Estrogen regulates endometrial cell cytoskeletal remodeling and motility via focal adhesion kinase

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Objective: To explore the effects of 17β-estradiol (E_2) on cytoskeletal remodeling and motility of endometrial stromal cells (ESC) and Ishikawa cells and to characterize the role of focal adhesion kinase (FAK) in these processes.
Design: In vitro study of cytoskeletal remodeling and cellular morphology and motility in ESC or Ishikawa cells.
Setting: University research center.
Patient(s): Endometrial samples obtained from women requiring endometrial biopsies.
Intervention(s): Treatments with E_2 and multiple inhibitors of signaling pathways.
Main Outcome Measure(s): Activation of FAK, actin remodeling, membrane morphology, cell motility, and invasion.
Result(s): Estrogen induces a rapid and concentration-related FAK phosphorylation in ESC and Ishikawa cells. In this time frame, FAK localizes to the plasma membrane at sites of focal adhesion complexes formation, as shown by immunofluorescence. Phosphorylation of FAK in the presence of estrogen depends on the recruitment of both estrogen receptor α and estrogen receptor β and of a rapid G protein–dependent signaling to c-Src and phosphatidylinositol 3-OH kinase. Activation of FAK in ESC and Ishikawa cells is required for estrogen-induced horizontal migration and invasion of three-dimensional matrices of endometrial cells.
Conclusion(s): Estrogen enhances cytoskeletal and membrane remodeling in ESC and Ishikawa cells by controlling FAK, thus resulting in enhanced cell motility and invasion. These findings may have clinical relevance for the development of new therapeutic strategies for the prevention or control of endometrial diseases. (Fertil Steril® 2010;■:■–■. ©2010 by American Society for Reproductive Medicine.)

Key Words: Estrogen, endometrial cells, focal adhesion kinase, actin cytoskeleton, focal adhesion complexes

Deregulated endometrial cell remodeling, proliferation, adhesion, and interaction with the extracellular matrix (ECM) are important to a variety of endometrial disorders, including infertility, endometriosis, and cancer (1, 2). All these processes are regulated by estrogen (E), in particular endometrial cancer development and progression (3, 4). Although in its early stages endometrial cancer is a well-curable disease, when metastasis occurs the prognosis is severe (5). Thus, understanding the molecular actions of E linked to metastasis is pivotal for the development of novel therapeutic strategies.

The metastatic process requires motility, invasion, and remodeling of ECM. A key ECM controller is focal adhesion kinase (FAK), a tyrosine kinase active at focal contact sites involved in cell attachment, migration, invasion, proliferation, and survival, all of which are crucial for cancer development and metastasis (6, 7). Focal adhesion kinase overexpression has been described in human cancers and is related to invasive potential and poor prognosis (8).

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Estrogens regulate several cytoskeletal components, particularly actin fibers (9–11). Through the control of actin organization, E regulates cell morphology and interaction with ECM and drives cell movement (9–11).

On the basis of these observations, we investigated the regulatory actions of 17β-estradiol (E_2) on FAK activity and normal or neoplastic endometrial cell cytoskeletal remodeling and motility.

MATERIALS AND METHODS

Cell Cultures and Treatments

Ishikawa human endometrial adenocarcinoma cell line and endometrial stromal cells (ESC) were cultured and treated as previously described (11). Endometrial stromal cells were obtained from women undergoing surgical procedures requiring endometrial biopsy (diagnostic or operative hysteroscopy, diagnostic or operative laparoscopic procedures). Parallel histologic examination confirmed normal endometrial tissue. No patients with endometriosis were included. These patients were included only when free of oral contraceptives, progestins, GnRH analogues or antagonists, or other hormonal medications for at least 3 months. Women with previous autoimmune, neoplastic, hepatic, or thyroid disorders were excluded from the study. All women were younger than 40 years and older than 17 years; the median ($\pm$SD) age was 29.5 $\pm$ 7 years. All samples were collected during the follicular phase, according to the last menstrual period and histologic examination of the samples. Institutional review board approval is not requested by the University of Pisa for such cases in which no additional sampling is required. Informed consent was obtained by the patients. Ishikawa cells were grown in phenol red–free minimum essential medium supplemented with L-glutamine (2 mM), 10% fetal bovine serum (FBS), and 1% nonessential amino acids. Endometrial stromal cells were cultured to subconfluence in phenol red–free Dulbecco's minimum essential medium with 10% FBS and antibiotics.

