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ORIGINAL RESEARCH

Estrogen receptor-α promotes endothelial cell motility through focal adhesion kinase

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ABSTRACT: Sex steroids play a key role in cell movement and tissue organization. Cell migration requires the integration of events that induce changes in cell structure such as protrusion, polarization and traction toward the direction of migration. These actions are driven by actin remodeling and are stabilized by the development of adhesion sites to extracellular matrix via transmembrane receptors linked to the actin cytoskeleton. Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that facilitates cell migration via the control of the turnover of focal adhesion complexes. In this work, we demonstrated that 17β-estradiol (E_2) regulates actin remodeling and cell movement in human umbilical vein endothelial cells through the recruitment of FAK. E_2 induces phosphorylation of FAK and its translocation toward membrane sites where focal adhesion complexes are assembled. This process is triggered via a Gα/Gβ protein-dependent, rapid extra-nuclear signaling of estrogen receptor-α (ERα) that interacts in a multiprotein complex with c-Src, phosphatidylinositol 3-OH kinase and FAK. Phosphorylation of FAK is fundamental for its activation, translocation to the plasmatic membrane and the subsequent formation of focal adhesion complexes. In conclusion, we found that ERα enhances endothelial cell motility through the dynamic control of actin arrangement and the formation of focal adhesion complexes. The identification of these processes broadens the understanding of the actions of estrogens on endothelial cells and could be relevant in physiological or pathological settings.

Key words: endothelial cells / estrogen / estrogen receptor / focal adhesion kinase / focal adhesion complexes

Introduction

The cardiovascular actions of estrogens are prominent, but incompletely understood (Mendelsohn and Karas, 2005; Arnal *et al.*, 2009; Luksha and Kublickiene, 2009; Simoncini, 2009). The presence of estrogen receptors (ERs) and of enzymes involved in estrogen synthesis in human blood vessels and endothelium is recognized (Diano *et al.*, 1999). Between the many actions of estrogens on the vascular wall, facilitation of endothelial recovery after injury has been one of the first to be established (Mendelsohn and Karas, 2005; Arnal *et al.*, 2009; Luksha and Kublickiene, 2009; Simoncini, 2009).

A number of studies show that estrogens enhance endothelial proliferation at sites of vascular injury, promoting endothelial recovery after structural damage (Mendelsohn and Karas, 2005; Arnal *et al.*, 2009; Luksha and Kublickiene, 2009). This has been related to the activation of both long-term genomic actions and rapid signaling by estrogens through ERs (Mendelsohn and Karas, 2005; Arnal *et al.*, 2009; Luksha and Kublickiene, 2009) or through the G-protein-coupled receptor 30 (GPR30) (Prossnitz and Maggiolini, 2009), but the mechanistic explanation of these processes has been scarcely investigated. The actin cytoskeleton forms the backbone of the cell, and its spatial organization is crucial for cell movement and migration. Modification of the form and positioning of actin fibers and their relationship with membrane-anchoring structures such as integrins and focal adhesion complexes allows cell movement in the extracellular environment (Pollard and Cooper, 2009). We have recently shown that estrogen controls actin remodeling in human endothelial cells and the development of specialized membrane structures such as ruffles and pseudopodia, thus inducing endothelial cell migration (Simoncini *et al.*, 2006). These phenomena depend on the activation of the actin-regulatory protein moesin (Simoncini *et al.*, 2006).

Related to this set of actions of estrogens, another important factor of cell-membrane remodeling and cell movement is the turnover of focal adhesion complexes. These structures are dynamic in nature and their formation and breakdown are regulated by different stimuli, particularly by tyrosine kinases (McLean *et al.*, 2005).

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A key player involved in the control of the interaction between cells and the extracellular matrix (ECM) is focal adhesion kinase (FAK), a tyrosine kinase that is localized to cellular focal contact sites (McLean et al., 2005). FAK plays a crucial role in the recruitment of signal transduction pathways initiated at sites of cell attachment or by growth-factor receptors (McLean et al., 2005). FAK activation is involved in cell attachment, migration, invasion, proliferation and survival, all of which are crucial for vascular remodeling and angiogenesis (Gabarra-Niecko et al., 2003; McLean et al., 2005). FAK is involved in the motility and survival of endothelial cells (Avraham et al., 2003). Moreover, deletion of FAK in endothelial cells leads to reduced tubulogenesis, cell survival, proliferation and migration *in vitro* (Shen et al., 2005; Angelucci and Bologna, 2007). On the other hand, endothelial overexpression of FAK promotes wound-induced angiogenesis as well as ischemia-induced neovascularization (Peng et al., 2004).

Phosphorylation of FAK on Tyr^{397} creates a high-affinity binding site for the c-Src homology 2 (SH2) domain of several proteins, including c-Src kinase (Schaller et al., 1994; Schlaepfer et al., 1994). The association of c-Src with FAK leads to a conformational change and to the activation of c-Src. The ensuing phosphorylation of FAK by c-Src on Tyr^{576} and Tyr^{577} within the FAK catalytic domain leads to the full enzymatic activity of FAK (Calalb et al., 1995). The FAK–c-Src signaling complex recruits and/or phosphorylates a number of other proteins and is involved in the regulation of adhesion, motility and invasion, as well as in cell growth and survival. As these processes are crucial components of cell migration, FAK might well be involved in the mechanisms through which estrogen might control the endothelial motility during re-endothelialization of damaged vessels or angiogenesis.

