectively. Immunostaining for P-ERK1/2 was performed by following the steps described above. Following development of P-ERK1/2 immunoreactivity by DAB, the slides were washed ×3 in TBS, blocked with 5% horse serum (Vector Labs) for 30 minute, and double-immunostained using mouse monoclonal anti-CD34 overnight at 4°C. After several washes, biotinylated horse anti-mouse or goat anti-rabbit (Vector Labs) IgG was added for 30 minute at RT. The antigen-antibody complex was detected using an

avidin-biotin-alkaline phosphatase kit (Vector Labs) for 30 minute at RT. The chromogen, Vector Red (Vector Labs) visualized CD34 immunoreactivity. Sections were then counterstained with hematoxylin. For negative controls, normal rabbit IgG or normal mouse IgG isotypes (Vector Labs) were used at the same primary antibody concentrations. Similar steps were applied for PCNA and CD34 double immunostaining of serial sections.

Immunoreactive T-ERK1/2 and P-ERK1/2 levels were semiquantitatively evaluated using the following intensity categories: 0, no staining; 1+, weak but detectable staining; 2+, moderate or distinct staining; 3+, intense staining to generate a histological score (HSCORE) value as described previously.[21] Each slide was evaluated by two investigators blinded to the type and source of the tissues. The average HSCORE of two examiners was used.

2.3 | HEEC cultures and experimental setup

Aliquots of frozen primary HEECs (n = 6 patients) previously isolated and characterized[20,22] were grown in EGM-2 MV medium containing 2% fetal bovine serum and endothelial supplements (Cambrex, Walkersville, MD, USA). Confluent HEECs were treated with vehicle (control) or 10^{-8} mol/L E_2 (Sigma-Aldrich) or 2 ng/mL IL-8 (R&D Systems, Minneapolis, MN, USA) or 2 ng/mL MCP-1 (R&D Systems) or 1 ng/mL vascular growth factor A (VEGF-A; R&D Systems) for 15 minute followed by protein extraction using a buffer (BioSource International, Camarillo, CA, USA) containing 3 mmol/L phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich) for immunoblotting analysis of T-ERK1/2 and P-ERK1/2 levels. To determine cell viability, confluent HEECs were seeded at 2×10^4 cells/well in 96-well plates and then treated for 48 hour with vehicle (control) or 20-μmol/L PD98059 (PD, specific ERK1/2 inhibitor) or normal peritoneal fluid (NPF, n = 3) or endometriotic peritoneal fluid (EPF, n = 3) from women with severe endometriosis. Severe endometriosis was diagnosed during laparoscopy based on the staging system designed by ASRM.[23] In this system, stage IV endometriosis is the same as severe endometriosis.

2.4 | Immunoblot analysis

The protein concentration was determined by a detergent-compatible protein assay (Bio-Rad, Hercules, CA, USA). Immunoblotting was performed as described.[24] Briefly, samples (20 μg) were loaded on 10% Tris-HCl Ready Gels (Bio-Rad), electrophoretically separated, and blotted onto nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% non-fat dry milk in TBS containing 0.1% Tween-20 (TBS-T) for 1 hour, then washed and incubated overnight with rabbit anti-P-ERK1/2 (Thr202/Tyr204; Cell Signaling Technology) in 5% non-fat dry milk. After several TBS-T washes, the membrane were incubated with peroxidase-conjugated anti-rabbit IgG (Vector Labs) and P-ERK1/2 signals were developed on a X-ray film (Denville Scientific, Holliston, MA, USA) using a chemiluminescence kit (Thermo-Scientific, Rockford, IL, USA). The membrane was sequentially stripped and reprobed with rabbit antibody for T-ERK1/2 (Cell Signaling Technology) or with rabbit antibody for β-actin (Cell Signaling Technology). Band intensities were quantified using ImageJ (NIH, Bethesda, MD, USA).

2.5 | 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay

Cell viability was assessed by colorimetric assays using an MTT kit (XTT proliferation kit; Cell Signaling Technology) as described previously.[11] Four hours before the end of each experiment, 10 μL of MTT solution was added onto each well of a 96-well plate containing control or PD- or VEGF- or NPF- or EPF-treated HEECs for 48 hour. The optical absorbance at 450 nm was read within 30 minute. The last column in each plate did not contain cells and was used as a blank.

2.6 | Tube formation assay

96-well culture plates coated with Matrigel (Matrigel® basement membrane matrix growth factor-reduced phenol-red free; Corning Inc., Corning, NY, USA) were incubated at 37°C for 30 minute to solidify the gel. In each well, 15×10^3 HEECs/100 μL (n = 4 with two replicates) with vehicle (control) or PD (2×10^{-5} mol/L) were pipetted onto Matrigel. Tube formation was photographed every 4 hour under a phase-contrast microscope (Zeiss, Jena, Germany). As reported[20,25,26], Angiogenesis Analyzer, an ImageJ (NIH) software plug-in digitally quantified in vitro angiogenesis parameters (Figure S1).

2.7 | Reverse transcription and quantitative real-time (q) PCR analysis

Total RNA from cultured HEEC was isolated using miRNeasy Mini kit according to the manufacturer's instructions (Qiagen, Germantown, MD, USA). Reverse transcription and q-PCR were performed as described.[24] The expression of VEGF, IL-8, and MCP-1 and β-actin were each determined by q-PCR using TaqMan Gene Expression Assays (VEGF, Hs00900055_m1; IL-8, Hs00174103_m1; MCP-1 Hs00161204_m1; and β-actin Hs99999903_m1 according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). All samples were run in triplicate, and the average was used for each sample. Expression of the target mRNAs was normalized to β-actin levels, and the $2^{-\Delta\Delta Ct}$ (cycle threshold) method was used to calculate relative expression levels. Results are reported as fold change in gene expression levels among the different groups.

