Co-operation between the AKT and ERK signaling pathways may support growth of deep endometriosis in a fibrotic microenvironment in vitro†

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STUDY QUESTION: How can deep endometriotic stromal cells proliferate and persist in a fibrotic environment?

SUMMARY ANSWER: The serine/threonine kinase AKT and extracellular regulated kinase (ERK) signaling pathways may co-operate to support growth of deep endometriotic lesions by enhancing endometriotic stromal cell proliferation and survival in a fibrotic microenvironment in vitro.

WHAT IS KNOWN ALREADY: Endometriosis, particularly deep infiltrating endometriosis, is characterized histologically by dense fibrous tissue that is primarily composed of type I collagen. This tissue may cause pelvic pain and infertility, which are major clinical issues associated with endometriosis. Proliferation of normal fibroblasts is tightly regulated, and fibrillar, polymerized type I collagen inhibits normal fibroblast proliferation. However, no studies to date have investigated how deep endometriotic stromal cells can proliferate and persist in a fibrotic environment.

STUDY DESIGN, SIZE, DURATION: Endometrial and/or endometriotic tissues from 104 patients (61 with and 43 without endometriosis) of reproductive age with normal menstrual cycles were analyzed. A total of 25 nude mice received a single injection of endometrial fragments from a total of five samples.

PARTICIPANTS/MATERIALS, SETTING, METHODS: We evaluated cell proliferation, caspase 3/7 activity, and the AKT and ERK signaling pathways in endometrial and endometriotic stromal cells on three-dimensional (3D) polymerized collagen matrices in vitro. In addition, to determine whether aberrant activation of the AKT and ERK pathways is involved during progression of fibrosis in endometriosis in vivo, we evaluated the expression of phosphorylated AKT and ERK1/2 in endometriotic implants in a nude mouse model of endometriosis. Finally, we evaluated the effects of MK2206 (an AKT inhibitor) and U0126 (a MEK inhibitor) on cell proliferation, caspase 3/7 activity, and phosphorylation of AKT and ERK1/2 of endometriotic stromal cells on 3D polymerized collagen matrices.

MAIN RESULTS AND THE ROLE OF CHANCE: Proliferation of endometriotic stromal cells was significantly less inhibited than that of endometrial stromal cells (P < 0.05) on 3D polymerized collagen. Levels of phosphorylated AKT, phosphorylated p70S6K and phosphorylated ERK1/2 were significantly higher in endometriotic stromal cells than in endometrial stromal cells at 24 h (P < 0.05) and at 72 h (P < 0.05) on 3D polymerized collagen. Phosphorylated AKT expression was significantly increased on Days 21 and 28 compared with those on Days 3 and 7 (all P < 0.05) in endometriotic implants during progression of fibrosis in a nude mouse model of endometriosis. Inhibition of AKT or ERK1/2 with MK2206 or U0126, respectively, did not significantly increase caspase 3/7 activity in endometriotic stromal cells on either two-dimensional or 3D collagen matrices. Western blot analysis showed that MK2206 alone decreased levels of phosphorylated AKT; however, it increased levels of phosphorylated ERK in endometriotic cells compared with vehicle-treated cells (both P < 0.05). In addition, U0126 treatment decreased levels of phosphorylated ERK; however, it resulted in increased levels of phosphorylated AKT in endometriotic stromal cells compared with vehicle-treated cells (both P < 0.05).

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**Introduction**

Endometriosis, particularly deep infiltrating endometriosis (DIE), is characterized histologically by dense fibrous tissue that is primarily composed of type I collagen (Nisolle and Donnez, 1997; Matsuzaki et al., 1999; Giudice and Kao, 2004). This tissue may cause pelvic pain and infertility, which are major clinical issues associated with endometriosis (Nisolle and Donnez, 1997; Matsuzaki et al., 1999; Giudice and Kao, 2004).

Proliferation of normal fibroblasts is tightly regulated, and fibrillar, polymerized type I collagen inhibits normal fibroblast proliferation (Schor, 1980; Rhudy and McPherson, 1988; Koyama et al., 1996). Such tight regulation is essential for physiological wound repair. Dysregulation of apoptosis in pathological wound healing may result in excessive scarring and fibrosis (Eilmore, 2007; Cox and Erler, 2011). The formation of endometriotic lesions may share characteristics with pathological wound healing (van Kaam et al., 2008). However, no studies to date have investigated how deep endometriotic stromal cells can proliferate and persist in a fibrotic environment. Endometriosis is an estrogen-dependent disease (Giudice and Kao, 2004). However, deep endometriosis usually does not respond well to hormone suppression therapy (Konincx et al., 2012). Studies aimed at clarifying the underlying mechanisms could potentially provide a novel strategy for more effective treatment of DIE.