We have studied the regulation of endothelial actin rearrangement and cell movement by estrogens and related these effects to the recruitment of FAK by ERα through an extra-nuclear signaling cascade.

Materials and Methods

Cell cultures and treatments

Human umbilical vein endothelial cells (HUVECs) were cultured as described previously (40). Before treatments, HUVECs were kept for 48 h in Dulbecco's modified Eagle's medium (DMEM) containing a commercial fetal bovine serum (FBS) deprived of steroids with charcoal stripping. All media were free of phenol-red. Before experiments investigating non-transcriptional effects, HUVECs were kept for 8 h in DMEM containing no FBS. Whenever an inhibitor was used, the compound was added 30 min before the active treatments. 17β-estradiol (E_2), pertussis toxin (PTX), PD98059 (PD) and wortmannin (WM) were purchased from Sigma-Aldrich (Saint-Louis, MO, USA); ICI 182,780 (ICI), 4,49,40-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) and 2,3-bis (4-hydroxyphenil)-propionitrile (DPN) were obtained from Tocris Cookson (Avonmouth, UK). 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo (3,4-*d*) pyrimidine, (PP2) was obtained from Calbiochem (EMD Biosciences, Germany). FAK inhibitor 14 (FAKi) and the GPR30 agonist G1 were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 17β-E_2 and ICI were dissolved in 100% ethanol. PTX was dissolved in phosphate-buffered saline (PBS). PD, WM and PP2 were dissolved in dimethylsulphoxide. PPT, DPN and FAKi were dissolved in water. The final concentration of the solvents was 1 µl of solvent per 1 ml of medium.

Immunoblottings

Cell lysates were separated by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE). Antibodies used were: FAK and phosphorylated FAK (p-FAK) (Tyr^{397}) (Transduction Laboratories, Lexington, KY, USA), actin (C-11), p-FAK (Tyr^{397}), ERβ (N-19), c-Src (sc-5266), p85α (N-18), $G\alpha_{13}$ (A-20), $G\alpha_{i1}$ (R-4), $G\beta_1$ (C-16) and GPR30 (K-19)-R (Santa Cruz Biotechnology), ERα (TE111, NeoMarkers, Union City, CA, USA). Primary and secondary Abs were incubated with the membranes, followed by three 5-min washings with tris-buffered saline-Tween 20. Immunodetection was carried out using enhanced chemiluminescence and was recorded with a quantitative digital imaging system (Quantity One; BioRad, Hercules, CA, USA), enabling us to assess saturation. Band densitometric analysis was performed on some blots using conditions ensuring analysis of signals in the linear range of detection.

Cell immunofluorescence

HUVECs were grown on coverslips and exposed to treatments. Cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton for 5 min. Blocking was performed with 3% normal serum for 30 min. Cells were incubated with antibodies against p-FAK (Tyr^{397}, Santa Cruz Biotechnology), Texas Red-phalloidin (Sigma-Aldrich). The nuclei were counterstained with or 4-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). Immunofluorescence was visualized using an Olympus BX41 microscope and recorded with a high-resolution DP70 Olympus digital camera. Membrane-localized Tyr^{397} p-FAK was assessed in 40 different cells in each experimental condition. Results were expressed as number of membrane-localized Tyr^{397} p-FAK (mean ± SD).

Co-immunoprecipitation assays

HUVECs were washed with ice-cold PBS and lysed with: 20 mM Tris–HCl (pH 7.4), 10 mM EDTA, 100 mM NaCl, 1% IGEPAL (octylphenoxypolyethoxyethanol), 1 mM Na_3VO_4, 50 mM NaF, 0.1 mg/l of phenylmethylsulphonyl fluoride (PMSF), 0.3 mg/l of aprotinin and 0.01% protease inhibitor mixture (Sigma-Aldrich Laboratories) before addition of the immunoprecipitating antibodies versus ERα, c-Src and FAK in 500 µl of lysis buffer for 1 h at 4°C with gentle rocking. Subsequently, 25 µl of 1:1 Protein-A-agarose was added and gently rocked for two additional hours at 4°C. The mixture was then centrifuged at 13 000*g* for 5 min at 4°C. The supernatant was removed, and the immunoprecipitates were washed with 500 ml of: 20 mM Tris–HCl (pH 7.4), 10 mM EDTA, 150 mM NaCl, 1% IGEPAL, 1 mM Na_3VO_4, 50 mM NaF, 0.1 mg/l PMSF, 0.3 mg/l aprotinin and 0.01% protease inhibitor mixture (Sigma-Aldrich Laboratories). Immunoprecipitated proteins were separated under reducing and denaturing conditions by 10% SDS–PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). Non-specific binding was blocked with 5% skim milk in PBS–Tween. Membranes were incubated with anti-FAK, p85α, ERα and c-Src antibodies.