2.8 | Statistical analysis

Data from immunohistochemistry, cell proliferation assay, Western blot analysis, and the majority of tube formation parameters were normally distributed (as determined by the Kolmogorov-Smirnov test); therefore, comparisons of samples were analyzed with Student's *t* test or one-way ANOVA followed by the post hoc Holm-Sidak test. As some of the data for tube formation parameters were not normally distributed, they were analyzed with nonparametric ANOVA on ranks (Kruskal-Wallis test) followed by the post hoc Student-Newman-Keuls test. Statistical

calculations were performed using SigmaStat (Jandel Scientific Corp., San Rafael, CA, USA). Statistical significance was defined as $P < .05$.

3 | RESULTS

3.1 | In situ regulation of ERK1/2 expression and phosphorylation in eutopic and ectopic HEECs

In both eutopic and ectopic endometrial specimens, T-ERK1/2 immunostaining was detected in the cytoplasm and nucleus of HEECs with no significant changes evident between proliferative or secretory phases. Moreover, T-ERK1/2 HSCOREs were not significantly different in HEECs between sections of eutopic vs ectopic specimens in either the proliferative (mean ± SEM: 214.03 ± 9.06 vs 198.74 ± 15.29; $P > .05$) or secretory phase (209.44 ± 14.63 vs 218.33 ± 13.74; $P > .05$; Figure 1).

P-ERK1/2 immunostaining was primarily nuclear in both eutopic and ectopic HEECs. Eutopic HEECs displayed moderate P-ERK1/2 immunostaining in both proliferative and secretory phases with no significant change between the phases, whereas ectopic HEECs exhibited strong P-ERK1/2 immunostaining in both proliferative and secretory specimens with no significant change between the phases (Figure 1). Ectopic HEECs displayed significantly higher P-ERK1/2 HSCOREs compared to eutopic HEECs in both proliferative (mean ± SEM: 229.97 ± 17.24 vs 116.42 ± 13.97; $P < .001$) and secretory phases (244.33 ± 11.24 vs 160.93 ± 12.57; $P < .001$).

Noteworthy, in the stroma of ectopic implants, some individual cells and/or cell clusters, morphologically resembling newly vascularized areas with endothelial progenitor cells, displayed the strongest P-ERK1/2 immunoreactivity, whereas such immunoreactivity was weaker in the endothelial cells of peritoneal tissue (Figure 2A-B). Further corroboration of the angioblastic nature of these cell clusters at the molecular level used double immunostaining, which revealed that the cell clusters exhibiting the strongest P-ERK1/2 immunoreactivity also co-express CD34 (an endothelial progenitor cell marker), thus confirming these cells as endothelial progenitor cells (Figure 2C-E). Furthermore, CD34 and PCNA double immunostaining confirmed that these cells are also strongly positive for PCNA, indicating their highly proliferative aspect that is associated with P-ERK1/2 immunostaining (Figure 2F-G).

3.2 | VEGF-induced ERK1/2 phosphorylation (activity) stimulates HEEC proliferation

Immunoblotting of confluent HEEC cultures treated for 15 minute with vehicle (control) or 10^{-8} mol/L E_2 or 2 ng/mL IL-8 or 2 ng/mL

