Extracellularly Signal-Regulated Kinase Activity in the Human Endometrium: Possible Roles in the Pathogenesis of Endometriosis

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Context: Endometriosis is an estrogen-dependent disease characterized by the presence of endometrial tissue outside of the uterine cavity, causing pelvic pain and infertility in 10% of reproductive-aged women. It is unclear why ectopic endometrium remains viable in only a subset of women. ERK1/2 plays key intracellular roles in activating cellular survival and differentiation processes.

Objective: We sought to determine ERK1/2 activity in patients with endometriosis and its possible roles in regulating endometrial cell survival.

Design: ERK1/2 phosphorylation and expression throughout the menstrual cycle were evaluated *in vivo* in normal and endometriotic human endometrium, and *in vitro* techniques assessed the steroidal regulation of ERK1/2 and its effect on endometrial cell survival.

Results: Total ERK1/2 remained constant in normal and endometriotic endometrium throughout the menstrual cycle. Phospho-ERK1/2 was high in the late proliferative and secretory phases in normal endometrium ($P < 0.05$). In endometriotic glandular cells, there was no cyclical variation in phospho-ERK1/2. In endometriotic stromal cells, there was also a reduction in phospho-ERK1/2 variation, with higher levels in the early-mid secretory phase ($P < 0.05$). In cultured endometrial stromal cells (ESCs), estrogen plus progesterone increased ERK1/2 phosphorylation within 15 min ($P < 0.05$). Although estrogen alone did not induce ERK1/2 phosphorylation in normal ESCs, there was a significant response to estrogen in ESCs isolated from eutopic endometriotic endometrium ($P < 0.05$). ERK1/2 inhibition in ESCs reduced proliferation and increased apoptosis ($P < 0.05$).

Conclusion: Abnormally high levels of ERK1/2 activity may be involved in endometriosis, possibly by stimulating endometrial cell survival. **(*J Clin Endocrinol Metab* 93: 3532–3540, 2008)**

Endometriosis is a gynecological disease characterized by the presence of endometrial tissue outside of the uterine cavity, causing pelvic pain and infertility in approximately 10% of reproductive-aged women. This disease is thought to be principally caused by the shedding of viable endometrial cells into the peritoneal cavity by retrograde menstruation, followed by their implantation and growth on the surface of pelvic organs (1). However, the pathogenesis of endometriosis remains poorly understood, and it is unclear why ectopic endometrial growth occurs in only a subset of women when retrograde menstruation and the presence of viable endometrial cells in peritoneal fluid is observed in most women (2, 3).

The MAPK family operates as key links in intracellular signaling pathways by phosphorylating specific serine/threonine residues of target molecules (4–6). The ERK1/2 is a MAPK subfamily that is activated by phosphorylation in response to a broad array of stimuli (7, 8). ERK1/2 localization is largely cytoplasmic but undergoes nuclear translocation upon activation.

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Abbreviations: BrdU, 5-Bromo-2′-deoxyuridine; E2, estradiol; ESC, endometrial stromal cell; P, progesterone; TBS, Tris-buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.

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Although the effects of ERK1/2 activation depend on cell type and stimulus, they typically involve the regulation of cellular proliferation, survival, and differentiation (8).

Normal endometrial growth is a highly regulated process that involves menstrual cycle-dependent changes in cellular survival, proliferation, and differentiation. These changes occur under the overall control of ovarian steroids, and we have previously shown that phosphorylation of intracellular signaling molecules such as p38 MAPK, Akt, and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is cycle dependent under steroidal control (9–11). We hypothesized that ERK1/2 is likewise under steroidal control and plays a role in regulating cycle-dependent endometrial changes. Furthermore, ectopic endometrial tissue in women with endometriosis, a steroid-dependent disease, requires the sustained survival and proliferation of implanted endometrial cells, and given the classic role played by ERK1/2 in these processes in other tissue types, we further hypothesized that ERK1/2 activity is abnormally regulated in the endometrial tissue of women with endometriosis.

Subjects and Methods

Tissue collection

Eutopic (n = 17) and ectopic (n = 34) endometria were obtained from women with endometriosis undergoing laparoscopy for infertility or pelvic pain. Endometriosis was confirmed by histopathological examination by two pathologists. Normal endometria were obtained from women (n = 36) undergoing laparoscopy or hysterectomy for benign gynecological conditions other than endometrial disease. These women had no visible pelvic inflammation or endometriosis at laparoscopy or laparotomy. The day of menstrual cycle was established according to menstrual history and confirmed by endometrial histology using the criteria of Noyes *et al.* (12) by two pathologists. Endometrial samples were grouped according to menstrual cycle phase: early-mid (d 1–10; n = 12, 6, and 11 for normal, eutopic, and ectopic tissues, respectively) and late (d 11–14; n = 6, 3, and 6, respectively) proliferative and early-mid (d 15–23; n = 12, 5, and 11, respectively) and late (d 24–28; n = 6, 3, 6, respectively) secretory. All specimens within groups were from different subjects.

Written, informed consent was obtained from each patient using consent forms and protocols approved by the Yale Human Investigation Committee.

Immunohistochemistry

Paraffin sections were deparaffinized and rehydrated and then boiled in 10 mM citrate buffer (pH 6.0) for 15 min for antigen retrieval. Sections were immersed in 3% hydrogen peroxide in methanol for 15 min to quench endogenous peroxidase activity, washed in Tris-buffered saline (TBS), and then blocked with 5% goat serum (Vector Laboratories, Burlingame, CA) in TBS for 30 min. Sections were then incubated with rabbit polyclonal anti-total ERK1/2 antibody (1:100; Biosource International, Camarillo, CA) and rabbit anti-phosphorylated (phospho)-ERK1/2 (pTpY185/187) antibody (1:100; Biosource) overnight at 4 C. Negative controls were nonspecific rabbit IgG at identical concentrations. Sections were washed, and biotinylated goat antirabbit antibody (Vector) was added at 1:400 for 30 min. The antigen-antibody complex was detected with a streptavidin-biotin-peroxidase kit (Vector). 3,3-Diamonibenzidine tetrahydrochloride dihydrate (Vector) was used as the chromogen, and sections were counterstained with hematoxylin.