Gene silencing with RNA interference

Synthetic, small interfering RNAs targeting ERRR-α (siRNAs SMARTpool ESR1), ER-β (siRNA SMARTpool ESR2) and control siRNAs (D-001810-01-05) were purchased from Dharmacon (Thermo Fisher Scientific Inc., USA). $G\beta_1$ and GPR30 siRNAs were from Santa Cruz Biotechnology. The siRNAs were used at the final concentration of 50–75 nM. HUVECs were treated 48 h after siRNAs transfection. Efficacy of gene silencing was checked with western analysis and found to be optimal at 48 h.

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Transfection experiments

Dominant negative constructs for $G\alpha_{i1}$ ($G\alpha_{i1}$ G202T) and $G\alpha_{13}$ ($G\alpha_{13}$ Q226L/D294N) were from the Guthrie cDNA Resource Center (www.cdna.org). The inserts were cloned in pcDNA3.1+. The plasmids (10 µg) were transfected into T47-D cells using Lipofectamine (Invitrogen, Carlsbad, CA, USA). Parallel cells were transfected with empty pcDNA3.1 + plasmid. Cells (60–70% confluent) were treated 24 h after transfection.

Cell-migration assays

Cell migration was assayed with razor-scrape assays as previously described (Simoncini *et al.*, 2006). Briefly, a razor blade was pressed through the confluent HUVECs monolayer into the plastic plate to mark the starting line. HUVECs were swept away on one side of that line. Cells were washed, and 2.0 ml of DMEM containing steroid-deprived FBS were added. Cytosine β-D-arabinofuranoside hydrochloride (Sigma-Aldrich) (10 µM), a selective inhibitor of DNA synthesis that does not inhibit RNA synthesis, was used 1 h before the test substance was added. Migration was monitored for 48 h. Every 12 h, fresh medium and treatment were replaced. Cells were digitally imaged and migration distance was measured with phase-contrast microscopy.

Statistical analysis

All values are expressed as (mean + SD). Statistical analysis of the data was performed using one-way analysis of variance followed by Tukey–Kramer multiple comparisons test. $P < 0.05$ was considered as statistically significant.

Results

E_2 rapidly activates FAK in HUVECs

Exposure of HUVECs to different concentrations of E_2 (from 10^{-10} to 10^{-6} M) for 20 min resulted in a phosphorylation of FAK at Tyr^{397} that was maximal at 10^{-10} M, and then decreased with increasing E_2 concentration (Fig. 1A). The total cell content of FAK did not change at any concentration tested (Fig. 1A). Since the quantitative effect of 10^{-10} M and that of 10^{-8} M E_2 were not statistically different, we chose to use 10^{-8} M in the subsequent experiments, to allow comparisons with previous studies on cytoskeletal controllers, where this concentration was used (Simoncini *et al.*, 2006). In addition, E_2 rapidly activated Tyr^{397} FAK phosphorylation in a time-dependent manner with a maximal effect at 20 min and then a decrease to basal levels after 60 min (Fig. 1B).

Estrogen activates FAK via ER-α

HUVECs express both ER isoforms, ERα and ERβ (Kim-Schulze *et al.*, 1998; Toth *et al.*, 2009). To identify which ER isoform is required for the signaling of estrogen to FAK, we treated HUVECs with E_2 or with the preferential ERα agonist PPT (1 nM) or with the ERβ agonist DPN (1 nM) in the presence or absence of the pure ER antagonist ICI (100 nM). FAK activation was detected in the presence of either E_2 or PPT, but not by DPN (Fig. 2A). ICI blocked the effect of E_2 and PPT, suggesting that ERα (and not ERβ) mediates the signaling of estrogen to FAK.

Because the ER selectivity of PPT and DPN is not absolute (at high concentrations, they cross-bind to the other receptor), we confirmed this result by silencing ERα and ERβ in endothelial cells with targeted siRNAs. Transfection of ERα siRNAs resulted in a marked reduction in ERα expression (Fig. 2C), along with a marked decrease in FAK phosphorylation on tyrosine 397 upon exposure to E_2 administration (Fig. 2B), in the absence of modifications of the expression of FAK (Fig. 2B). On the contrary, silencing of ERβ did not influence FAK phosphorylation by E_2 (Fig. 2B and D).