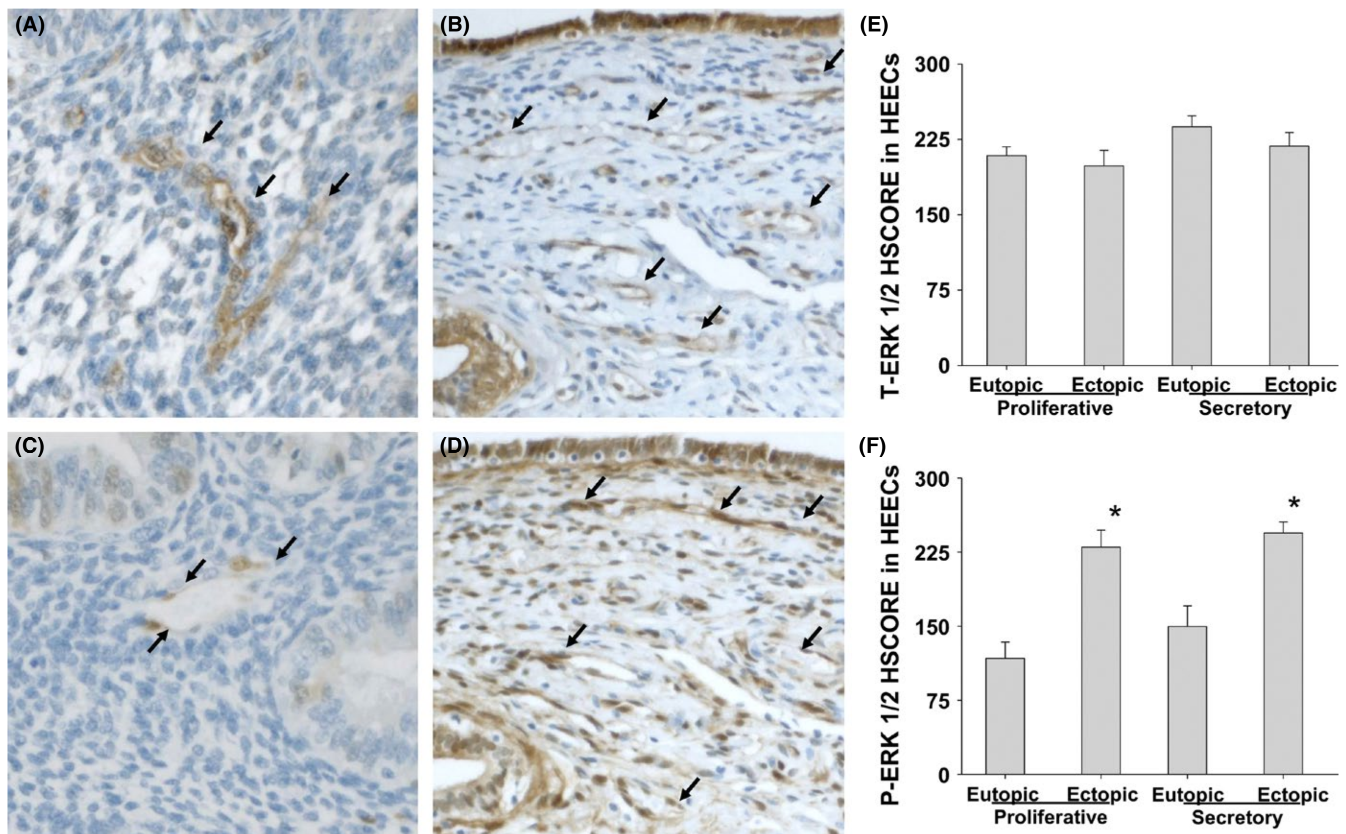

FIGURE 1 Increased phosphorylated extracellular signal-regulated kinase (ERK)1/2 immunoreactivity in human endometrial endothelial cells (HEECs) in ectopic endometrial specimens. T-ERK1/2 (A, B) and P-ERK1/2 (B, C) immunoreactivity in HEECs (arrows) in paired eutopic (A, C) and ectopic (B, D) endometrial specimens obtained from the secretory phase. Graphs represent histological score (HSCORE) values in HEECs for T-ERK1/2 (E) and P-ERK1/2 (F) in proliferative eutopic (n = 7) vs ectopic (n = 6) and secretory eutopic (n = 9) vs ectopic (n = 5) specimens. Bars represent mean ± SEM. *$P < .005$ compared to menstrual cycle-matched eutopic specimens

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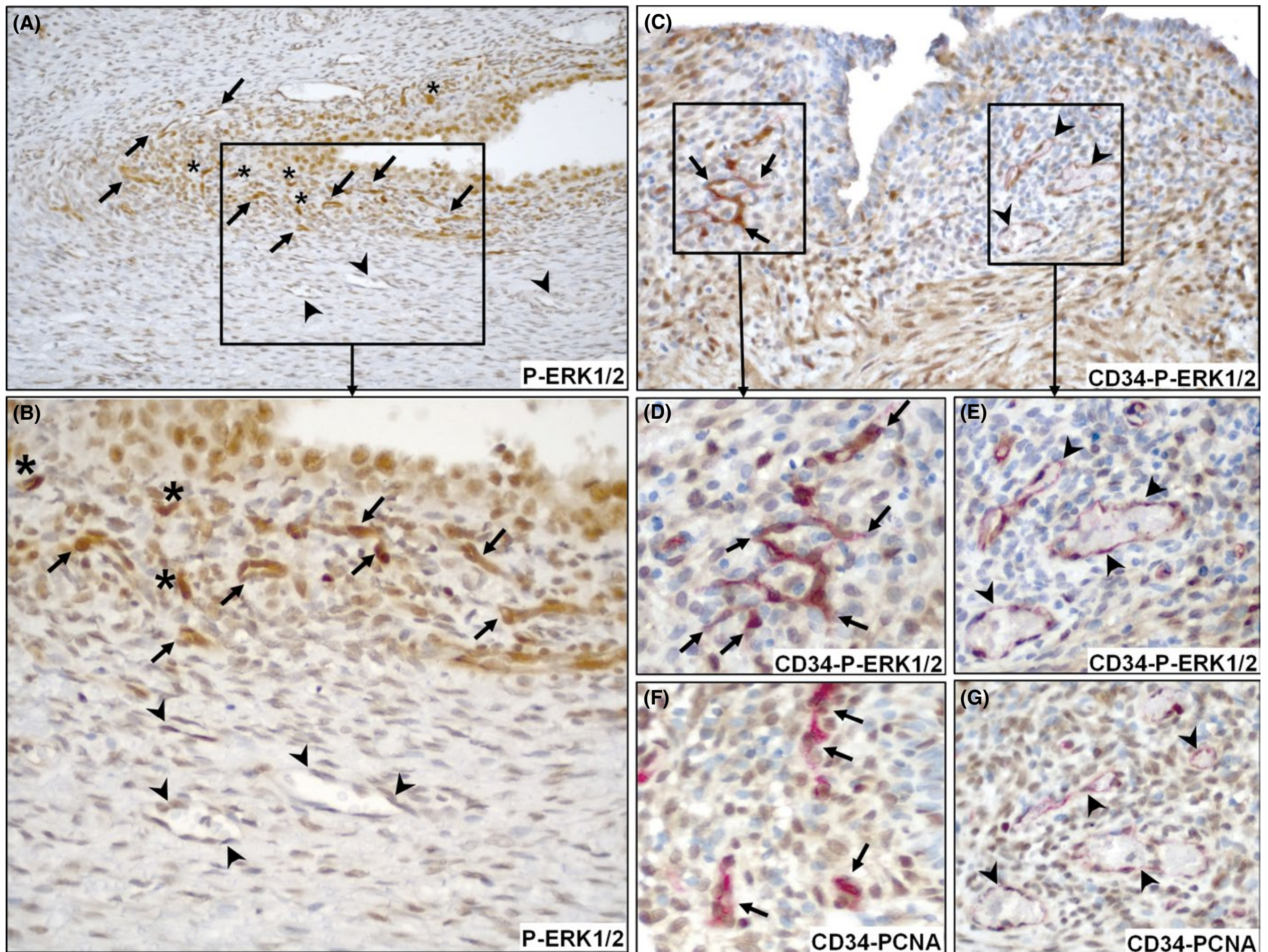

FIGURE 2 P-ERK1/2 involves in endothelial progenitor cell (angioblast) differentiation and proliferation. Several individual cells (asterisks) and cell clusters of vascular-like structures (presumptive vessels; arrows) display the strongest P-ERK1/2 immunoreactivity in the ectopic endometrium, whereas endothelial cells in distal to ectopic tissue (arrowheads) exhibit weak to moderate P-ERK1/2 immunoreactivity (A, B). CD34 (red) and P-ERK1/2 (brown) double immunostaining is seen in ectopic endometrial specimens (C-E) with stronger ERK1/2 (brown) immunoreactivity in CD34 immunoreactive (red) endothelial progenitor cells (arrows; C or D) vs mature vascular endothelial cells (arrowheads in C or E). Representative photomicrographs of double-immunostained ectopic endometrial specimens show stronger co-expression of CD34 (red) and PCNA (brown) in endothelial progenitor cells (arrows; F) than in mature vascular endothelial cells (arrowheads; G)

MCP-1 or 1 ng/mL VEGF revealed that VEGF exerts the strongest effect on ERK1/2 phosphorylation compared to weak to moderate effects elicited by E_2 or MCP-1 or IL-8 (Figure 3A). Subsequent experiments determined that compared to control, the impact of VEGF on P-ERK1/2 levels is statistically significant and inhibited by the ERK1/2-specific inhibitor PD98059 (2×10^{-5} mol/L; Figure 3B).