Staining intensities were semiquantitatively evaluated using the following categories: 0, no staining; 1+, weak; 2+, moderate; 3+, intense staining. For each tissue, a histological score (HSCORE) value was derived by using the formula HSCORE = $\Sigma Pi\,(i + 1)$, where i represents an intensity score and Pi is its corresponding percentage of cells. In each slide, five randomly selected areas were evaluated under a light microscope (×100 magnification), and the HSCOREs were determined at different times by two investigators blinded to the type and source of the tissues. The intraindividual and interindividual coefficients of variation were 11 and 12%, respectively, for the HSCORE evaluation. The average score of two was used.

Isolation and culture of human endometrial stromal cells (ESCs)

Portions of the normal and eutopic endometria as obtained above were used for normal cell and ESC culture, respectively. Endometrial tissues were minced with a sterile blade and digested in Hanks' balanced salt solution (Sigma-Aldrich, St. Louis, MO) containing collagenase B (1 mg/ml, 15 U/mg; Roche, Indianapolis, IN), DNase I (0.1 mg/ml, 1500 U/mg; Roche), penicillin (200 U/ml), and streptomycin (200 mg/ml) for 60 min at 37 C. Dispersed endometrial cells were filtered through a wire sieve (73-μm-diameter pore; Sigma-Aldrich) and cultured in DMEM Ham's F-12 (1:1 vol/vol; Sigma-Aldrich) containing fetal bovine serum (10% vol/vol; Invitrogen Corp., Carlsbad, CA) and antibiotics-antimycotics (1% vol/vol; Life Technologies, Inc., Rockville, MD) until grown to preconfluence (6–8 d) or confluence (7–10 d) in a standard 95% air/5% CO_2 incubator at 37 C.

Experimental setup

ESCs after first passage were previously assayed using specific cell-surface markers and were found to contain 0–7% epithelial cells, no detectable endothelial cells, and 0.2% macrophages (13, 14). Before all culture experiments, ESCs were incubated with serum-free, phenol red-free DMEM Ham's F12 with 2% BSA (Sigma-Aldrich) and 1% antibiotics-antimycotics for 24 h.

Confluent normal ESCs were incubated with vehicle, estradiol (E2; 10^{-8} M), progesterone (P; 10^{-7} M), or E2+P for 5- and 15-min treatments. Confluent ESCs from eutopic endometriotic or normal endometrium were incubated with vehicle or E2 (10^{-8} M) for 10 min.

All experiments were performed at least three times using cells from at least three different patients.

Western blot analysis

Total protein extraction and Western blotting was performed as previously described (9). After PAGE and protein blotting, the membrane was blocked with 5% nonfat dry milk in TBS containing 0.1% Tween 20 for 1 h. Subsequently, the membrane was incubated for 1 h with anti-phospho-ERK1/2 antibody (1:1000 in 3% BSA), washed, incubated for 1 h with peroxidase-labeled secondary antibody (Vector), and then washed again. Signals were developed using a chemiluminescence kit (Amersham Biosciences, Piscataway, NJ). The membrane was stripped and reprobed for 1 h with anti-total ERK1/2 antibody (1:1000 in 3% BSA), with the remaining steps performed as above. Band intensities were quantified using computer densitometry analysis (ImageJ; National Institutes of Health, Bethesda, MD).

Immunocytochemistry

Confluent normal ESCs were treated with vehicle, E2 (10^{-8} M), P (10^{-7} M), or E2+P for 15 min and then fixed in 4% paraformaldehyde at 4 C for 20 min and washed with TBS. Cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature and washed. Immunocytochemical analysis, including HSCORE evaluation, was performed according to the protocol used for immunohistochemistry, starting from the endogenous peroxidase quench.

5-Bromo-2′-deoxyuridine (BrdU) incorporation assay

Preconfluent normal ESCs in 96-well plates were treated with vehicle [dimethylsulfoxide (DMSO)] or a specific ERK1/2 inhibitor, PD98059 (20 μM; EMD Biosciences, San Diego, CA), for 24 h. PD98059 at 20–25

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μM has been typically used in experiments involving cultured ESCs (15–17). BrdU incorporation into newly synthesized DNA was assessed using a BrdU cell proliferation assay kit (Chemicon International, Temecula, CA). Briefly, BrdU solution was added to each well 12 h before termination of each experiment, and then cells were washed in PBS, fixed, and washed again. Cells were then incubated with anti-BrdU antibody for 1 h, washed, and incubated with peroxidase-conjugated secondary antibody for 30 min. After washing, cells were exposed to tetramethylbenzidine peroxidase substrate. Plates were read with a multiwell plate reader (Thermomax; Molecular Devices Corp., Menlo Park, CA). Data are expressed in OD units, with higher values indicating greater BrdU incorporation. Each experiment involved replicates of 12 wells per treatment.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay

Preconfluent normal ESCs were treated with vehicle (DMSO) or PD98059 (20 μM) for 24 h, and the TUNEL assay was performed using a cell death detection kit (Roche, Mannheim, Germany) as previously described (18) to detect DNA strand breaks. Positive signals were confirmed by 4,6′-diamidino-2-phenylindole staining for histological signs of apoptosis, as described previously (14). Quantification of apoptotic cells was performed by counting the number of TUNEL-positive cells per 100 total cells under a light microscope in five randomly selected fields per slide.