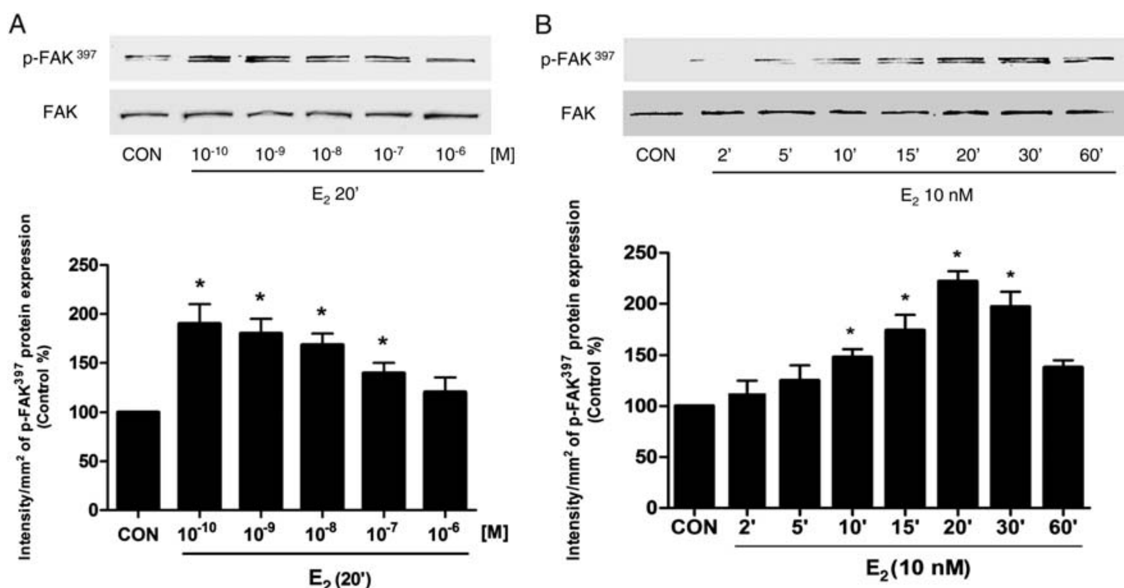

Figure 1 E_2 activates FAK in endothelial cells. (**A** and **B**) Show the dose- and time-dependent FAK activation in HUVECs after treatment with E_2. Total cell amount of wild-type (FAK) or Tyr^{397}-p-FAK^{397} are shown with western blot. Phospho-FAK^{397} densitometry values were adjusted to FAK intensity, then normalized to the control sample. *$P < 0.05$ versus corresponding control.

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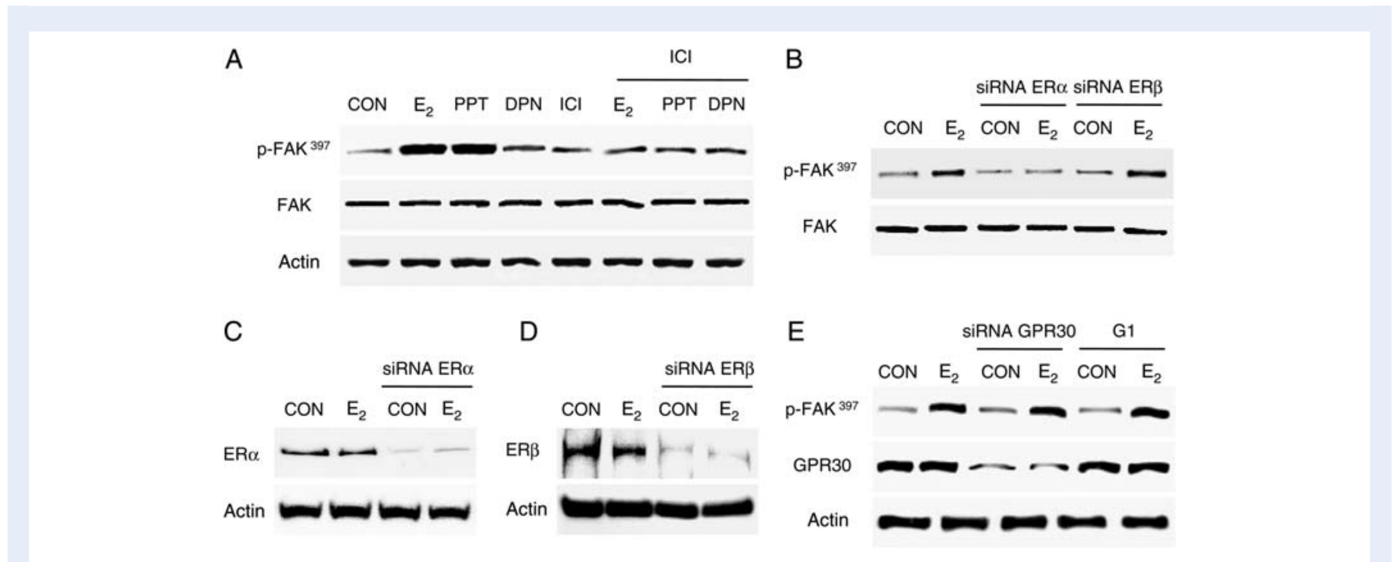

Figure 2 Estrogen signals to FAK via ERα. (**A**) Endothelial cells were exposed for 20 min to 10 nM E_2 or with the preferential ERα agonist PPT (1 nM) or with the ERβ agonist DPN (1 nM) in the presence or absence of the pure ER antagonist ICI (100 nM). Phosphorylation of FAK was assayed with western analysis. (**B–D**) HUVECs were transfected with siRNA versus ERα (siRNA ERα) or ERβ (siRNA ERβ) or with vehicle, and protein analysis for ERα, ERβ, actin, total immunoreactive FAK (FAK) or phospho-FAK397 was performed on cell lysates after treatment for 20 min with 10 nM E_2. (**E**) HUVECs were transfected with siRNA versus GPR30 (siRNA GPR30) and treated for 20 min with vehicle, E_2 or with the GPR30 agonist, G1 (10^{-6} M). CON denotes control. All experiments were repeated three times and consistent results were obtained and representative images are shown.