In parallel experiments, a cell proliferation assay was performed to complement our in situ observation of highly proliferative HEECs in endometrial implants as well as to determine whether ERK1/2 activity mediates HEEC proliferation. This analysis revealed a significant increase in HEEC proliferation after 48-hour treatment with VEGF vs control ($P < .01$) and that PD98059 completely blocks this VEGF effect on HEECs (Figure 3C).

As the components of peritoneal fluid contribute to the development of endometriosis, parallel proliferation assays were performed in HEECs treated with NPF or EPF with or without the ERK1/2 inhibitor. Compared to NPF, EPF treatment resulted in a significantly higher HEEC proliferation index, which was blocked by co-treatment with the ERK1/2 inhibitor ($P < .05$; Figure 3D), providing in situ evidence for the involvement of the ERK1/2 signaling pathway in endometriotic HEEC proliferation.

3.3 | ERK1/2-mediated regulation of in vitro tube formation in HEECs

In addition to proliferation, a well-established in vitro tube formation assay was used to assess the role of P-ERK1/2 activation on later stages of angiogenesis such as tubulogenesis (endothelial cell tube formation), vessel fusion, branching, and/or stabilization following treatment of HEECs seeded on growth factor-reduced Matrigel (vehicle) ±PD98059 or ±VEGF or ±VEGF + PD. Tube formation was photographically monitored every 4 hour under a phase-contrast microscope. The ImageJ plug-in Angiogenesis Analyzer measured