FIGURE 1

(**A**) Ishikawa cell treatment with E_2 (10^{-10}–10^{-7} M) and with E_2 (10^{-9} M) in the presence of ICI 182,780 or (**B**) with E_2 (10^{-9} M) for 5, 10, 15, 30, or 60 minutes. p-FAK Tyr^{397}, p-FAK Tyr^{576}, FAK, and actin are shown. *$P \leq .05$ vs. control.

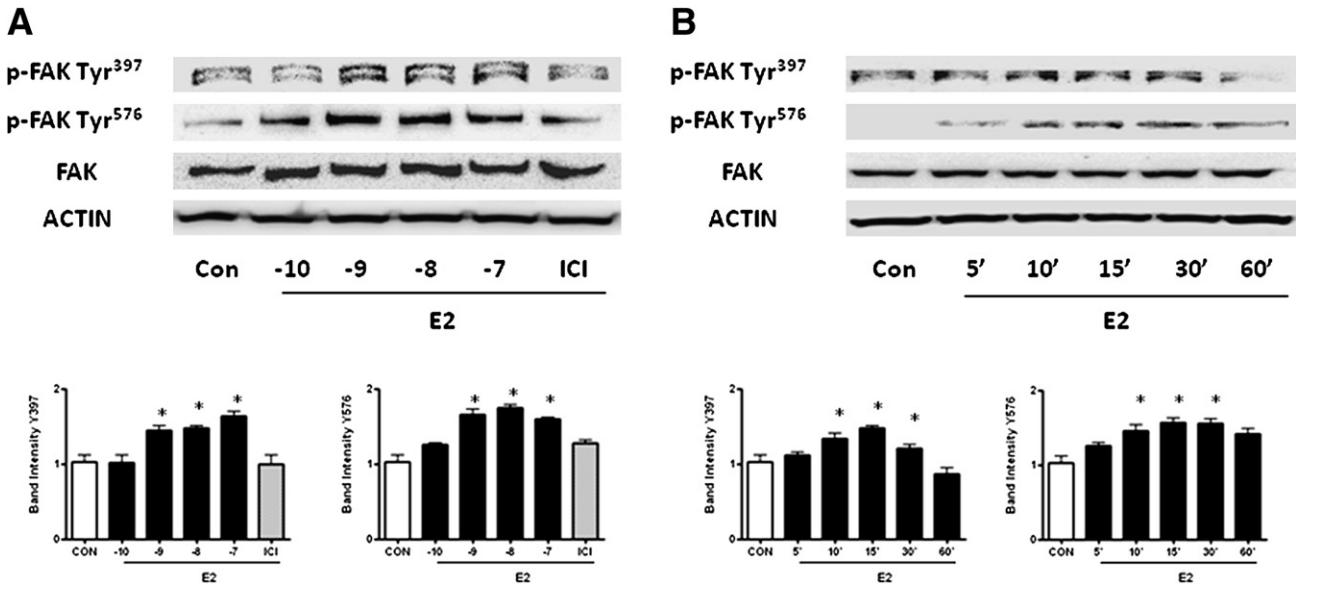

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FIGURE 2

Ishikawa cell treatment with E_2 in the presence or absence of ICI 182,780 (ICI) and (**A**) Texas Red-phalloidin linked to actin fibers, p-FAK Tyr^{397} linked to fluorescein isothiocyanate conjugate, (**B**) vinculin fibers linked to Texas Red, and p-FAK Tyr^{397} linked to fluorescein isothiocyanate conjugate. Yellow arrows indicate membrane ruffles and pseudopodia.