To determine the possible role of GPR30, also known as the membrane ER in E_2 signaling to FAK, we have used both G1 (GPR30 agonist) and GPR30 siRNAs, and we showed that GPR30 does not mediate the effects of E_2, since the siRNA does not block E_2-mediated FAK phosphorylation, and G1 does not trigger FAK phosphorylation nor does it potentiate the effect of E_2 (Fig. 2E).

ER signals to FAK through a G protein, c-Src and PI3K-dependent signaling pathway

In search for the signaling pathways through which ER leads to FAK activation, we interfered with a number of signaling cascades that linked ER to FAK. PD (5 mM), an inhibitor of mitogen-activated protein kinases (MAPK), did not alter the E_2-induced FAK phosphorylation (Fig. 3A). Instead, the G-protein-inhibitor, PTX, (100 ng/ml), the c-Src kinase inhibitor (PP2, 10 μM) and an inhibitor of phosphatidylinositol 3-OH kinase (PI3K), WM, (30 nM), significantly inhibited FAK Tyr^{397} phosphorylation induced by E_2, indicating that ERα recruits a G protein, c-Src and PI3K to FAK in endothelial cells (Fig. 3A).

To determine the specific G protein involved in the FAK phosphorylation during E_2 exposure, we blocked $G\alpha_i$ and $G\alpha_{13}$, by transfection of a dominant negative construct and silenced Gβ with targeted siRNAs. With western analysis we found that, in the presence of E_2, ERα signals to FAK via $G\alpha_i$ and Gβ, while $G\alpha_{13}$ is not involved in FAK activation (Fig. 3B).

ERα forms a multi-protein complex with c-Src, PI3K and FAK

To further characterize the signaling partnership between ERα, c-Src, PI3K and FAK, we performed co-immunoprecipitation assays in endothelial cells. With multiple immunoprecipitation assays, we found that, in the presence of E_2, ERα increases its interaction with c-Src, p85α and FAK and that this interaction is disrupted by the use of ICI (Fig. 4A). The E_2-dependent formation of a multiprotein complex between ERα, Src, p85α and FAK was confirmed by separate immunoprecipitations (Fig. 4B and C). This is consistent with the findings of a previous study (Le Romancer et al., 2008) and suggests that estrogen increase the interaction via a direct association of ERα with c-Src, PI3K and FAK and consequent formation of a multi-protein complex.

E_2 induces a rapid rearrangement of the actin cytoskeleton and the formation of focal adhesion complexes in HUVECs

In order to ascertain the role of FAK in the formation of focal adhesion complexes, we examined the co-localization of actin and Tyr^{397}-p-FAK. Steroid and serum-deprived HUVECs were treated with E_2 (10 nM) for 20 min with or without the ER antagonist ICI (100 nM), the c-Src kinase inhibitor (PP2 (10 μM), the PI3K inhibitor WM (30 nM) and the FAKi 1 μM, and actin and p-FAK Tyr^{397} localization were visualized with cell immunofluorescence. In untreated cells, p-FAK and actin fibers were diffusely distributed throughout the cytoplasm (Fig. 5A). Treatment with E_2 induced a rapid actin translocation from the cytoplasm toward the cell membrane were it co-localized with p-FAK, in association with specialized membrane structures and formation of focal adhesion complexes (Fig. 5A). These morphological changes were blunted when the cells were treated with the ER antagonist ICI, PP2, WM and FAKi (Fig. 5A and B). In support of a role of ERα in the rapid activation of FAK by E_2, a ligand-dependent co-localization of ERα and Tyr^{397}-p-FAK was seen (Fig. 5C). This is consistent with our recent observation in breast-cancer cells (Sanchez et al., 2010).

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Activation of FAK by estrogen mediates migration of endothelial cells

To address the question of the relevance of estrogen signaling to FAK for HUVEC motility, we pre-treated HUVECs with cytosine arabinoside (1-(β-D-arabinofuranosyl) cytosine hydrochloride-Ara-C, 100 μM), an inhibitor of DNA-strand separation that prevents cell division (so to dissect the actions of estrogen on movement from those on cell proliferation), and we performed horizontal migration assays. As we previously demonstrated with different types of cells, including HUVECs (Simoncini et al., 2006; Flamini et al., 2009a, b), treatment with E_2 (10 nM) significantly increased the mean length of migration (Fig. 6A). Cell migration was inhibited by ICI, PP2, WM and by FAKi (Fig. 6A and B).