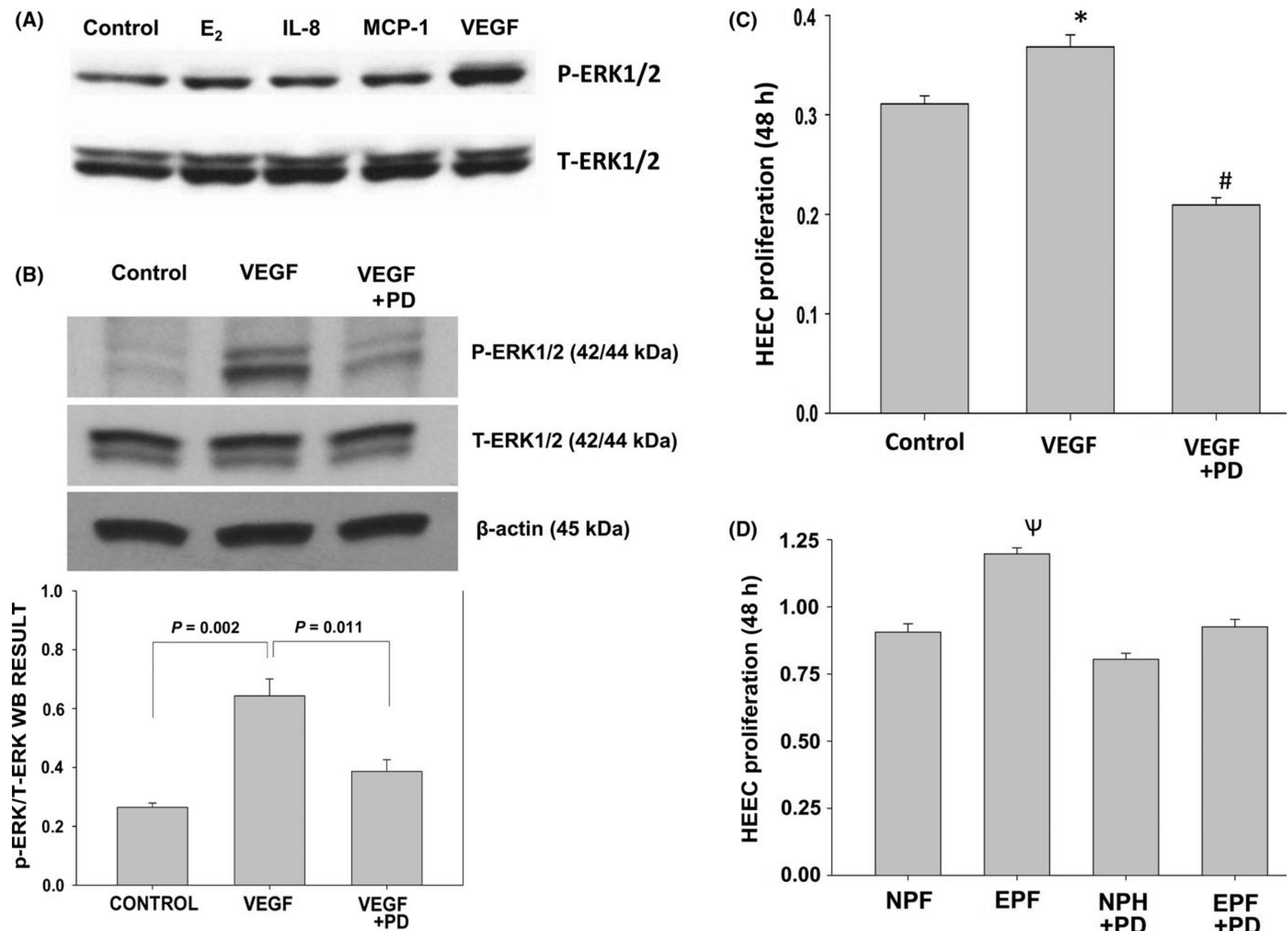

FIGURE 3 Vascular growth factor (VEGF)- and endometriotic peritoneal fluid-induced increase in P-ERK1/2 expression involves human endometrial endothelial cells (HEEC) proliferation. P-ERK1/2 levels in HEECs treated with control or estradiol (E_2; 10^{-8}M) or interleukin-8 (IL-8; 2 ng) or monocyte chemotactic protein-1 (monocyte chemo-attractant protein [MCP]-1; 2 ng) or vascular endothelial growth factor (VEFG; 1 ng) (A). Regulation of P-ERK levels (B) and proliferation (C) in HEECs treated with control or VEFG (1 ng) or vascular growth factor (VEGF) + PD98059 (VEGF + PD; 2×10^{-5} mol/L) for 30 min and 48 h, respectively. Bars represent mean ± SEM. $^{*}P < .001$ vs control or VEGF + PD; $^{\#}P < .001$ vs control. HEECs were treated with normal peritoneal fluid (NPF) or endometriotic peritoneal fluid (EPF) or NPF + PD98059 (2×10^{-5} mol/L) or EPF + PD98059 (2×10^{-5} mol/L). Bars represent mean ± SEM (n = 3). $^{\Psi}P < .001$ vs NPF or NPF + PD or EPF +PD

several angiogenesis parameters (Figure S1). HEECs seeded on growth factor-reduced Matrigel began to form tube-like structures, branches, junctions, and a mesh-like network at 4 hour, attaining the highest levels at 12 hour for all the parameters. Addition of PD98059 did not significantly affect HEEC tube formation and/or branching during this period (Figure 4). However, at 24 hour and thereafter, HEECs seeded on Matrigel exhibited disruption of these newly formed tubes as well as disintegration of their branches. Unexpectedly, PD98059 inhibited such tube disruption (regression) as well as branch disintegration, which attained statistical significance at 48 hour of incubation (Figure 4). In parallel experiments, VEGF treatment of HEECs did not significantly increase Matrigel-induced tube formation at any evaluated time point. Furthermore, the addition of PD98059 did not significantly alter VEGF-HEEC tube formation and/or branching. Similar to HEEC tube formation on Matrigel alone, disruption of tube-like structures as well as disintegration of branches was observed after 24 hour of VEGF treatment with the addition of PD98059 inhibiting such tube disruption as well as branch disintegration (Figure 4).

As VEGF did not significantly induce tube formation by HEECs seeded on Matrigel, P-ERK1/2 immunoblotting was performed in HEECs treated with Matrigel ±PD98059 or ±VEGF (10 ng/mL) or ±VEGF + PD98059 for 30 minute to determine whether VEGF generates an additional effect on P-ERK 1/2 levels in HEECs grown on Matrigel. This analysis revealed that compared to Matrigel alone, VEGF did not further increase HEEC P-ERK 1/2 levels and that PD98059 inhibited P-ERK 1/2 levels in HEECs grown on either Matrigel alone or Matrigel + VEGF (Figure 5).

3.4 | ERK1/2-mediated regulation of cytokine expression in HEECs

To evaluate the contribution that enhanced P-ERK1/2 activity exerts on expression of the pro-angiogenic molecules MCP-1, IL-8, or VEGF