Cell proliferation assay

Preconfluent normal ESCs in 96-well plates were treated with vehicle (DMSO) or PD98059 (5 μM and 20 μM) in phenol red-free DMEM containing 2% fetal bovine serum. After 24 h of treatment, ESC number was determined by a colorimetric assay using the CellTiter 96 AQueous nonradioactive cell proliferation assay kit (Promega Corp., Madison, WI), as previously described (19). Data are expressed in OD units, with higher values indicating greater numbers of metabolically active cells.

Statistical analysis

All *in vitro* data were normally distributed (Kolmogorov-Smirnov test) and analyzed with Student's *t* test or one-way ANOVA followed by *post hoc* Holm-Sidak testing, where appropriate. HSCORE data, except for ectopic glandular cells, were normally distributed and analyzed by one-way ANOVA with *post hoc* Holm-Sidak testing. HSCOREs for ectopic glandular cells were analyzed with nonparametric ANOVA on ranks (Kruskal-Wallis test) followed by *post hoc* Student-Newman-Keuls testing. Statistical calculations were performed using SigmaStat 3.00 (SPSS Inc., Chicago, IL). Statistical significance was defined as $P < 0.05$.

Results

ERK1/2 phosphorylation in normal endometrium is menstrual cycle dependent

Normal endometrial tissues were subjected to immunohistochemical analysis for total and phospho-ERK1/2 (Figs. 1 and 2). Isotype-matched negative control antibody corresponding to total and phospho-ERK1/2 incubations revealed no staining (Fig. 1, I and J, respectively). Total ERK1/2 was both cytoplasmic and nuclear and showed high levels of staining without variation in all phases of the menstrual cycle in both endometrial glandular and stromal cells (Fig. 1, A and B).

Phospho-ERK1/2 staining in normal endometrium was mostly nuclear (Fig. 1, C and D). Glandular cells showed weak

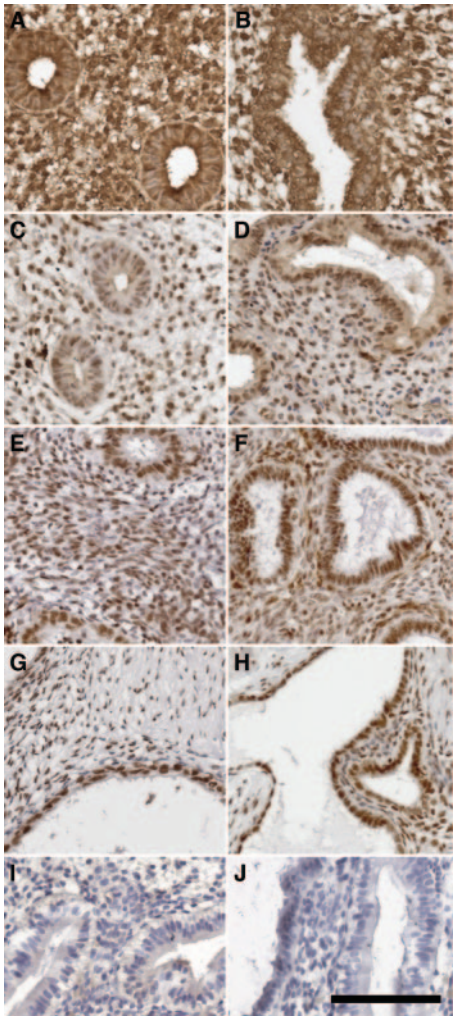

FIG. 1. Immunoreactive total ERK1/2 and phospho-ERK1/2 in endometrial stromal and glandular cells from normal, eutopic, and ectopic endometrium. Representative micrographs are shown of immunohistochemical staining for phospho- and total ERK1/2. Total ERK1/2 staining, shown in normal endometrium from the early-mid proliferative and early-mid secretory phases (A and B, respectively), was both cytoplasmic and nuclear. Phospho-ERK1/2 staining was mostly nuclear and is shown in tissues of early-mid proliferative and early-mid secretory phases from normal endometrium (C and D, respectively), eutopic endometriotic endometrium (E and F, respectively), and ectopic endometrium (G and H, respectively). Isotype-matched negative control antibody corresponding to total ERK1/2 (I) and phospho-ERK1/2 (J) incubations are shown. *Scale bar*, 100 μm (A–H).

phospho-ERK1/2 immunoreactivity specifically in the early-mid proliferative phase (Fig. 1C), which was significantly lower than the consistently high levels seen in the rest of the cycle (Figs. 1D and 2A; $P < 0.05$).

In normal stromal cells, phospho-ERK1/2 was weakest in the early-mid proliferative phase (Figs. 1C and 2B; $P < 0.05$ *vs.* all other phases), reaching the highest levels of staining in the late proliferative phase (Fig. 2B; $P < 0.05$ *vs.* all other phases), and decreased to moderate levels throughout the secretory phase (Figs. 1D and 2B).

ERK1/2 phosphorylation in women with endometriosis is abnormally up-regulated

Eutopic and ectopic endometrial tissues from women with endometriosis were immunostained for phospho-ERK1/2 and