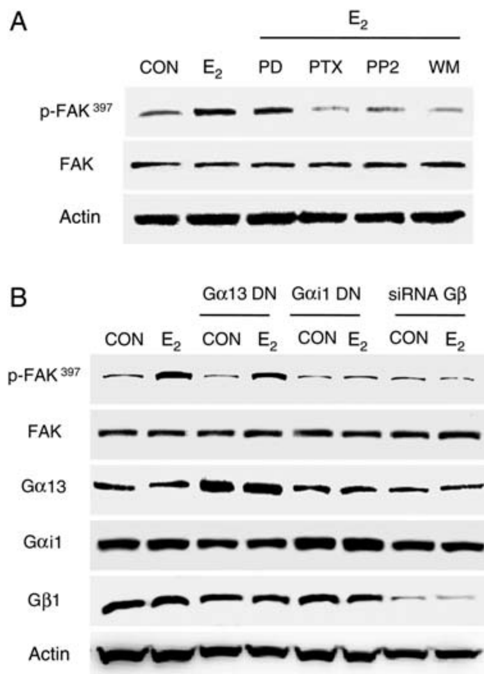

Figure 3 ERα signaling to FAK requires $G\alpha_i/G\beta$. (**A**) HUVECs were exposed for 20 min to 10 nM E_2, in the presence or absence of the G-protein-inhibitor PTX (100 ng/ml), the c-Src inhibitor PP2 (0.2 μM), the PI3 kinase inhibitor WM; (30 nM) or MAPK inhibitor PD (5 mM), and Tyr^{397} FAK phosphorylation was assayed with western analysis. (**B**) HUVECs were treated with E_2 (10 nM) after transfection with dominant-negative $G\alpha_{13}$ or $G\alpha_i$ constructs or siRNAs versus $G\beta_1$. $G\alpha_{13}$, $G\alpha_i$, $G\beta_1$, actin, FAK or phospho-FAK397 were assayed in cell extracts. CON represents control. All experiments were performed three times and representative blots are presented.

Discussion

The main result of this work is that estrogen enhances the motility of endothelial cell through the control of the focal adhesion complex regulator, FAK. This finding is in line with emerging evidence that highlights how steroid hormones are powerful regulators of the plasticity of cell membrane and cytoskeleton (Sanchez and Simoncini, 2010). Such actions are being appreciated as potentially important means of regulation of cell motility by steroid hormones in different settings, such as endothelial remodeling (Simoncini et al., 2006), cancer progression (Flamini et al., 2009b; Fu et al., 2010) or neuronal branching (Sanchez et al., 2009).

In endothelial cells, correct regulation of cell motility is thought to be important to retain a healthy endothelium, allowing an effective regeneration of damaged or dysfunctional cells (Simoncini et al., 2006). Furthermore, it is also important during angiogenesis (Peng et al., 2004; Shen et al., 2005). In addition to this, estrogens are thought to be important players in the control of atherosclerosis development and progression (Arnal et al., 2009), and their withdrawal at the time of menopause or, on the opposite, their replacement with hormone therapies is known to affect function and morphology of endothelial cells (Kublickiene et al., 2008). For these reasons, understanding the mechanistic basis through which estrogens control the interaction between the cell and the extracellular environment heralds profound biological and medical implications.

We previously showed that the sex steroid estrogen promotes endothelial cell migration through the control of the actin-binding protein moesin (Simoncini et al., 2006). We here found that estrogen also contributes to the motility of endothelial cells by regulating FAK.

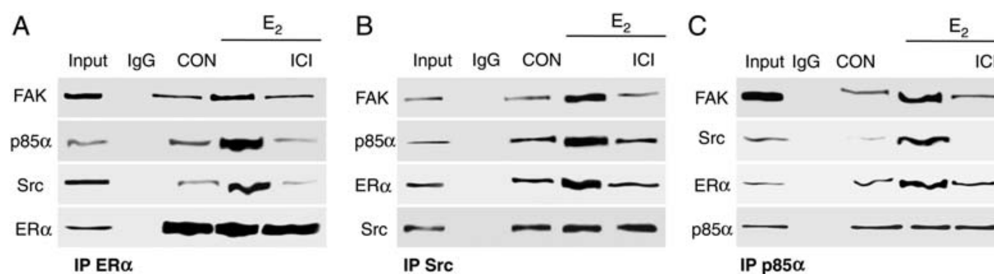

Figure 4 ERα signals to FAK via interaction with Src and PI3K. Endothelial cells were exposed to 10-nM E_2 for 20 min, in the presence or absence of the ER antagonist ICI (100 nM). Cell protein extracts were immunoprecipitated with an Ab. versus. ERα (**A**), c-Src (**B**) and p85α (**C**). The IPs were assayed for co-immunoprecipitation of ERα, c-Src, p85α and FAK. The membranes were re-blotted for the immunoprecipitated protein to show equal loading. Protein extracts without immunoprecipitation (input) were used to identify the correct band. The experiments were performed in triplicates and representative images are shown.