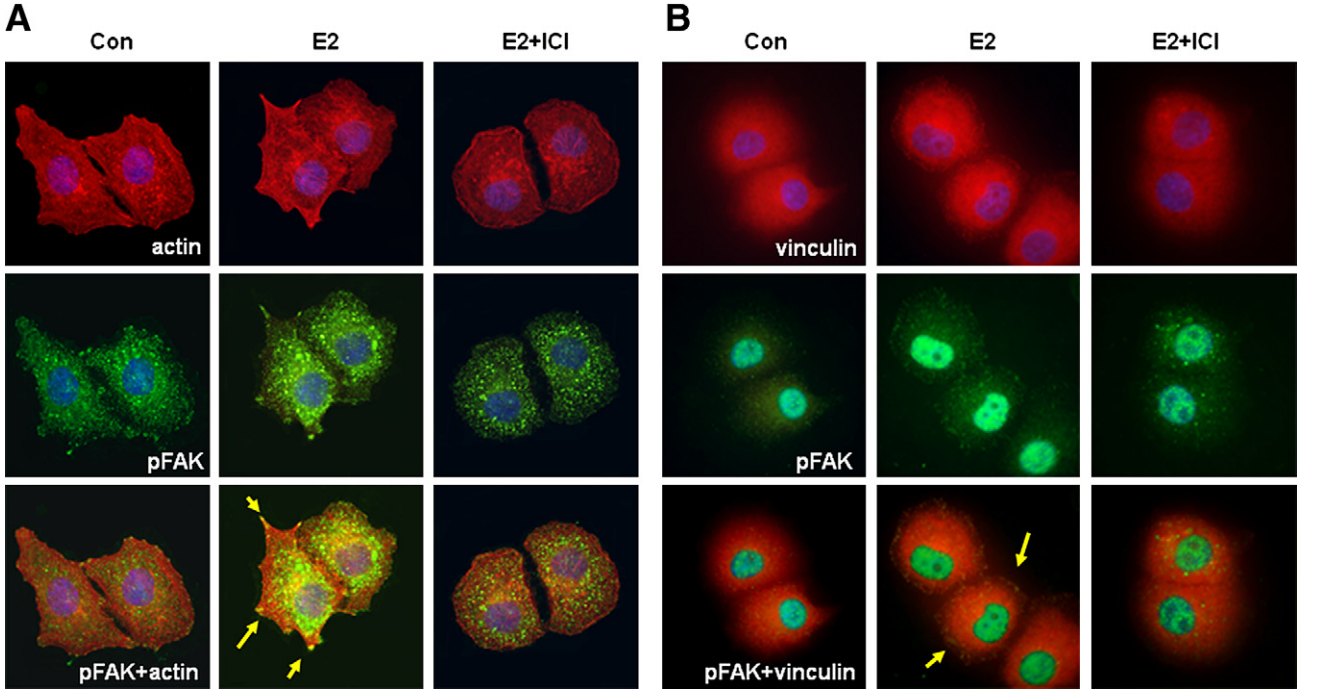

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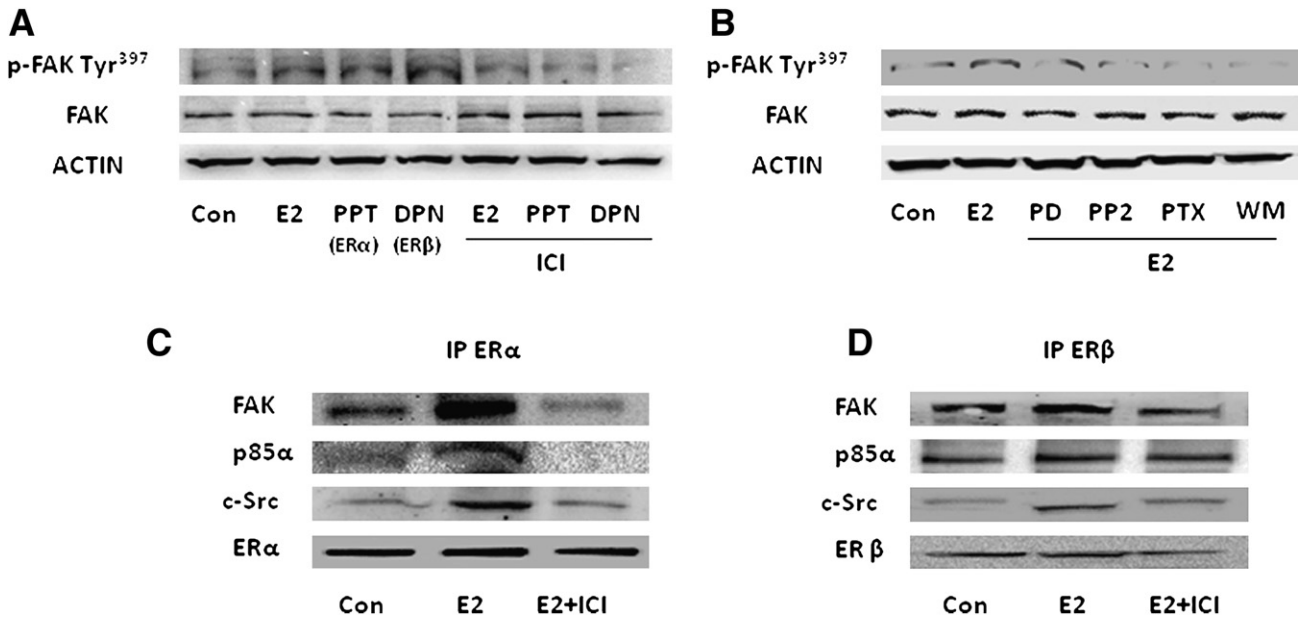
FIGURE 3