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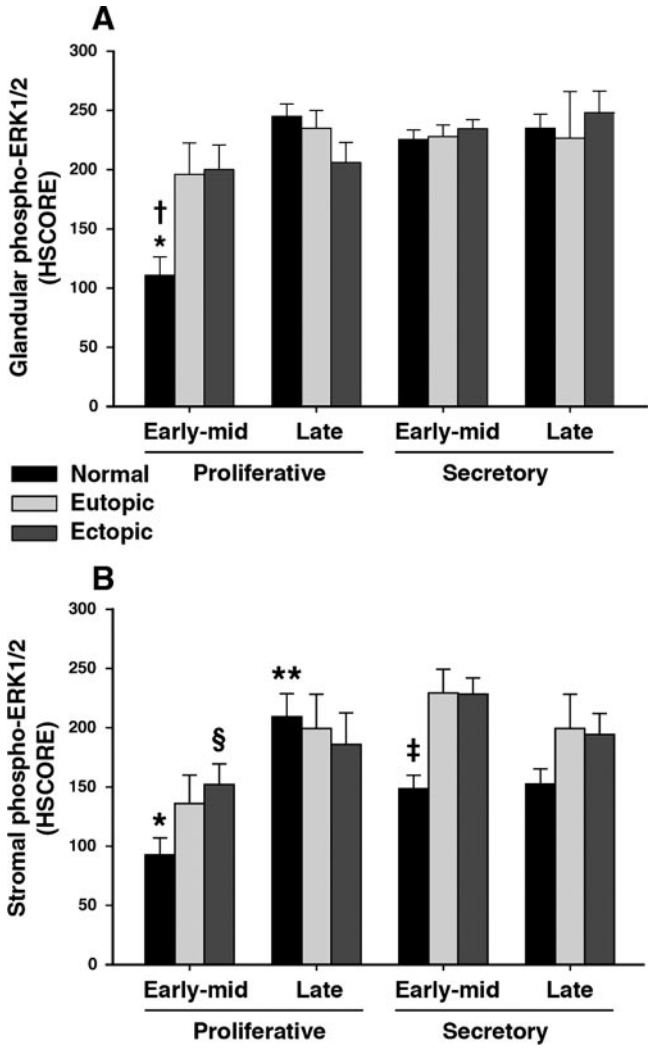

FIG. 2. HSCORE analysis of phospho-ERK1/2 immunostaining in endometrial stromal and glandular cells from normal, eutopic, and ectopic endometrium. Staining intensities of immunoreactive phospho-ERK1/2 were evaluated by HSCORE for endometrial glandular (A) and stromal (B) cells. In normal endometrium, phospho-ERK1/2 staining in the early-mid proliferative phase was significantly lower than that of all other phases, in both glandular and stromal cells (*, $P < 0.05$ *vs.* all other normal phases). In normal stromal cells, phospho-ERK1/2 in the late proliferative phase was significantly higher than all other phases (**, $P < 0.05$ *vs.* all other normal phases). In eutopic endometriotic endometrium, both glandular and stromal cells did not show any cycle variation in phospho-ERK1/2 staining. In ectopic endometrium, glandular cells did not show any phospho-ERK1/2 cycle variation, whereas stromal cells showed lower phospho-ERK1/2 in the early-mid proliferative phase than the early-mid secretory phase (§, $P < 0.05$). In the early-mid proliferative phase, glandular phospho-ERK1/2 in eutopic and ectopic tissues was significantly higher than that in normal tissues (†, $P < 0.05$). In the early-mid secretory phase, stromal phospho-ERK1/2 in eutopic and ectopic tissues was significantly higher than that in normal cells (‡, $P < 0.05$).

total ERK1/2. Total ERK1/2 was cytoplasmic and nuclear, showing consistently high levels of staining in all phases of the menstrual cycle, in both eutopic and ectopic endometrial glandular and stromal cells. Total ERK1/2 staining was similar in normal and endometriotic tissues.

Phospho-ERK1/2 was mostly nuclear in both eutopic (Fig. 1, E and F) and ectopic (Fig. 1, G and H) endometrium. Eutopic and ectopic glandular cells showed persistently high levels of phospho-ERK1/2 in all phases of the menstrual cycle, with no significant variation between phases (Fig. 2A). In the early-mid proliferative phase, phospho-ERK1/2 levels in eutopic and ectopic glandular cells (Fig. 1, E and G, respectively) were significantly higher than that in normal glandular cells (Figs. 1A and 2A; $P < 0.05$). Throughout the rest of the cycle, phospho-ERK1/2 staining was similar between normal, eutopic, and ectopic glandular cells.

Eutopic stromal cells showed moderate-to-strong phospho-ERK1/2 staining throughout the menstrual cycle, with no statistically significant differences between cycle phases (Figs. 1, E and F, and 2B). Likewise, ectopic stromal cells showed moderate-to-strong phospho-ERK1/2 levels throughout the menstrual cycle, with the only significant variation detected between the early-mid proliferative (Fig. 1G) and early-mid secretory (Fig. 1H) phases (Fig. 2B; $P < 0.05$). In the early-mid secretory phase, phospho-ERK1/2 levels in eutopic and ectopic stromal cells (Fig. 1, F and H, respectively) were significantly higher than that in normal stromal cells (Figs. 1D and 2B; $P < 0.05$). In other phases of the menstrual cycle, phospho-ERK1/2 exhibited similar levels of staining between normal, eutopic, and ectopic stromal cells.

Results from the HSCORE analysis are summarized in Table 1.

Estrogen plus progesterone rapidly increases ERK1/2 phosphorylation in normal human ESCs

To investigate the effects of ovarian steroids on ERK1/2 activation, cultured ESCs from normal endometrium were treated with vehicle, 10^{-8} M E2, 10^{-7} M P, or E2+P in 5- and 15-min treatment periods and analyzed by Western blot (Fig. 3). Total ERK1/2 levels did not change with time or treatment. No differences among the treatments were observed after 5 min. At 15 min of treatment, there was a marked reduction in basal (control) phospho-ERK1/2 compared with that at 5 min, and E2 or P alone showed no difference compared with the control. However, phospho-ERK1/2 after 15-min E2+P treatment was signifi-

TABLE 1. Summary of phospho-ERK1/2 immunohistochemical observations

	Phospho-ERK1/2 expression	
	Cycle-dependent regulation	**Aberrant regulation *vs.* normal**
Endometrial glands		
Normal	Low in EP *vs.* all other phases[a]	
Eutopic	None	Increased in EP[a]
Ectopic	None	Increased in EP[a]
Endometrial stroma		
Normal	Low in EP *vs.* all other phases[a] High in LP *vs.* all other phases[a]	
Eutopic	None	Increased in ES[a]
Ectopic	Low in EP *vs.* ES[a]	Increased in ES[a]

EP, Early-mid proliferative phase; ES, early-mid secretory phase; LP, late proliferative phase.