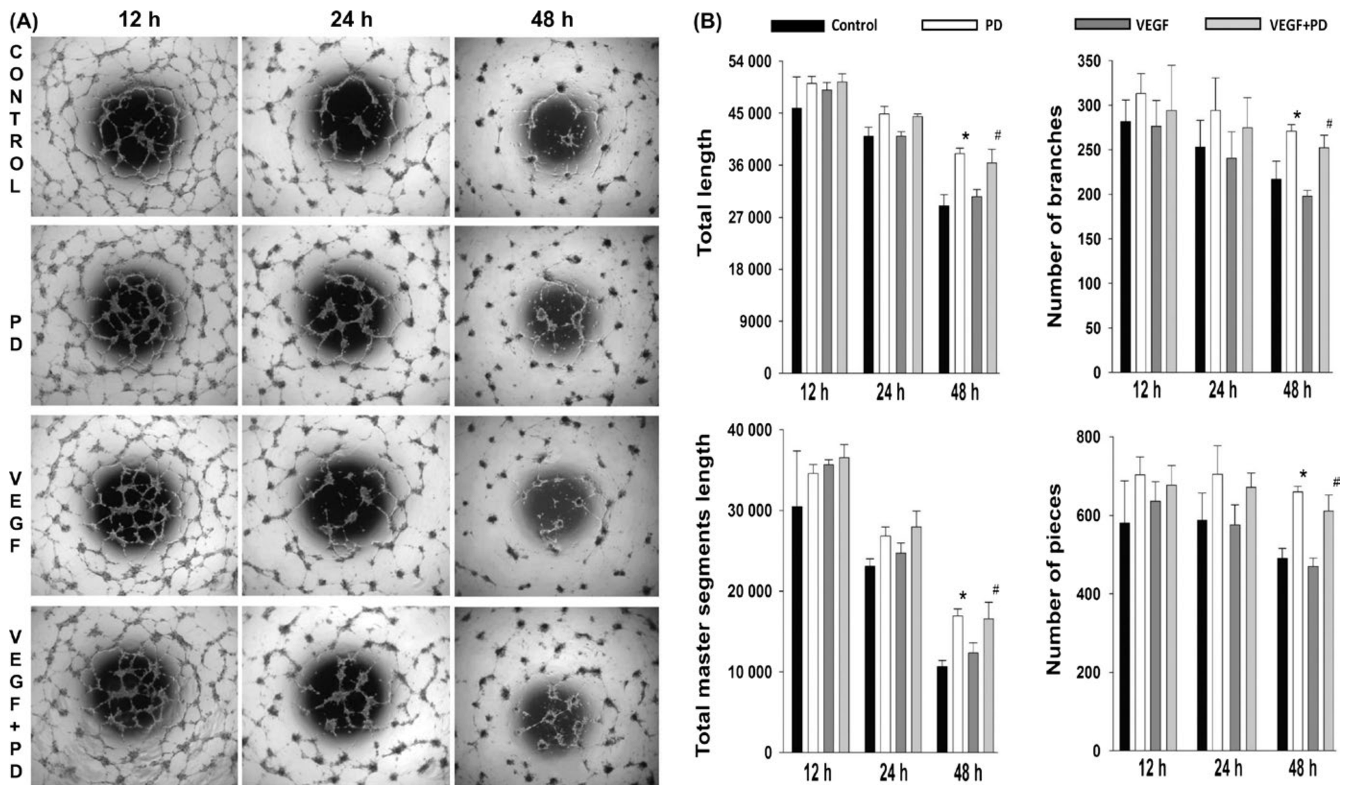

FIGURE 4 Regulation and stabilization of human endometrial endothelial cells (HEEC) tube formation by extracellular signal-regulated kinase (ERK)1/2 activity. HEECs grown on growth factor-reduced Matrigel (control) ±PD98059 (PD) or ±VEGF (10 ng/mL) or ±VEGF + PD for 48 h. Tube formation was photographed under phase-contrast microscopy after 12 h, 24 h, and 48 h (A). Graphs display ImageJ-quantified angiogenic parameters found to be significantly different among the groups, which include total length (sum of length of segments, isolated elements and branches), total master segment length (sum of the length of the detected master segments), number of branches in the analyzed area, and number of pieces (sum of number of segments, isolated elements, and branches detected) (B). The analyzed area in each well was 5002624 pixels, and the measurement unit is presented in pixels. Bars represent mean ± SEM (n = 4). $^{*}P < .05$ vs control at 48 h; $^{\#}P < .05$ vs vascular growth factor (VEGF)

in endothelial cells, confluent HEEC cultures treated with vehicle (control) or VEGF (10 ng/mL) or VEGF ± PD98059 for 6 hour were evaluated by qRT-PCR. This analysis revealed that compared to control, VEGF significantly induces both IL-8 and MCP-1 mRNA ($P < .001$), but not VEGF mRNA levels (Figure 6A-C), and that PD98059 blocked VEGF-induced MCP-1 mRNA increases, but not increases in IL-8 mRNA levels. Unexpectedly, PD98059 induced VEGF mRNA levels in these HEEC cultures (Figure 6C).

4 | DISCUSSION

Survival of ectopic endometrial implants necessitates a suitable local environment to both protect against cell-mediated immune attack and provide a vascular network to perfuse developing extra-uterine endometrial tissues.[13] Levels of angiogenic and growth factors in the peritoneal fluid of patients with endometriosis have been shown to correlate with the morphological severity of the disease.[27,28] In addition to genetic and hormonal changes, paracrine and autocrine growth-promoting factors as well as pro-angiogenic and pro-inflammatory cytokines contribute to the development of endometriosis by impairing immune regulation of endometrial tissue.[3,27,29]

A recent study by Luo et al[30] showed enhanced Toll-like receptor 4 (TLR4) expression the ectopic endometrium and stimulation of IL-8 secretion by TLR4 ligands in ERK1/2 and p38 MAPK-dependent manner in endometriotic stromal cells. Moreover, a previous report from our laboratory demonstrated the expression and regulation of T-ERK1/2 and P-ERK1/2 in endometrial stromal and glandular cells in women with or without endometriosis.[11] That study showed that T-ERK1/2 expression remained constant in stromal and glandular cells of eutopic and ectopic endometria throughout the menstrual cycle. Similarly, levels of P-ERK1/2 expression were unaffected in glandular cells in both eutopic and ectopic endometrial tissues. However, significantly lower P-ERK1/2 expression was observed in stromal cells from the early-mid-proliferative phase compared to the early-mid-secretory phase.[11]

The current observations indicating no menstrual cycle-dependent change in T-ERK1/2 levels in either eutopic or ectopic HEECs parallel our prior findings in endometrial stromal and glandular cells.[11] Moreover, the absence of a menstrual cycle-dependent change in HEEC P-ERK1/2 levels is similar to P-ERK1/2 expression in endometriotic glandular cells. However, unlike stromal and glandular cells,