The α1(1) and α2(1) integrins are major type I collagen receptors (Gullberg et al., 1992; Giancotti and Ruoslahti, 1999). Upon ligand binding, integrins activate cell survival pathways, including those that involve phosphatidylinositol 3'-kinase (PI3K) and the serine/threonine kinase AKT, as well as the mitogen-activated protein kinase (MAPK)/extracellular regulated kinase (MAPK/ERK) (Giancotti and Ruoslahti, 1999). Aberrant activation of both signaling pathways appears to play a critical role in drug resistance in cancer (Aoudjat and Vuori, 2012). Previous studies showed the involvement of both signaling pathways in the pathophysiology of endometriosis (Ngô et al., 2010; Leconte et al., 2011; Yin et al., 2012; Eaton et al., 2013; Kim et al., 2014). Previous in vitro studies on 2D culture systems and animal experiments suggest that inhibition of the PI3K/AKT/mammalian target of rapamycin (mTOR) or (rapidly accelerated fibrosarcoma) RAF/mitogen-activated protein kinase (MEK)/ERK pathways might be effective in the treatment of endometriosis (Ngô et al., 2010; Leconte et al., 2011; Yin et al., 2012; Eaton et al., 2013; Kim et al., 2014). Thus, we hypothesized that the AKT and ERK signaling pathways may be involved in the proliferation and survival of endometriotic stromal cells in a fibrotic environment.

Studies have demonstrated that monomeric collagen (two-dimensional: 2D) and polymerized (fibrillar) collagen (three-dimensional: 3D) activate distinct pathways in normal fibroblasts: cell proliferation in monomeric collagen but prevention of cell proliferation in polymerized (fibrillar) collagen (Schor, 1980; Rhudy and McPherson, 1988; Koyama et al., 1996). Three-dimensional culture systems can mimic in vivo tissues better than conventional 2D culture systems on hard plastic or glass substrates (Pampaloni et al., 2007). To investigate interactions between collagen type I and endometriotic stromal cells in vitro, 3D culture systems are necessary.

The objective of the present study was to evaluate whether the AKT and ERK signaling pathways influence proliferation and survival of endometriotic stromal cells in a fibrotic environment.

We evaluated cell proliferation, caspase 3/7 activity, and the AKT and ERK signaling pathways in endometrial and endometriotic stromal cells on 3D polymerized collagen matrices in vitro. In addition, to determine whether aberrant activation of the AKT and ERK pathways is involved during progression of fibrosis in endometriosis in vivo, we evaluated the expression of phosphorylated AKT and phosphorylated ERK1/2 in endometriotic implants in a nude mouse model of endometriosis. Finally, we evaluated the effects of MK2206 (an AKT inhibitor) and U0126 (a MEK inhibitor) on cell proliferation, caspase 3/7 activity, and phosphorylation of AKT and ERK1/2 of endometriotic stromal cells on 3D polymerized collagen matrices.

**Materials and Methods**

**Patients**

Patients aged 20–37 years undergoing laparoscopy for endometriosis were recruited at CHU Clermont-Ferrand, France, for the present study. None of the women had received hormonal therapy and none used intrauterine contraception for at least 6 months prior to surgery. Recruited patients had regular menstrual cycles (26–32 days) with confirmation of their menstrual history. Endometrial and/or endometriotic samples from 61 patients who had histological evidence of DIE with or without ovarian endometriosis were used for the present analysis. In addition, endometrial tissues from 43 patients without endometriosis (patients with uterine fibroma: n = 19, patients with tubal infertility: n = 24) were obtained. The clinical characteristics of patients are shown in Supplementary Table SI. The research protocol was approved by the Consultative Committee for Protection of Persons in Biomedical Research (CPP) of the Auvergne (France) region. Informed written consent was obtained from each patient prior to tissue collection.