[a] $P < 0.05$.

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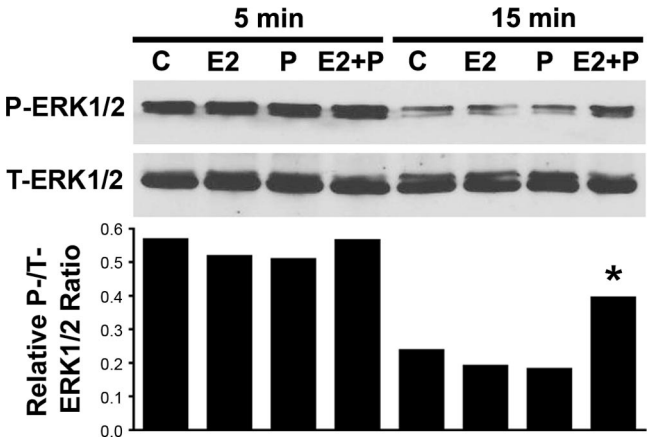

FIG. 3. Western blot analysis of short-term steroid effects on ERK phosphorylation in cultured normal ESCs. Confluent ESCs isolated from normal endometrium were incubated with vehicle (C), E2 (10^{-8} M), P (10^{-7} M), or E2+P for 5- and 15-min treatment periods. The ratio of phospho- to total ERK1/2 was significantly increased by treatment of E2+P at 15 min, compared with all other 15-min treatments (*, $P < 0.05$ *vs.* C, E2, and P at 15 min). Treatment with E2 or P alone had no effect on ERK1/2 phosphorylation. Representative results are shown.

cantly higher compared with other treatments of the same time course ($P < 0.05$).

To confirm these results, immunocytochemistry was performed on cultured ESCs treated with E2, P, or E2+P for 15 min (Fig. 4). Phospho-ERK1/2 was mostly nuclear (Fig. 4, A–E), whereas total ERK1/2 was both cytoplasmic and nuclear (Fig. 4, F–I), as seen *in vivo*. HSCORE analysis revealed significantly increased P-ERK1/2 staining in the nuclei of cells treated with E2+P (Fig. 4D), compared with treatments of vehicle or E2 or P alone (Fig. 4, A, B, and C, respectively; $P < 0.05$). Total ERK1/2 did not show any change with treatment (Fig. 4, F–I). Notably, phospho-ERK1/2 immunoreactivity was intense in dividing cells (Fig. 4E), regardless of treatment. Isotype-matched negative control antibody for phospho-ERK1/2 and total ERK1/2 revealed no staining (Fig. 4, J and K, respectively).

Estrogen rapidly stimulates ERK1/2 phosphorylation in endometriotic but not normal ESCs

To investigate whether estrogen abnormally activates ERK1/2 in ESCs from women with endometriosis, cultured ESCs isolated from eutopic endometrium of women with and without endometriosis were treated with E2 (10^{-8} M) or vehicle for 10 min and then subjected to Western blot analysis (Fig. 5A). Total ERK1/2 levels were similar between normal and endometriotic ESCs ($P = 0.93$). In normal ESCs, there was no significant difference in phospho- to total ERK1/2 ratios between estrogen and vehicle treatments (mean ± SEM, 0.69 ± 0.01 *vs.* 0.67 ± 0.02, respectively; $P = 0.42$), whereas in endometriotic ESCs, phospho- to total ERK1/2 ratios were significantly higher in treatment with estrogen compared with vehicle (Fig. 5B; mean ± SEM, 0.81 ± 0.03 *vs.* 0.60 ± 0.03, respectively; $P < 0.05$). The ratio of estrogen- to vehicle-stimulated ERK1/2 phosphorylation was significantly higher in endometriotic ESCs compared with normal ESCs, with that of the latter being near parity (Fig. 5C; mean ± SEM, 1.36 ± 0.09 *vs.* 1.03 ± 0.02, respectively; $P < 0.05$).

ERK1/2 activity enhances ESC survival

To investigate the role of ERK1/2 in the viability of cultured normal ESCs, the 2-(4-sulfophenyl)-^{2}H-tetrazolium (MTS) assay was used to assess viable cell number after treatment with vehicle or the specific ERK1/2 inhibitor PD98059 (5 and 20 μM). Although 5 μM PD98059 did not result in a difference in metabolically active cells compared with vehicle, 20 μM PD98059 resulted in a significant, 50% decrease (Fig. 6A; $P < 0.05$).