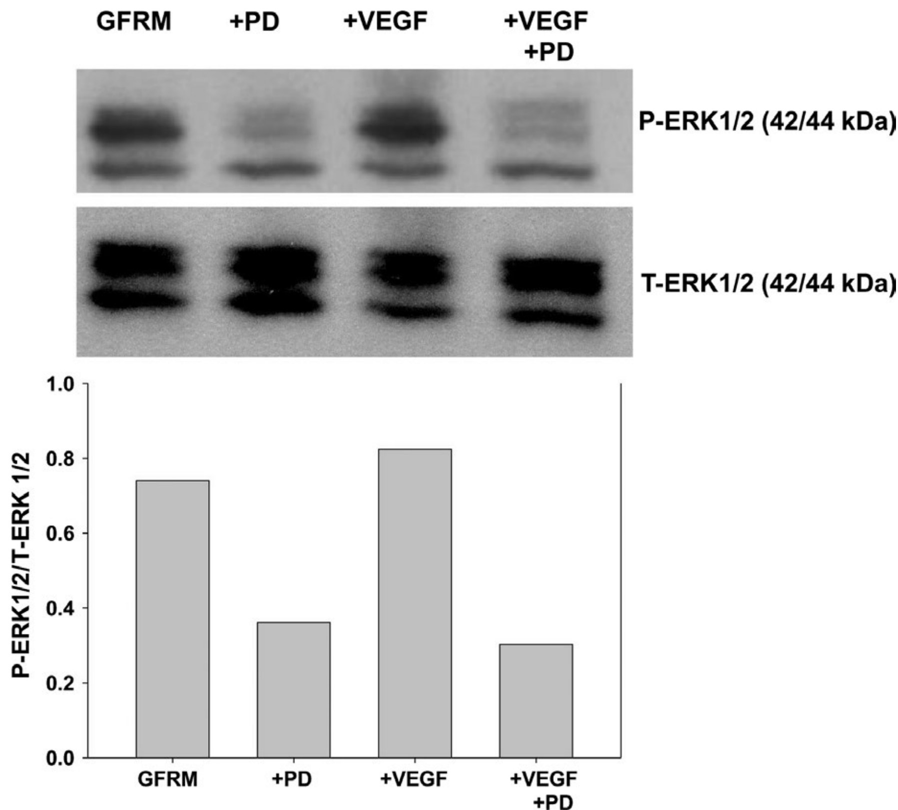

FIGURE 5 Regulation of extracellular signal-regulated kinase (ERK)1/2 activity (phosphorylation) by Matrigel. Representative immunoblotting in human endometrial endothelial cells (HEEC) cultures (n = 3) treated with growth factor-reduced Matrigel (GFRM) ±PD98059 (PD) or ±VEGF (10 ng/mL) or ±VEGF + PD for 30 min. Immunoblot bands for P-ERK1/2 and total ERK1/2 MAPK were quantified using ImageJ. Note that there was no additional increase in P-ERK1/2 levels by vascular growth factor (VEGF) treatment of HEECs seeded on GFRM

higher P-ERK1/2 phosphorylation in ectopic vs eutopic HEECs in both proliferative and secretory phases suggests that continuous activation of ERK1/2 MAPK signaling in HEECs may promote angiogenesis in ectopic implants. This premise is supported by our in situ observation of the presence of individual and/or clusters of endothelial progenitor cells as detected by CD34 expression (an endothelial progenitor cell marker as confirmed in our studies[31]) and complemented by the accompanying strongest P-ERK1/2 levels observed in these endothelial progenitors compared with any other cell type present in the ectopic implants.

Angiogenesis involves endothelial cell differentiation, proliferation, and migration to form new vessels from pre-existing vessels.[32] Endometriotic tissues also require these steps[33] to ultimately connect endometriotic vessels with the vascular network of the targeted ectopic endometrial nidation sites (peritoneum, ovarian surface, etc.). Moreover, aberrant neo-angiogenesis associated with pathological tissue growth has been documented in endometriosis.[33,34] The current in situ observation does not show the source of these CD34(+) endothelial progenitor cells in the ectopic endometria. However, the distal location of CD34(+) cells from pre-existing vessels observed in this study suggests that rather than originating from existing endothelial cells, these cells likely differentiate from stem cells (mesenchymal stem cells or angioblasts) located among the ectopic stromal cells. Further studies are required to uncover their exact source of these progenitor cells.

Regardless of origin, these presumptive vascular structures must ultimately connect to pre-existing vessels in resident tissue (peritoneum, etc.) to provide adequate perfusion of the developing extra-uterine endometrial tissues. The strong P-ERK1/2 immunostaining in these CD34(+) cells supports the involvement of ERK1/2 activity in the revascularization steps of ectopic endometrial tissue, that is, by modulating endothelial progenitor cell differentiation, proliferation, and/or migration. Moreover, the in situ observation of higher PCNA immunostaining in CD34(+) cells is consistent with the role of ERK1/2 activity in inducing their proliferation.

Expression of aromatase by endometriotic stromal cells accounts for the local increase in E_2 production in ectopic implants.[35] Moreover, the peritoneal fluid of women with endometriosis contains significantly higher levels of pro-angiogenic and/or pro-inflammatory cytokines VEGF, IL-8, and MCP-1.[17,36,37] Although the current study found VEGF to be the strongest stimulator of P-ERK1/2 activity, each of these factors exhibits a mild to moderate increase in P-ERK1/2 levels in cultured HEECs. Therefore, elevated levels of VEGF, IL-8, MCP-1, and E_2 in endometriosis suggest that their combined effect could additively or synergistically enhance endothelial P-ERK1/2 levels, supporting our in situ observation of amplified ERK1/2 phosphorylation in endometriotic endothelial cells. Furthermore, the current study also demonstrates that VEGF induces HEEC proliferation in an ERK1/2-dependent manner, as this proliferative effect of VEGF was completely blocked by PD98059. Supporting this observation, significantly greater induction of HEEC proliferation by endometriotic vs normal peritoneal fluid as well as blocking this effect by the ERK1/2 inhibitor PD98059 provides in situ confirmation that peritoneal factors from women