(**A**) Ishikawa cell treatment with E_2, PPT, or DPN in the presence or absence of ICI 182,780 (ICI). (**B**) Ishikawa cell treatment with E_2 in the presence or absence of different inhibitors. (**C**) Ishikawa cell treatment with E_2 in the presence or absence of ICI 182,780. Protein extracts were immunoprecipitated with an antibody against ERα, and the IPs were assayed for coimmunoprecipitation of FAK, p85α, and c-Src. (**D**) Ishikawa cell treatment with E_2 in the presence or absence of ICI 182,780. Protein extracts were immunoprecipitated with an antibody against ERβ, and the IPs were assayed for coimmunoprecipitation of FAK, p85α, and c-Src. IP = immunoprecipitate.



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Ishikawa and ESC were expanded in medium containing normal FBS. Twenty-four hours before treatments all cells were shifted to medium containing charcoal-stripped FBS with no detectable amounts of sex steroids. Before experiments investigating nontranscriptional effects, Ishikawa or ESC were kept in culture medium containing no FBS for 8 hours. Estrogen receptor (ER)α and ERβ expression was confirmed by Western analysis in all primary endometrial cell cultures and in Ishikawa cells.

Immunoblottings

Cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Antibodies used were as follows: FAK and p-FAK (Tyr^{397}) (Transduction Laboratories, Lexington, KY), ERβ (N-19), c-Src (sc-5266), and p85α (Santa Cruz Biotechnology, Santa Cruz, CA), p-FAK (Tyr^{576}; Upstate, Lake Placid, NY), ERα (TE111; NeoMarkers, Union City, CA), and vinculin (V284; Calbiochem-Novabiochem, San Diego, CA).

Cell Immunofluorescence

Fixed cells on coverslips were incubated with p-FAK Tyr^{397}, Texas Red–phalloidin (Sigma-Aldrich, St. Louis, MO), or vinculin. Immunofluorescence was visualized using an Olympus BX41 microscope (Olympus, Tokyo, Japan).

Coimmunoprecipitation Assays

Ishikawa cells were harvested as previously described (11). Equal amounts of cell lysates were incubated with 1 μg of precipitating Ab (ERα, ERβ) overnight at 4°C. Twenty-five microliters of a 1:1 protein A-agarose were added, and the samples were rolled at 4°C for another hour. The samples were washed and resuspended in 50 μL of 2× Laemmli buffer for immunoblotting.

Transfection Experiments

Small interfering RNA (SiRNA) targeting FAK (siRNA SMARTpool FAK) and a control siRNA (D-001810-01-05) were from Dharmacon (Thermo Fisher Scientific, Waltham, MA). Endometrial stromal cells were transfected with 50–75 nM siRNAs using Lipofectamine (Invitrogen, Carlsbad, CA). Endometrial stromal cells were treated 48 hours after transfection. Gene silencing was optimal at 48 hours.

Cell Migration Assays

Cell migration was tested with razor scrape assays as previously described (11). Migration was monitored for 48 hours. Cells were digitally imaged, and migration distance was measured.

Cell Invasion Assay

Cell invasion was assayed with the BioCoat Growth Factor Reduced Matrigel Invasion Chamber (BD Biosciences, San Jose, CA) (11). Invading cells were photographed at 100× magnification. Cells were counted in the central field of triplicate membranes.

Statistical Analysis

All values are expressed as mean ± SD. Statistical analysis was performed using analysis of variance followed by Tukey-Kramer multiple comparisons test. A P value of $<.05$ was considered statistically significant.