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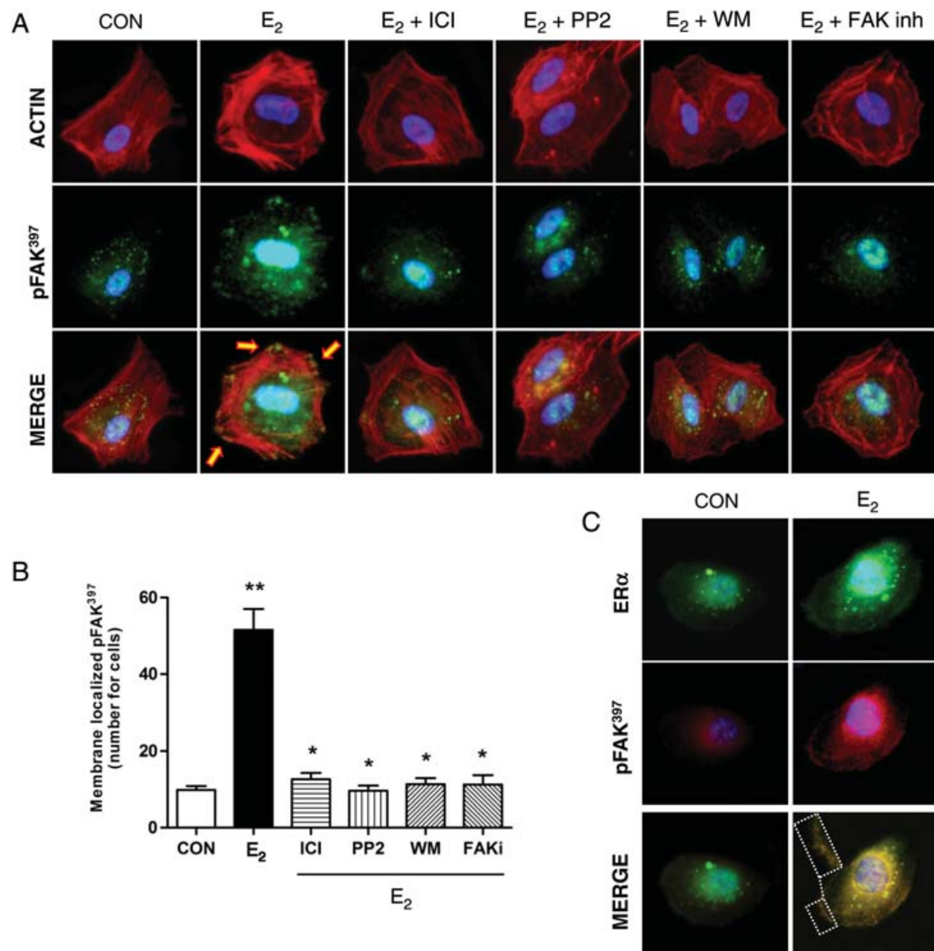

Figure 5 E_2 induces FAK phosphorylation and formation of focal adhesion complexes. (**A**) HUVECs were treated with E_2 (10 nM) for 20 min in the presence or absence of the ER antagonist ICI (100 nM), the c-Src kinase inhibitor (PP2, 10 μM), the PI3K inhibitor WM (30 nM) and the FAKi (1 μM). Cells were stained with anti-phospho-Tyr^{397} FAK (p-FAK^{397}) linked to fluorescein isothiocyanate (FITC), actin was stained with phalloidin linked to Texas Red and nuclei were counterstained with DAPI. Yellow arrows indicate membrane-localized p-FAK^{397}. CON stands control. (**B**) The bar graphs show the quantification of the membrane-localized Tyr^{397} phospho-FAK in different conditions. $*P < 0.05$ versus E_2, $**P < 0.05$ versus control. Membrane-localized Tyr^{397} p-FAK complexes were counted in 40 different cells for condition. (**C**) Cells were stained with anti-ERα linked to FITC or with anti-phospho-Tyr^{397} FAK (p-FAK^{397}) linked to Texas Red and nuclei were counterstained with DAPI. Yellow signal indicates areas of co-localization. All the experiments were repeated three times and consistent results were obtained, and the representative images are shown.

This is consistent with previous publications, showing that estrogens control motility in endothelial cells through the control of endothelial nitric oxide synthase and FAK (Tan et al., 1999), as well as with our recent identification of a control of FAK by ERs in breast-cancer cells (Sanchez et al., 2010). Based on our present and previous results (Sanchez et al., 2010), the interaction of ERα with FAK and other signaling intermediates, such as c-Src and PI3K, would be important for the activation of FAK, and would likely happen at or near the cell membrane.

FAK is a non-receptor tyrosine kinase that recruits Src family kinases and phosphatidyl-inositol-3-OH kinase via autophosphorylation and is a pivotal modulator of adhesion turnover (Reiske et al., 1999; Thamilselvan et al., 2007). The precise mechanism of FAK activation has remained elusive for many years. This is likely due to the fact that FAK can be activated by multiple inputs and in different manners (McLean et al., 2005). Modulation of FAK by G-protein-initiated pathways has been previously established in other settings (Cohen-Hillel et al., 2009). This paper shows that in the presence of E_2, ERα recruits a $G\alpha_i/G\beta$-dependent signaling that triggers the formation of a multi-protein complex where ERα, c-Src, PI3K and FAK interact. Within this complex, FAK is hyperphosphorylated on Tyr^{397}, inducing the formation of focal adhesion complexes. We here identify the recruitment of c-Src and PI3K by estrogen, and we show that this step is required for FAK phosphorylation on Tyr^{397}. This is consistent with previous reports showing that the c-Src/PI3K pathway is implicated in Tyr^{397} FAK phosphorylation (Reiske et al., 1999; Thamilselvan et al., 2007). Based on our results and on those from previous reports (Le Romancer et al., 2008), estrogen seems to control FAK tyrosine phosphorylation through c-Src kinase (McLean et al., 2005). The current understanding is that