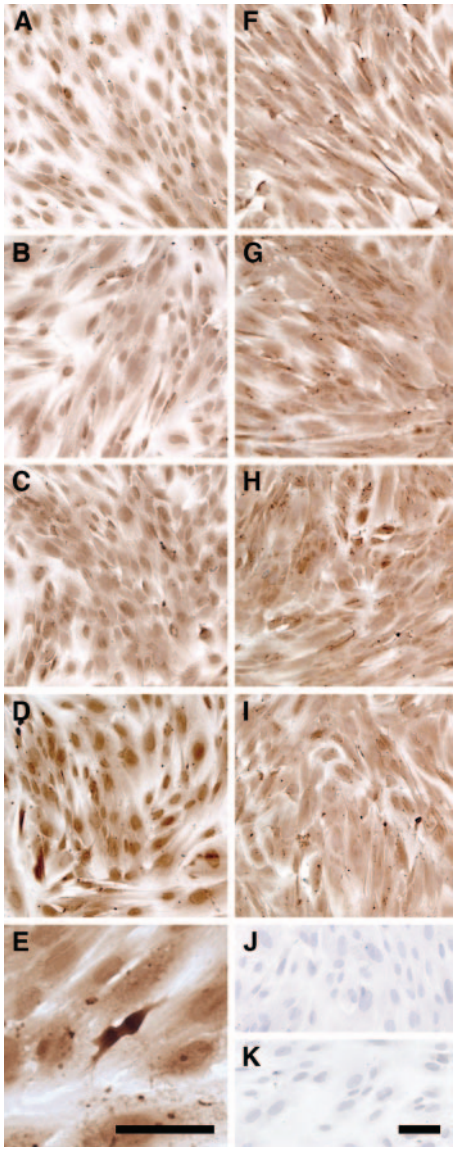

FIG. 4. Immunoreactive phospho- and total ERK1/2 in cultured normal ESCs in response to short-term steroid treatment. Confluent normal ESCs were immunostained for phospho-ERK1/2 (A–E) and total ERK1/2 (F–I) after 15-min treatments with vehicle (A and F, respectively), E2 (10^{-8} M; B and G, respectively), P (10^{-7} M; C and H, respectively), or E2+P (D and I, respectively). Phospho-ERK1/2 was mostly nuclear, whereas total ERK1/2 was both cytoplasmic and nuclear. HSCORE analysis revealed that treatment with E2+P significantly increased nuclear phospho-ERK1/2 staining compared with vehicle ($P < 0.05$), whereas all other treatments showed similar staining as vehicle. Total ERK1/2 levels were the same in all treatments. Regardless of treatment, dividing ESCs revealed intense staining for phospho-ERK1/2 (E). Immunostaining with isotype-matched negative control antibody for phospho- and total ERK1/2 incubations revealed no staining (J and K, respectively). Representative micrographs are shown. *Scale bar*, 40 μm; the *scale bar* in K indicates the scale for all *panels* except E.

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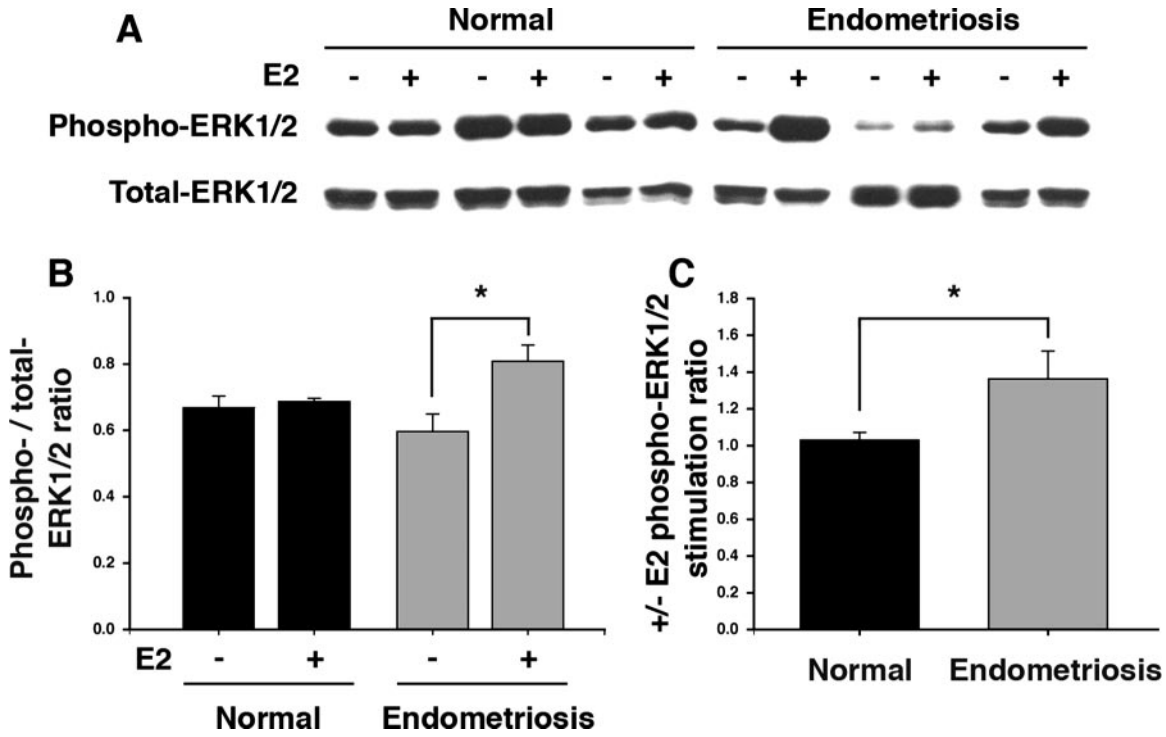

FIG. 5. Comparison of estrogen-stimulated ERK1/2 phosphorylation in normal and endometriotic ESCs. Cultured ESCs isolated from normal endometrium or eutopic endometrium of women with endometriosis were incubated with vehicle or E2 (10^{-8} M) for 10 min and then subjected to Western blot analysis for phospho-ERK1/2 and total ERK1/2. A, Experiments with eutopic ESCs cultured from six different subjects (three without and three with endometriosis) are presented, with each pair of bands representing an experiment using cells from one subject. Although the ratio of phospho- to total ERK1/2 in normal ESCs was similar between E2 and vehicle treatment, in endometriotic ESCs, it was significantly increased upon E2 treatment compared with vehicle (B; *, $P < 0.05$). The ratio of E2- to vehicle-induced phospho-ERK1/2 stimulation was near parity in normal ESCs but was significantly higher in endometriotic ESCs (C; *, $P < 0.05$).