**Cell culture**

Endometrial and endometriotic stromal cells were isolated as previously described (Matsuzaki and Darcha 2013a,b, 2014). Isolated cells were
plated onto Primaria flasks (BD, Le Pont-De-Clair, France) in phenol red-free Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (Life Technologies, Cergy Pontoise, France) containing 10% charcoal-stripped fetal bovine serum (FBS) (Sigma-Aldrich, Lyon, France), 100 U/ml penicillin (Sigma-Aldrich), 0.1 mg/ml streptomycin (Sigma-Aldrich), and 0.25 μg/ml amphotericin B (Sigma-Aldrich) and incubated at 37 °C in 95% air/5% CO2. When the cells reached confluence, the first passages were used for experiments. Immunofluorescence staining was performed to determine the purity of the isolated endometrial and endometriotic stromal cells using monoclonal antibodies for human cytokeratin (a marker of epithelial cells) (MNFI 16, 1:100, DAKO, Glostrup, Denmark), vimentin (a marker of stromal cells) (V9, 1:100, DAKO), CD10 (a marker of stromal cells) (F-4, 1:25, Santa Cruz Biotechnology, Santa Cruz, CA, USA), smoothelin (a marker of smooth muscle cells) (MAB3242, 1:200, Merck Millipore, Molsheim, France) (Hinz et al., 2007; Paner et al., 2009), factor VIII (a marker of endothelial cells) (1:100, DAKO), and CD 45 (a marker of bone marrow-derived cells) (1:100, DAKO), as previously described (Matsuzaki and Darcha 2013a, 2014). The results showed that the purity of stromal cells was more than 99% (Supplementary Table SII).

**Collagen matrices**

Three-dimensional polymerized collagen matrices (final concentration = 2 mg/ml) were prepared as previously described (Matsuzaki and Darcha 2013b, 2014). The collagen solution was allowed to polymerize for 2 h at 37 °C. For 2D monomeric collagen matrices, 96-well plates were coated with 100 μg/ml type 1 collagen solution (BD, Le Pont de Clair, France).

**Cell proliferation assays**

Cells (endometrial stromal cells from patients with [n = 21] or without [n = 16] endometriosis, endometriotic stromal cells [n = 16]) were cultured for 24 h in serum-free culture. Cells were then seeded on 96-well plates coated with 50 μg/ml polymerized collagen or monomeric collagen, at a density of 5 × 10³ cells per well in 100 μl culture media. These cells were cultured at 37 °C for 3 or 6 days. Cell proliferation assays were performed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) (Promega, Charbonnières-les-Bains, France), as previously described (Matsuzaki and Darcha 2013a, 2014). The ratio of cell proliferation on polymerized collagen (3D) to monomeric collagen (2D) was calculated. All experiments were performed in triplicate.

**Caspase 3/7 activity assays**

Caspase 3/7 activity was measured by using the Apo-ONE Homogeneous Caspase-3/7 assay kit according to the manufacturer’s instructions (Promega). Cells (endometrial stromal cells from patients with [n = 21] or without [n = 16] endometriosis, endometriotic stromal cells [n = 16]) were cultured for 24 h in serum-free culture. A total of 5 × 10³ endometrial or endometriotic stromal cells were then seeded on 96-well plates coated with 50 μg/ml polymerized collagen in 100 μl serum-free medium. After 24 h, 100 μl of the mixture of caspase substrate and Apo-ONE Caspase-3/7 buffer were added and incubated for 16 h at room temperature. Fluorescence was then measured with the Fluoroskan Ascent Microplate Fluorometer (Thermo Scientific, Illkirch, France) at 480 nm Ex/530 nm Em. All experiments were performed in triplicate.

**Immunofluorescence staining for Ki67**

Cells (endometrial stromal cells from patients with [n = 21] or without [n = 16] endometriosis, endometriotic stromal cells [n = 16]) were seeded onto 8-well Lab-Tek slides (BD) coated with 100 μg/ml polymerized collagen or monomeric collagen, at a density of 2 × 10⁵ cells per well in 200 μl culture media (2% charcoal-stripped FBS) for 24 or 72 h. Immunofluorescence staining for Ki67 was performed according to the published protocol by Lee et al. (2005). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Slides were analyzed with a Leica TCS SPE confocal laser-scanning microscope (Leica Microsystems, Nanterre, France). The cellular proliferation index (percentage of Ki67-positive cells among the total number of DAPI-stained nuclei) was calculated from five random high-power fields through each section.

**Immunohistochemistry for phosphorylated AKT and phosphorylated ERK**

Menstrual endometrium of patients with (n = 20) and without (n = 16) endometriosis were used for immunohistochemistry. Immunohistochemical staining was performed on paraffin sections with a rabbit monoclonal antibody directed against phosphorylated AKT (pAKT) (1:50, Cell Signaling Technology, Danvers, MA, USA), or a rabbit monoclonal antibody directed against phosphorylated ERK (pERK) (1:400, Cell Signaling Technology). The staining scores for pAKT or pERK in stromal cells were computed using a computerized image analysis system, as previously described (Matsuzaki et al., 2010; Matsuzaki and Darcha 2013b, 2014).