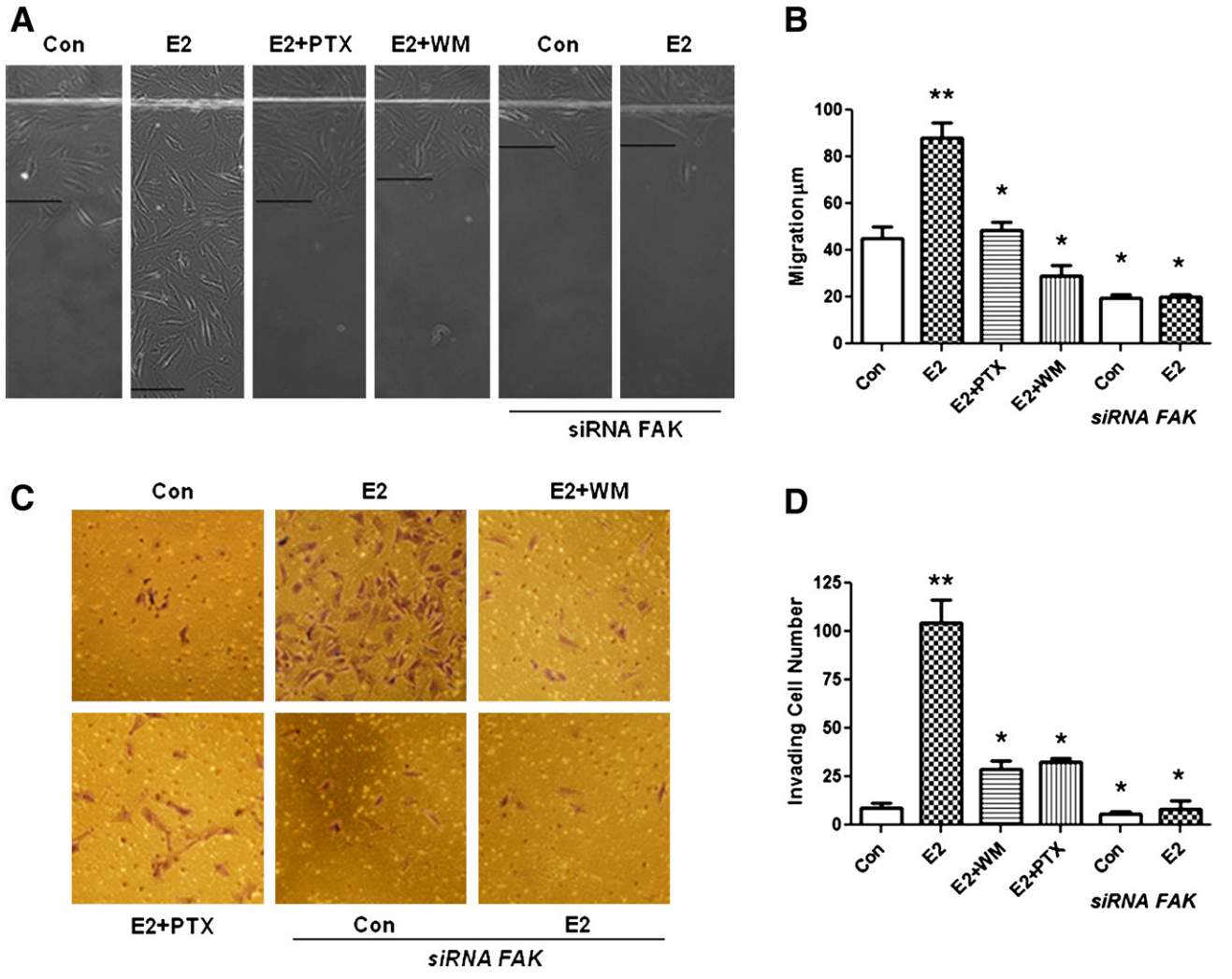
RESULTS

E_2 Rapidly Activates FAK

Ishikawa cells exposed to different concentrations of E_2 (0.1–100 nM) for 15 minutes resulted in a concentration-related phosphorylation of FAK at Tyr^{397} and Tyr^{576}. The ER antagonist ICI 182,780

FIGURE 4

Treatment of ESC with E_2 (48 hours) in the presence or absence of WM or PTX or transfect with FAK siRNA. (**A**) Horizontal migration images. (**B**) Mean migration length quantitative analysis. (**C**) Matrigel chamber images. (**D**) Mean number of invading cells. Data are expressed as mean ± SD of three separate experiments. $^{**}P \leq .05$ vs. control; $^{*}P \leq .05$ vs. E_2.



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(100 nM) prevented this action (Fig. 1A). In addition, E_2 rapidly activated Tyr^{397} and Tyr^{576} FAK phosphorylation in a time-dependent manner (Fig. 1B).