To determine whether this positive role of ERK1/2 activity on ESC number is due to regulation of proliferation and/or apoptosis, BrdU and TUNEL assays were used to assess DNA synthesis and breakage, respectively, after PD98059 (20 μM) treatment. ESCs treated with PD98059 showed a significant, 17% decrease in BrdU uptake (cell proliferation) compared with vehicle ($P < 0.05$; Fig. 6B), and a significant, 89% increase in TUNEL-positive (apoptotic) cell number compared with vehicle (Fig. 6C; $P < 0.05$).

Discussion

Aberrations in the regulation of endometrial physiology may lead to endometriosis (20). Because the endometrium is under ovarian steroid control, much insight into endometriosis may be gained through understanding this steroidal regulation in both health and disease. Such understanding is particularly important given that current treatments of endometriosis typically involve the continued administration of progesterone or estrogen with progesterone.

In the present study, we demonstrate that ERK1/2 exhibits abnormally high levels of activation in women with endometriosis throughout the menstrual cycle. This is consistent with microarray analyses reporting that genes activating the ERK1/2 MAPK signaling cascade are up-regulated in patients with endometriosis (21, 22).

Strikingly, we found that estrogen stimulates ERK1/2 phosphorylation in endometriotic but not normal ESCs. Because endometriosis is estrogen dependent, this abnormal responsiveness may explain the high phospho-ERK1/2 levels in women with endometriosis. The inflammatory environment in endometriosis, high in cytokines such as IL-1β and TNF-α (23), may also be a cause of the high ERK1/2 phosphorylation, as supported by a recent study in which IL-1β, TNF-α, and oxidative stress stimulated ERK1/2 phosphorylation in cultured ectopic ESCs (17). Moreoever, a study by Burney *et al.* (24) reported that mitogen-inducible gene 6 (MIG6), an inhibitor of epidermal growth factor receptor (EGFR) signaling and MAPK activation, is significantly down-regulated in eutopic endometriotic tissues, suggesting that altered ERK1/2 activity may also be due to aberrant growth factor signaling modulation.

Our *in vitro* data implicating ERK1/2 in the maintenance of ESC viability suggests that high ERK1/2 activity in endometriosis contributes to an aberrant regulation of endometrial cell viability, thus playing a crucial role in the pathogenesis of this disease. Reduced apoptosis has been observed in eutopic compared with normal endometrium (25, 26), and ectopic endometrium has been found to have lower rates of apoptosis compared with matched eutopic endometrium (26, 27). Reduced apoptosis may contribute to the survival of desquamated endometrial cells and give rise to inadequate macrophage-mediated clearance of peritoneal endometrial cells. A possible involvement of ERK1/2 in endometrial c-*fos* and c-*jun* expression (16) suggests pathways through which ERK1/2 may regulate viability, and these gene families have been associated with endometriosis (28). Hirota *et al.* (15) have shown that protease-activated receptor 2 (PAR2) ac-

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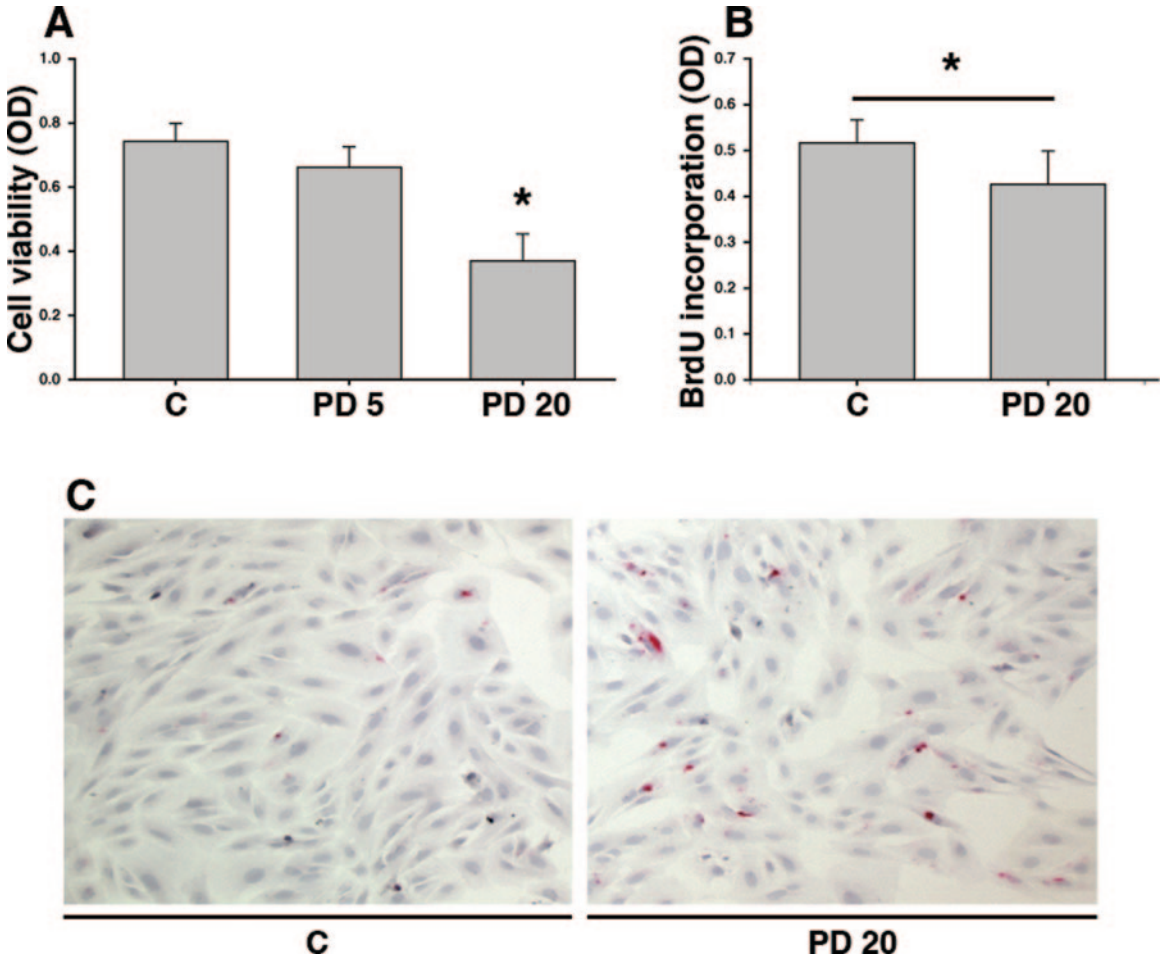