**Western blot analysis**

Endometrial and endometriotic stromal cells from the same patients (n = 6) were cultured for 24 h in serum-free culture media. Then, cells were seeded on 6-well plates (2 × 10³ cells per dish) coated with 1000 μl polymerized collagen in culture media (2% charcoal-stripped FBS) for 24 or 72 h. Collagen gels were digested in a collagenase (Life Technologies) solution at 100 U/ml at 37 °C for 60 min.

Cell lysates were isolated using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific). Twenty micrograms of total protein lysates were loaded onto 4–10% sodium dodecyl sulphate-polyacrylamide gels and transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). Blots were processed in the SNAP i.d. Protein Detection System User Guide (Merck Millipore), as previously described (Matsuzaki and Darcha 2013a, 2014). The primary antibodies are shown in Supplementary Table SIII. Protein-antibody complexes were detected as previously described (Matsuzaki and Darcha 2013a, 2014). The western blot bands were quantified using ImageJ Software (version 1.41).

**Mouse model for endometriosis**

The nude mouse model of endometriosis was used as previously described (Matsuzaki and Darcha 2013b, 2014). Institutional review board approval at the University of Auvergne was obtained for the current animal study. Proliferative endometrial tissues from one patient without endometriosis were implanted into five mice simultaneously on Day 0. A total of 25 nude mice received a single injection of endometrial fragments from a total of five patients without endometriosis and mice were killed (n = 5 per time point) on Days 3, 7, 14, 21 or 28 for collection of endometriotic implants.

**In vitro model for menstruation**

Endometrial stromal cells of patients with (n = 8) and without (n = 8) endometriosis derived from the proliferative phase were cultured on 60-mm dishes (2 × 10³ cells per dish). When the cells were nearly confluent, they were washed, and the medium was replaced with serum-free culture media with 1 μM progesterone (Sigma-Aldrich, Lyon, France) + 10 nM estradiol (E2) (Sigma) for 14 days. The medium was changed every 2 days. Then, cells were washed and incubated without progesterone/E2 for an additional 6 days. Cell lysates were isolated for western blot analysis on Days 0, 3 and 6.
**Treatment of endometriotic stromal cells with MK2206 or U0126**

Endometriotic stromal cells from a total of 10 patients were cultured for 24 h in serum-free culture media. For cell proliferation and caspase 3/7 activity assays, cells were seeded on 96-well plates (5 × 10^3 cells per well) coated with polymerized collagen or monomeric collagen. For western blot analysis, cells were cultured for 2 h to allow for cell adhesion and spreading in culture media (2% charcoal-stripped FBS). Then, cells were treated with a designated concentration of MK2206 (Selleck Chemicals, Munich, Germany), U0126 (Selleck Chemicals) or vehicle (dimethylsulphoxide: DMSO) for 72 h for cell proliferation assays, 6 h for the caspase 3/7 activity assays, and 24 h for western blot analysis.

**Statistical analysis**

The STATA program version 12 (StataCorp, College Station, TX, USA) was used for statistical analysis. Comparisons between different groups were made using one-way analysis of variance following Scheffe’s method, the Mann–Whitney U-test, or the Wilcoxon matched pairs signed-ranks test. Statistical significance was defined as P < 0.05.

**Results**

**Cell proliferation assay**

No significant difference in deep endometriotic stromal cell proliferation was observed between the proliferative (n = 8) and secretory (n = 8) phases. Thus, we analyzed all of the data from deep endometriotic stromal cells irrespective of menstrual phase. The ratio of cell proliferation on 3D to 2D of endometrial stromal cells from patients with deep endometriosis derived from the menstrual and secretory phases was significantly lower compared with deep endometriotic stromal cells at Day 3 (Fig. 1A). The ratio of cell proliferation on 3D to 2D in endometrial stromal cells derived from the menstrual, proliferative and secretory phases was significantly lower compared with deep endometriotic stromal cells on Day 6 (Fig. 1A). In addition, the ratio of cell proliferation on 3D to 2D in endometrial stromal cells derived from the menstrual phase was significantly lower than that of the proliferative phase on Days 3 and 6 (Fig. 1A). No significant difference in the ratio of cell proliferation of endometrial stromal cells was observed between patients with and without endometriosis (Supplementary Fig. S1A).

**Ki67 expression**

No significant difference in the cellular proliferation index (percentage of Ki67-positive cells among the total number of DAPI-stained nuclei) for deep endometriotic stromal cells was observed between the proliferative (n = 8) and secretory (n = 8) phases. Thus, we analyzed all of the data from endometriotic stromal cells irrespective of menstrual phase. The cellular proliferation index was significantly lower in endometrial stromal cells of patients with deep endometriosis on polymerized collagen compared with that on monomeric collagen at both 24 and 72 h (Fig. 1B and C). In contrast, no significant difference in cellular proliferation index was observed between endometriotic stromal cells on polymerized collagen and monomeric collagen either at 24 or 72 h (Fig. 1B and C). No significant difference in cellular proliferation index of endometrial stromal cells was observed between patients with and without endometriosis (Supplementary Fig. S1C and D).