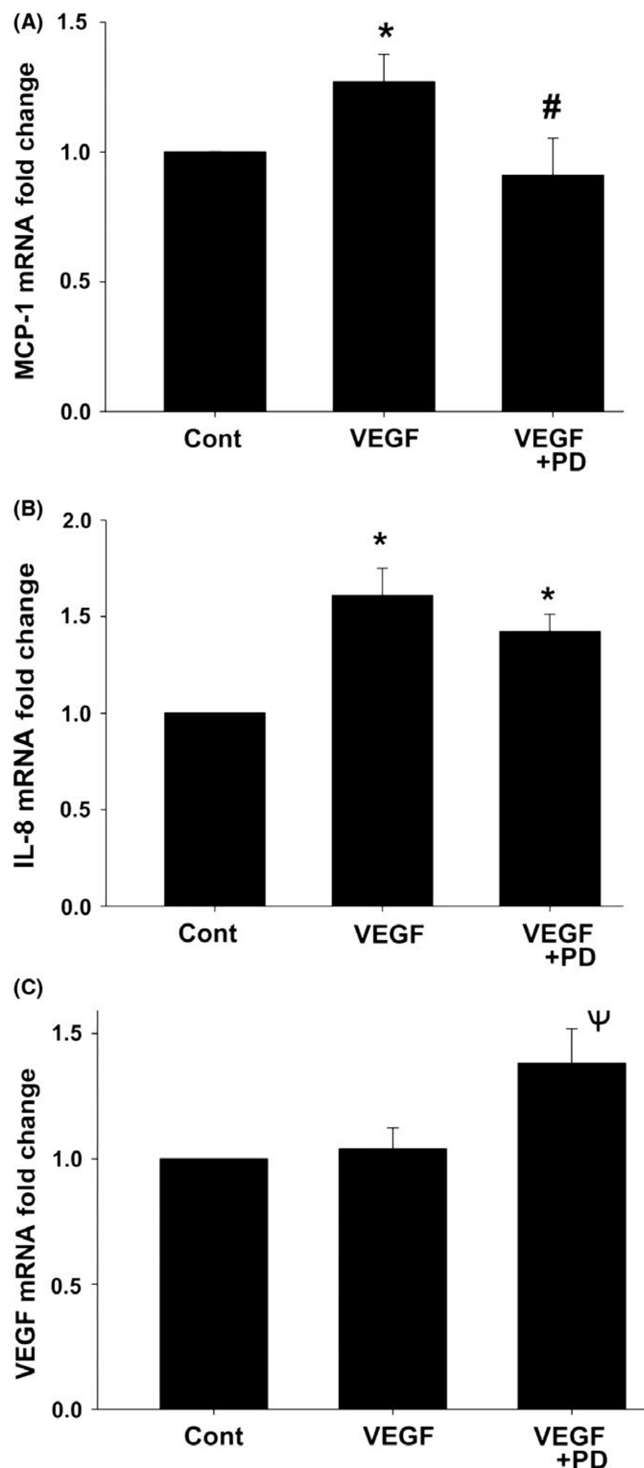

FIGURE 6 Regulation of angiogenic cytokines by extracellular signal-regulated kinase (ERK)1/2 signaling. monocyte chemo-attractant protein (MCP)-1 (A), IL-8 (B), and vascular growth factor (VEGF) (C) mRNA levels of n human endometrial endothelial cells (HEECs) treated with vehicle (control) or VEGF (10 ng/mL) or VEGF + PD98059 (PD, 2×10^{-5}M). Bars represent mean ± SEM (n = 6). $^{*}P < .05$ vs control; $^{\#}P < .05$ vs VEGF; $^{\Psi}P < .05$ vs control or VEGF

with endometriosis stimulate angiogenesis via endothelial cell proliferation/differentiation by triggering ERK1/2 signaling in ectopic implants.

In addition to endothelial cell proliferation, angiogenesis requires tubulogenesis (endothelial cell tube formation), branching, and stabilization.[32] Although several studies shown that inhibition of ERK1/2 activity does not affect tube formation by human umbilical vein[38] and dermal[39] or lung[40] microvascular endothelial cells, none of these previous studies evaluated the role of ERK1/2 activity in vascular stabilization following in vitro tube formation. Similar to those previous studies,[38-40] we found that PD98059-inhibition of ERK1/2 activity is ineffective on Matrigel- and/or Matrigel + VEGF-induced HEEC tube formation. As this study found that the role of ERK1/2 activity is limited to induction of endothelial cell proliferation, seeding sufficient endothelial cells on Matrigel required to mediate in vitro tube formation may mask the ERK1/2 proliferative effect during the 12 hour necessary to complete tube formation.

Conversely, our unexpected observation that ERK1/2 inhibition reduced tube degradation as well as branch disintegration at 24 hour and thereafter suggests that inhibition of ERK 1/2 activity is required to stabilize and/or mature newly formed vessels following angiogenesis. Support for this supposition is provided by the observation of weaker P-ERK1/2 immunoreactivity in endothelial cells of mature vessels compared to CD34 (+) endothelial progenitor cells in endometriotic tissues. These observations suggest that use of ERK1/2 inhibitors may prevent the development of endometriosis (ie, at the early stage), but is likely to be less effective in combating deep endometriosis, which contains an established vascular network within the resident tissue. A recent study[41] supports our observation that mRNA levels of the hypoxia-inducible factors (HIF)1 or HIF2α, protease-activated receptors (PARs) 1-4, and VEGF-A are significantly higher in ovarian endometriosis, but not in deep infiltrating endometriosis compared to endometrium from women without endometriosis.

The current observations in endothelial cells in combination with our previous observations in stromal and glandular cells suggest that increased ERK1/2 activity promotes endometriotic tissue growth by inducing proliferation of these cells in ectopic endometrium. Our in situ results show that angiogenesis requires an initial increase in ERK1/2 activity, which then subsides during the later stages of vascular stabilization. Thus, PD98059-enhanced VEGF mRNA expression in HEECs likely mediates vascular stabilization required to maintain vascular integrity reflected in our in vitro tube formation assay. However, further investigations are required to uncover the mechanism(s) mediating this PD98059 effect. Moreover, ERK1/2-dependent induction of MCP-1 in HEECs by VEGF suggests that ERK1/2 MAPK signaling contributes to monocyte recruitment into ectopic endometrial sites. In support of this observation, earlier studies showed that the ERK inhibitors PD98059, U0126, and A771726 decrease endometriosis progression in a mouse model.[42,43] Similarly, administration of Sorafenib, an inhibitor of ERK phosphorylation, to endometriosis-developed mice significantly decreased ectopic endometrial tissue volume and diameter compared to their initial size.[44]

In conclusion, the current study provides the first demonstration of the following: (i) increased ERK1/2 activity in ectopic endometriotic endothelial cells in situ and (ii) the effect of ERK1/2 on HEEC survival and angiogenic capacity in vitro by revealing that ERK1/2 activity stimulates

HEEC proliferation and reduces vascular stabilization. Moreover, the current study also provides evidence that increased ERK1/2 phosphorylation may be involved in endothelial progenitor cell differentiation and proliferation as confirmed by CD34 and PCNA immunoreactivity, respectively. Future studies are required to investigate the source and regulation of these endothelial progenitor cells in ectopic implants, which can serve as target cells of anti-angiogenic therapy in endometriosis.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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