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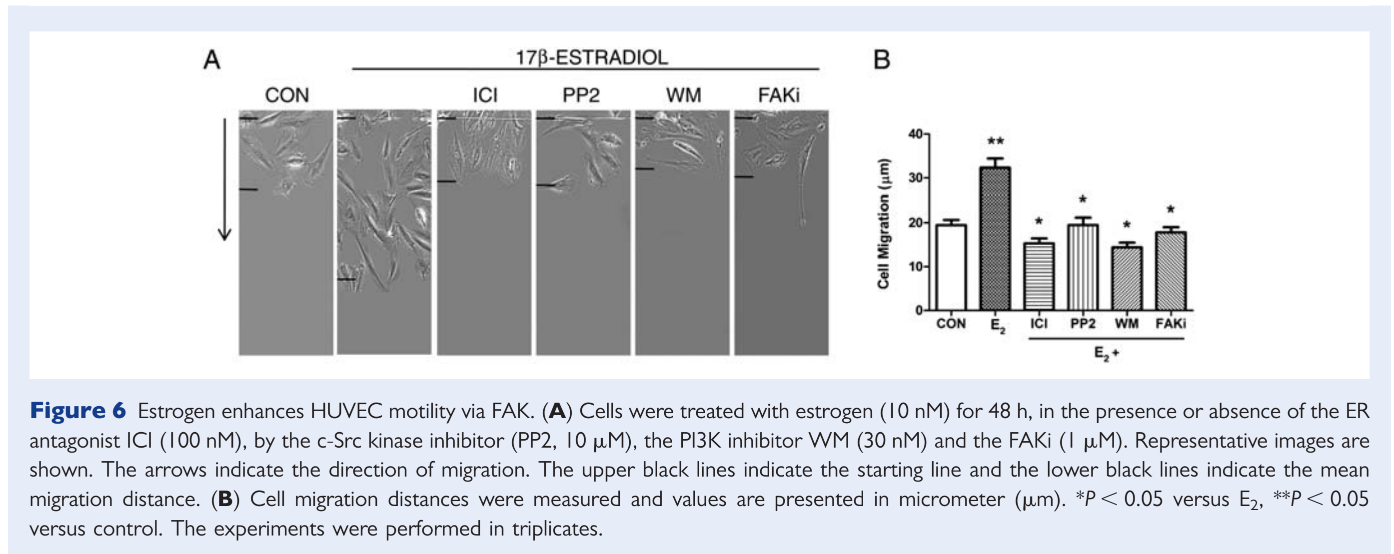

Figure 6 Estrogen enhances HUVEC motility via FAK. (**A**) Cells were treated with estrogen (10 nM) for 48 h, in the presence or absence of the ER antagonist ICI (100 nM), by the c-Src kinase inhibitor (PP2, 10 μM), the PI3K inhibitor WM (30 nM) and the FAKi (1 μM). Representative images are shown. The arrows indicate the direction of migration. The upper black lines indicate the starting line and the lower black lines indicate the mean migration distance. (**B**) Cell migration distances were measured and values are presented in micrometer (μm). $*P < 0.05$ versus E_2, $**P < 0.05$ versus control. The experiments were performed in triplicates.

autophosphorylation on Tyr^{397} creates a docking site for Src and other SH2-containing proteins (McLean *et al.*, 2005). Src would thus trigger the phosphorylation of the other Tyr residues, modulating FAK activity (McLean *et al.*, 2005).

FAK activation in endothelial cells is involved in the rapid and dynamic remodeling of the actin cytoskeleton induced by estrogen and in the formation of specialized cell membrane structures that mediate the interaction with the ECM and cell movement. This data suggest that FAK is an important mediator of cell motility induced by estrogens in endothelial cells.

In conclusion, we show that estrogen promotes HUVEC remodeling and motility by increasing the formation of focal adhesion complexes through the activation of FAK. In the presence of estrogen, ERα recruits $G\alpha_i/G\beta$ proteins, resulting in the formation of a multiprotein complex involving c-Src, PI3K and FAK, in the context of which FAK phosphorylation at Tyr^{397} takes place. Beyond its relevance in endothelial cells, the characterization of this novel mechanism of action may offer an insight into a number of processes regulated by estrogens that involve movement of endothelial cells, such as angiogenesis, vascular remodeling or atherosclerosis.

Authors' roles

A.M.S. carried out the majority of the experiments and drafted the manuscript. M.I.F. performed immunofluorescence and migration assays. S.Z. and S.G. performed cell culture and treatments. A.R.G. was instrumental in obtaining the fund for this study and is involved in drafting the manuscript. T.S. received the fund for the project, planned and supervised the experiments and wrote the paper.

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