**Caspase 3/7 activity**

No significant difference in Caspase 3/7 activity in deep endometriotic stromal cell was observed between the proliferative (n = 8) and secretory (n = 8) phases. Thus, we analyzed all of the data from endometriotic stromal cells irrespective of menstrual phase. Caspase 3/7 activity in endometrial stromal cells of patients with deep endometriosis derived from the menstrual endometrium was significantly higher than that of endometrial stromal cells from the proliferative and secretory endometrium, and compared with that of endometriotic stromal cells (Fig. 1D). No significant difference in caspase 3/7 activity of endometrial stromal cells was observed between patients with and without endometriosis (Supplementary Fig. S1B).

**Analysis of the AKT and ERK1/2 signaling pathways in endometrial and endometriotic stromal cells from patients with endometriosis on polymerized collagen matrices**

Levels of phosphorylated AKT, phosphorylated p70S6K and phosphorylated ERK1/2 were significantly higher in endometrial stromal cells compared with those of endometrial stroma cells at 0, 24 and 72 h (Fig. 2A–C). At 24 h, levels of phosphorylated AKT were significantly decreased compared with those at 0 h, and then increased at 72 h in endometriotic stromal cells (Fig. 2A). No significant difference was observed between 0 and 72 h (Fig. 2A). In contrast, levels of phosphorylated AKT in endometrial stromal cells were significantly decreased at both 24 and 72 h compared with those at 0 h (Fig. 2A). No significant difference in levels of phosphorylated p70S6K was observed in endometrial stromal cells at 0, 24 and 72 h (Fig. 2A). In endometrial stromal cells, levels of phosphorylated p70S6K were significantly decreased at 72 h compared with those at 0 h (Fig. 2B). Levels of phosphorylated ERK1/2 were significantly increased at 24 h compared with those at 0 h in both endometriotic and endometrial stromal cells (Fig. 2B). In addition, levels of phosphorylated ERK1/2 decreased to similar levels as 0 h in both endometriotic and endometrial stromal cells at 72 h (Fig. 2C).

**Phosphorylated AKT and phosphorylated ERK1/2 expression in a mouse model of endometriosis**

No positive phosphorylated AKT immunostaining was observed in endometriotic implants on Day 3 (Fig. 3A and C). Immunostaining scores for phosphorylated AKT in stromal cells of endometriotic implants were significantly increased on Days 21 and 28 compared with those on Days 3 and 7 (Fig. 3A and C). Immunostaining scores for phosphorylated ERK1/2 stromal cells of endometriotic implants were significantly increased on Day 7 compared with that on Day 3; scores on Days 14, 21 and 28 were significantly decreased and were similar to Day 3 levels (Fig. 3B and D).

**Levels of phosphorylated AKT and phosphorylated ERK1/2 in an in vitro menstruation model**

Levels of phosphorylated AKT and ERK1/2 were significantly higher in endometrial stromal cells of patients with endometriosis compared with those without endometriosis on Days 0, 3, and 6 after E2 and progesterone withdrawal (Fig. 4A and B). In endometrial stromal cells of
patients with endometriosis, levels of phosphorylated AKT and ERK1/2 were significantly higher on Days 3 and 6 compared with those on Day 0 after E2 and progesterone withdrawal (Fig. 4A and B). In endometrial stromal cells of patients without endometriosis, levels of phosphorylated AKT were significantly higher on Day 3 compared with those on Days 0 and 6, whereas levels of phosphorylated ERK1/2 were significantly higher on Days 3 and 6 compared with those on Day 0 after E2 and progesterone withdrawal (Fig. 4A and B).

Phosphorylated AKT and phosphorylated ERK1/2 expression in menstrual endometrium of patients with and without endometriosis

Nuclear and/or cytoplasmic phosphorylated ERK and phosphorylated AKT expression was observed in both epithelial and stromal cells. Immunostaining scores for phosphorylated ERK and phosphorylated AKT in