FIG. 6. Effect of ERK1/2 inhibition on ESC viability, proliferation, and apoptosis. A, Cultured normal ESCs were incubated with vehicle (C) and the specific ERK1/2 inhibitor PD98059 for 24 h and then assayed for MTS reduction to quantify the number of viable cells. Treatment with 5 μM PD (PD 5) did not affect cell number, whereas treatment with 20 μM (PD 20) resulted in a significant, 50% reduction in viable ESCs (*, $P < 0.05$ *vs.* C). B, Cultured normal ESCs were incubated with vehicle and 20 μM PD for 24 h and then assayed for BrdU incorporation to quantify active DNA synthesis. PD treatment resulted in a significant, 17% decrease in dividing cells (*, $P < 0.05$ *vs.* C). C, Representative micrographs of cultured normal ESCs, incubated with vehicle and 20 μM PD for 24 h and then subjected to TUNEL analysis to detect DNA breakage, with positive signals in *red*. Treatment with PD98059 at a concentration of 5 μm (PD 5) did not affect cell number, whereas treatment at a concentration of 20 μm (PD 20) resulted in a significant, 89% increase in number of cells undergoing apoptosis ($P < 0.05$).

tivates ERK1/2 and thereby stimulates proliferation in cultured ectopic ESCs. Moreover, statins have been suggested to have a therapeutic effect on endometriosis through inhibiting ESC growth, possibly by decreasing ERK1/2 activation (29).

ERK1/2 may also stimulate inflammatory cytokine and prostaglandin production in endometriosis. ERK1/2 is involved in the regulation of IL-8 secretion and cyclooxygenase-2 (COX-2) expression induced by IL-1β in endometriotic cultured ESCs (17), both of which may contribute to endometriosis (30, 31). The involvement of ERK1/2 in endometrial fibronectin expression may also play a role in ectopic implantation (16).

We have further demonstrated that ERK1/2 activity varies throughout the menstrual cycle in normal endometrium. Cycle-dependent ERK1/2 expression in normal endometrium has been previously described by Ozaki *et al.* (32), who reported that ERK1/2 expression is highest in the secretory phase, with expression mostly occurring in glandular cells, and almost no staining was found in stromal cells. However, our *in vivo* analysis evaluated both the expression and activity of ERK1/2 and demonstrated clear staining in not only glandular but also stromal cells. Our *in vitro* results and several studies (15, 16, 29, 33–35) have also demonstrated ERK1/2 in cultured ESCs. Moreover, we found that total ERK1/2 is not cycle dependent, suggesting that changes in phospho-ERK1/2 staining were due to variations in phosphorylation stimulation rather than ERK1/2 expression.

Cycle-dependent ERK1/2 activation further suggests that this signaling pathway is under ovarian steroid control. This is supported by our *in vitro* results showing that treatment of ESCs with estrogen and progesterone together, but neither alone, results in increased ERK1/2 activity. This effect may be due to stimulation of phosphorylation or inhibition of dephosphorylation, with the latter suggested by the fact that basal ERK1/2 activity was markedly reduced within 15 min, but not when the cells were treated with E2+P together. Furthermore, a previous study found that E2+P pretreatment is needed to activate ERK1/2 in a human endometrial adenocarcinoma cell line (36). These observations suggest that the higher levels of ERK1/2 activation in the secretory phase, a time when estrogen and progesterone are both high, may be due at least in part to these steroids. In contradiction, Gentilini *et al.* (33) reported that es-

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trogen alone increased ERK1/2 phosphorylation in ESCs, whereas progesterone alone had no effect, although they did not look at the effect of these steroids together. Another study showed that treatment of a rat uterine stromal cell line with the progestin R5020 induced rapid ERK1/2 activation (37). The actual steroidal regulation of ERK1/2 in the endometrium therefore requires greater elucidation, and it is possible that estrogen facilitates other factors that activate ERK1/2, which may also explain the high phospho-ERK1/2 levels observed in the late proliferative phase in lieu of a direct estrogenic effect. Molecules that rapidly activate endometrial ERK1/2 include chorionic gonadotropin (38), relaxin (35), and GnRH analog and TGF-β1 (16).

High ERK1/2 activity during the secretory phase may be involved in maintaining endometrial viability during this time. ERK1/2 may also have possible roles in decidualization, glandular differentiation, and ESC migration (33, 34, 39).

In conclusion, ERK1/2 is activated at abnormally high levels in the eutopic and ectopic endometrium of women with endometriosis and exhibits menstrual cycle-dependent activation in normal endometrial stromal and glandular cells. We observed that estrogen alone stimulates ERK1/2 phosphorylation in endometriotic but not normal ESCs and that ERK1/2 is involved in the positive regulation of ESC viability. These findings tentatively hint at a possible mechanism for the pathogenesis of the disease, whereby cells that are not normally responsive to estrogen are instead activated via ERK1/2 signaling and thereby remain viable and implant into the peritoneum after retrograde menstruation. Future investigations targeting the ERK1/2 pathway may be fruitful for developing a novel treatment of endometriosis.

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