E_2 Induces Rapid Rearrangement of Actin Cytoskeleton and Formation of Focal Adhesion Complexes in Endometrial Cells

Ishikawa cells were treated with E_2 (1 nM) with or without ICI 182,780 (100 nM) for 15 minutes, and actin, Tyr^{397} p-FAK, and vinculin were visualized. Treatment with E_2 induced a rapid actin and vinculin translocation from the cytoplasm toward the cell membrane, where these two proteins colocalized with p-FAK, in association with pseudopodia (Fig. 2A and B). These morphologic changes were blunted with ICI 182,780 (Fig. 2A and B). The same experiments were repeated in ESC with equal results (data not shown).

ER Signals to FAK Through a G Protein, c-Src, and PI3K-Dependent Signaling Pathway

Ishikawa cells express both ERα and ERβ (11). Treatment of Ishikawa cells with E_2 or with the preferential ERα agonist 4,49,40-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT; 1 nM) or with the ERβ agonist 2,3-bis(4-hydroxyphenil)-propionitrile (DPN; 1 nM) resulted in FAK activation (Fig. 3A), and ICI 182,780 blocked the effect of all three ligands (Fig. 3A).

We interfered with signaling cascades linked to ER or FAK. PD98059 (5 mM), an inhibitor of mitogen-activated protein kinases, did not alter E_2-induced FAK phosphorylation (Fig. 3B). The c-Src kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo (3,4-d) pyrimidine (PP2) (10 μM), the G protein inhibitor pertussis toxin (PTX; 100 ng/mL), and the inhibitor of phosphatidylinositol 3-OH kinase (PI3K) wortmannin (WM, 30 nM), significantly inhibited Tyr^{397} FAK phosphorylation induced by E_2 (Fig. 3B).

With coimmunoprecipitation assays we found that in Ishikawa cells E_2 triggers a ligand-dependent direct association of ERα with FAK, c-Src, and the p85α PI3K regulatory subunit (Fig. 3C). In the presence of E_2 ERβ interacts with FAK and c-Src but not with p85α (Fig. 3D).

FAK Activation by Estrogen Mediates Endometrial Cell Migration and Invasion

Estradiol (1 nM) significantly increased the mean length of migration of ESC (Fig. 4A and B). Cell migration was inhibited by PTX (100 ng/mL) and by WM (30 nM) (Fig. 4A and B). Silencing of FAK with siRNAs fully prevented the effects of E_2 (Fig. 4A and B) as well. In addition, ESC showed an enhanced invasion of three-dimensional matrices in the presence of E_2, and this effect was also blocked by WM, PTX, or FAK silencing (Fig. 4C and D). Similar responses to E administration on horizontal migration and matrix invasion were found with Ishikawa cells (data not shown).

DISCUSSION

The main finding of this study is that E enhances the motility of normal and neoplastic endometrial cells through the regulation of FAK. Focal adhesion kinase activation is necessary for actin and membrane remodeling induced by E that mediates the interaction with ECM and cell movement (12, 13). Such actions of E have been recently reported in endometrial cancer (14). This suggests that FAK is an important mediator of cell motility and invasion induced by E in Ishikawa and ESC, and may in part explain the actions of this steroid as a promoter of endometrial cancer.

This article suggests two mechanisms by which E activates FAK in endometrial cells: [1] a G protein–mediated signaling to FAK; or [2] a multiprotein complex in which ERs, c-Src, PI3K, and FAK interact. However, PI3K is recruited only in the presence of ERα, and not of ERβ, consistent with previous reports (15). In the first model a G protein seems to recruit c-Src and then PI3K, which eventually phosphorylates FAK at Tyr^{397}. Interactions of ERα and G proteins such as Gα_{13}, Gα_i, or Gβ have been characterized (9–11), and modulation of FAK by G protein–initiated pathways has been established as well (16). In the second model, multiprotein complexes composed either of ERα/c-Src/PI3K or ERβ/c-Src would activate FAK. Because ERβ does not recruit FAK it is probably c-Src that acts as a bridge between ERs and FAK (17). The finding of the formation of a multiprotein complex in which FAK and PI3K interact is consistent with other reports (18, 19).

In conclusion, we show that E promotes endometrial cell remodeling and motility by increasing the formation of focal adhesion complexes. Our results provide original mechanistic insights into the effects of E on normal and neoplastic endometrial cells and may be helpful in the development of new strategies against endometrial diseases.

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