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**Figure 1** Cell proliferation, Ki67 expression and caspase 3/7 activity on three-dimensional (3D) polymerized collagen matrices in endometrial and endometriotic stromal cells of patients with deep endometriosis. (A) The ratio of cell proliferation of endometrial and endometriotic stromal cells on 3D polymerized versus two-dimensional (2D) monomeric collagen. The ratio of cell proliferation is calculated as the 3D/2D ratio. *P < 0.05 versus menstrual and secretory endometrium, **P < 0.05 versus menstrual, proliferative and secretory endometrium. One-way analysis of variance following Scheffé’s method. M: menstrual endometrium (n = 5); P: proliferative endometrium (n = 8); S: secretory endometrium (n = 8); ENDO: Deep endometriosis (n = 16). (B) Cellular proliferation index (percentage of Ki67-positive cells among the total number of 4′,6-diamidino-2-phenylindole stained nuclei) of endometrial and endometriotic stromal cells on 3D polymerized versus 2D monomeric collagen. *P < 0.05 versus 2D monomeric collagen at 24 h, **P < 0.05 versus 2D monomeric collagen at 72 h. The Wilcoxon matched pairs signed-ranks test. M: (n = 5); P: (n = 8); S: (n = 8); ENDO: (n = 16). (C) Representative photomicrographs of Ki67 expression in endometrial and endometriotic stromal cells on 2D monomeric and 3D polymerized collagen. Scale bar: 100 μm. (D) Caspase 3/7 activity in endometrial and endometriotic stromal cells on 3D polymerized collagen. *P < 0.05 versus proliferative and secretory endometrium, and endometriosis. One-way analysis of variance following Scheffé’s method. M: (n = 5); P: (n = 8); S: (n = 8); ENDO (n = 16). Numeric values are presented as the mean + SEM.
stromal cells were significantly higher in menstrual endometrium of patients with endometriosis compared with those of patients without endometriosis (Figs. 5A–D).

Effects of MK2206 and U0126 treatment on cell proliferation, caspase 3/7 activity, and phosphorylation of AKT and ERK1/2 in endometriotic stromal cells

A dose-dependent decrease in cell proliferation following treatment with MK2206 or U0126 was observed in endometriotic stromal cells in all cases (Fig. 6A and B). The mean IC$_{50}$ of MK2206 was 11.6 ± 2.6 μM (mean ± SD) in 3D culture systems and 11.3 ± 2.1 μM in 2D culture systems. The mean IC$_{50}$ of U0126 was 38.7 ± 6.7 μM in 3D culture systems and 35.4 ± 4.7 μM in 2D culture systems. No significant difference in the IC$_{50}$ value of either MK2206 or U0126 was observed between cells cultured on 3D polymerized collagen and 2D monomeric collagen. Treatment with MK-2206 or U0126 alone did not induce a significant increase in caspase 3/7 activity in cells cultured either on 3D polymerized collagen or 2D monomeric collagen (Fig. 6C and D). In MK2206-treated cells, levels of phosphorylated AKT were significantly decreased; however, levels of phosphorylated ERK1/2 were significantly increased compared with those of vehicle-treated cells (Fig. 6E and F). In U0126-treated cells, levels of phosphorylated ERK1/2 were significantly decreased; however, levels of phosphorylated AKT were significantly increased compared with those of vehicle-treated cells (Fig. 6E and F).

Discussion

The present study showed that cell proliferation on 3D collagen matrices was significantly less inhibited in endometriotic stromal cells than in endometrial stromal cells. The western blot analysis showed aberrant activation of the AKT and ERK signaling pathways in endometriotic stromal cells on 3D collagen matrices. The in vitro findings suggested that aberrant activation of the AKT and ERK signaling pathways might support growth...
Figure 3  Expression of phosphorylated AKT and phosphorylated ERK1/2 in endometriotic implants in a mouse endometriosis model. (A and B) Staining score for phosphorylated AKT (A) or phosphorylated ERK1/2 (B) staining in stromal cells of endometriotic implants of mice on Day 3 (n = 5), Day 7 (n = 5), Day 14 (n = 5), Day 21 (n = 5) or Day 28 (n = 5). Day 0: Proliferative endometrial tissues from patients without endometriosis (n = 5). Numeric values are presented as the mean ± SEM. *P < 0.05 versus on Day 3 and on Day 7. #P < 0.05 versus on Days 3, 14, 21 and 28. The Mann–Whitney U-test. (C and D) Representative photomicrographs of the endometrium stained with phosphorylated AKT (C) or phosphorylated ERK (D) of endometriotic implants of mice on Day 3 (a, c) or on Day 21 (b, d). Scale bars: (a, c: 200 μm; b, d: 50 μm). Original magnification: a, c: ×100; b, d: ×400.

Figure 4  Effects of withdrawal of estrogen and progesterone on total and phosphorylated AKT, and total and phosphorylated ERK1/2 in endometrial stromal cells of patients with and without endometriosis. (A and B) Western blot-detected levels of phosphorylated AKT (A) or ERK1/2 (B) in protein extracts of endometrial stromal cells of patients with (n = 8) or without (n = 8) endometriosis on Days 0, 3 or 6 after withdrawal of estrogen and progesterone. Total AKT or ERK1/2 was used as a loading control in the respective panels. Numeric values are presented as the mean ± SEM. *P < 0.05 versus Day 0. #P < 0.05 versus Day 6. The Wilcoxon matched pairs signed-ranks test. aP < 0.05 versus patients without endometriosis. The Mann–Whitney U-test. Representative photomicrographs of western blot analysis of total and phosphorylated AKT (A), and total and phosphorylated ERK1/2 in endometrial stromal cells from patients with and without endometriosis. Endo (+): endometrial stromal cells of patients with endometriosis. Endo (−): endometrial stromal cells of patients without endometriosis.
of deep endometriotic lesions by enhancing endometriotic stromal cells persistence and survival in a fibrotic environment. Xia et al. (2008) showed that normal lung fibroblast interaction with polymerized collagen via β1 integrin suppressed the PI3K–Akt–S6K1 pathway and cell proliferation. In contrast, idiopathic pulmonary fibrosis fibroblasts interaction with polymerized collagen via β1 integrin activated Akt–S6K1 and increased cell proliferation. We speculate that a pathological pattern of β1 integrin signaling in response to polymerized collagen might also occur in endometriotic stromal cells. Further studies are required to fully confirm our speculation.

Although the pathogenesis of endometriosis remains unclear, one of the best supported theories may be the implantation theory (Giudice and Kao, 2004). Endometriosis originates from retrograde menstruation of endometrial tissue, which may then implant into the peritoneal cavity (Giudice and Kao, 2004). Type I collagen is distributed diffusely in the normal peritoneal stroma (Witz et al., 1998). The present study demonstrated significantly higher caspase 3/7 activity in menstrual endometrial stromal cells compared with proliferative or secretory endometrial stromal cells on 3D collagen matrices. Studies have shown that endometrium of patients with endometriosis may differ biochemically from that of patients without endometriosis (Matsuzaki et al., 2005, 2010; Kyama et al., 2006, 2008; Burney et al., 2007). The menstrual endometrial tissues of patients with endometriosis may have a greater ability to survive in the peritoneal cavity, and then to attach, proliferate, and invade the peritoneum (Kyama et al., 2006, 2008; Matsuzaki and Darcha, 2012). The present study showed no significant difference in either cell proliferation or caspase 3/7 activity in stromal cells derived from menstrual endometrium between patients with and without endometriosis. However, levels of phosphorylated AKT and phosphorylated ERK were significantly higher in menstrual endometrium in vivo and in endometrial stromal cells after E2 and progesterone withdrawal in vitro in patients with endometriosis compared with those of patients without endometriosis. These findings suggest that menstrual stromal cells of patients with and without endometriosis may undergo apoptosis in the peritoneal cavity, but menstrual endometrium of patients with endometriosis may have a greater ability to survive in a new collagen-rich microenvironment, the peritoneum, partly through aberrant activation of the AKT and ERK signaling pathways. Further studies are required to investigate whether aberrant activation of the AKT and ERK signaling pathways is involved in the initial development of endometriosis.

If aberrant activation of the AKT and ERK signaling pathways supports growth of deep endometriotic lesions, as speculated in the
present study, inhibition of these signaling pathways may decrease cell proliferation and induce apoptosis of deep endometriotic stromal cells in a fibrotic microenvironment. Therefore, we further investigated the effects of treatment with an AKT inhibitor (MK2206) or a MEK inhibitor (U-0126) on cell proliferation and caspase 3/7 activity in endometriotic stromal cells on 3D collagen matrices. In the present study, we did not investigate their effects on cell proliferation in endometrial stromal cells because levels of phosphorylated AKT and those of phosphorylated ERK1/2 were decreased in endometrial stromal cells on 3D collagen matrices. The results showed that either MK2206 or U0126 was used as a loading control in the respective panels. Studies showed that cross-talk and feedback loops exist between the PI3K/AKT/mTOR and RAF/MEK/ERK pathways (Mendoza et al., 2011; De Luca et al., 2012; Saini et al., 2013). Inhibition of the PI3K/AKT/mTOR pathway results in activation of the RAF/MEK/ERK pathway (Serra et al., 2011); similarly, inhibition of the RAF/MEK/ERK pathway results in activation of the PI3K/AKT/mTOR pathway in breast cancer (Hoeiflich et al., 2009). Previous clinical trials in various cancers showed that specific blocking of only one of the kinases involved in the PI3K/AKT/mTOR or RAF/MEK/ERK pathways has been associated with limited responses (Saini et al., 2013). Cotargeting the PI3K/AKT/mTOR and RAF/MEK/ERK pathways has resulted in enhanced tumor effects in vitro and in vivo in various types of cancer (Carracedo et al., 2008; Kinkade et al., 2008; Guenther et al., 2013). Endometriosis is a benign disease. However, studies have shown that endometriosis shares many aspects with cancer, such as invasion, metastasis, angiogenesis and apoptosis resistance. A variety of signaling pathways may be involved in promoting the development and progression of the disease.
of the disease. The present in vitro findings suggest that the AKT and ERK signaling pathways, both of which are important survival pathways, may compensate for each other, resulting in apoptosis resistance in endometriotic stromal cells. Further studies are required to determine whether cotargeting the PI3K/AKT/mTOR and RAF/MEK/ERK pathways could induce apoptosis in endometriosis.

However, a previous study showed by western blot analysis that either MK2206 or U0126 treatment increased cleaved caspase 3 in a dose-dependent manner in endometriotic stromal cells derived from ovarian endometriosis in a 2D culture system (Eaton et al., 2013). We speculated that sensitivity to MK2206 or U0126 treatment might be different between endometriotic stromal cells derived from deep endometriosis and ovarian endometriosis. Thus, we further investigated the effects of MK2206 and U0126 on caspase 3/7 activity in stromal cells derived from ovarian endometriosis. We observed that caspase 3/7 activity was not significantly increased by either MK2206 or U0126 treatment in endometriotic stromal cells of ovarian endometriosis (Supplementary Fig S2). However, the results of previous studies as well as those of the present study included only small numbers of samples; thus, individual differences between women might be important. Further studies are required to investigate whether either MK2206 or U0126 treatment could induce apoptosis in ovarian endometriosis.

Three-dimensional cultures are more closely related to in vivo conditions than are 2D cultures. However, a tremendous gap remains between 3D cell cultures and in vivo tissues. Thus, we investigated whether aberrant activation of AKT and ERK occurred in endometriotic implants during progression of fibrosis in a nude mouse model of endometriosis. In our mouse model of endometriosis, we previously showed that fibrosis was not yet evident on Day 7 after endometrial tissue implantation, and that fibrosis was established on Day 14, and then remained relatively stable until Day 28 (Matsuzaki and Darcha 2013b, 2014). The present animal experiment showed that phosphorylated AKT expression was not detected on Day 3, but was increased on Day 14 and continued to increase until Day 28 in endometriotic implants. This experiment suggested that AKT activation might occur during progression of fibrosis. On the contrary, phosphorylated ERK1/2 expression was significantly increased on Day 7 compared with that on Day 3, but was significantly decreased on Days 14, 21 and 28. Thus, it remains to be clarified whether the ERK pathway signaling may play a role in endometriotic implants during progression of fibrosis. However, the present in vitro experiments demonstrated that levels of phosphorylated ERK1/2 were increased, when levels of phosphorylated AKT were increased at 24 h in endometriotic stromal cells. In addition, when levels of phosphorylated AKT were increased at 72 h, levels of phosphorylated ERK1/2 were decreased in endometriotic stromal cells in vitro. These findings suggested the balanced cross-talk between the AKT and ERK pathways in a fibrotic environment in vitro (Aksamitiene et al., 2012). The cross-talk might lead to activation of compensatory signaling in vitro, when only the AKT pathway or the ERK pathway was inhibited (Aksamitiene et al., 2012). Similarly, the present mouse experiments showed that phosphorylated ERK1/2 expression was increased, when phosphorylated AKT expression was low on Day 7. Then, phosphorylated AKT expression was increased, when phosphorylated ERK1/2 expression was decreased in endometriotic implants during progression of fibrosis. Therefore, we speculate that there may be a balanced cross-talk between the AKT and ERK pathways in endometriotic implants during progression of fibrosis in vivo. Further animal experiments are required to investigate whether the AKT and ERK signaling pathways co-operate to support growth of endometriotic lesions in a fibrotic microenvironment in vivo.

In conclusion, the results of the present study suggest that the AKT and ERK signaling pathways may co-operate to support growth of deep endometriotic lesions by enhancing endometriotic stromal cell proliferation and survival in a fibrotic microenvironment in vitro.

**Supplementary data**

Supplementary data are available at http://humrep.oxfordjournals.org/.

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**Authors’ roles**

S.M. was involved in concept and design, sample collection, experiments, acquisition of data, analysis and interpretation of data, drafting the article and critical revision of the article. C.D. was involved in concept and design, analysis and interpretation of data and critical revision of the article. All authors read and approved the final version of the paper.

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**Conflict of interest**

None declared.

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