Cytonuclear Estrogen Receptor Alpha Regulates Proliferation and Migration of Endometrial Carcinoma Cells

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Objective: The effects of estrogen on cells are mediated by the estrogen receptor α (ERα) which localizes at the peri-membrane, cytoplasm, and the nucleus of cells. Therefore, we intended to investigate how cytonuclear ERα plays its roles in different cellular activities.
Methods: We used amino acid substituted ERα that localized at the cytoplasm and nucleus but has no direct DNA-binding activities. ERα-negative endometrial carcinoma cells (ERα-) were stably transfected with plasmid of human ERα carrying a substituted phenylalanine at position 445 with alanine (ERα-F445A). Treated with 17β-estrogen (E2) or bazedoxifene (BDF), cell proliferation, migration, and expression of kinases related to ERα signal transduction pathways were observed.
Results: E2 (40 nM) significantly activated proliferation in ERα-F445A cells, but not in ERα- cells. Similarly, E2 significantly activated cell migration in ERα-F445A cells, rather than that in ERα- cells. While no obvious change in the amount of the non-phosphorylated mammalian target of Rapamycin (mTOR), the expression of mTOR phosphorylated at serine 2448 decreased, which was recovered in presence of 17β-estrogen (E2) in the ERα-F445A cells. On the other hand, the expression of focal adhesion kinase (FAK) phosphorylated at tyrosine at 297 was attenuated in the ERα-F445A cells treated with E2.
Conclusion: It is suggested that the cytonuclear ERα-F445A induces phosphorylation of kinases in downstream pathways, which regulate cell proliferation and migration.

Key words: Estrogen receptor alpha; Migration; Endometrium; focal adhesion kinase (FAK); mammalian target of Rapamycin (mTOR)

INTRODUCTION

Endometrial cancer is the most common type of cancer in female reproductive tract (https://seer.cancer.gov/data/citation.html, 2018). The majority of endometrial cancers have potential to be activated by estrogen through estrogen receptor alpha (ERα) [1, 2]. Therefore, further studies should be devoted to further understanding of how ERα functions in endometrial cancer cells [3].

Although estrogens are classically considered as female reproductive hormones, they exert various cellular functions in almost all of the tissues in mammalian species. ERα is one of the main factors through which estrogens carry out their pleiotropic functions [4]. It is once suggested that estrogens predominantly bind to the ERα in the cytoplasm, and then the ERα translocate to the nucleus, where it directly or indirectly binds to DNA and regulate the transcription of the target genes [5–8].

In addition to the nuclear ERα, compelling evidence presently demonstrate that ERα in cytoplasm and at cell membrane also play critical roles in both normal endometrium and cancer cells [3]. Studies show that the ERα is palmitoylated in cytoplasm and translocated to the caveolae at cell membrane [9, 10], which constitutes approximately 10% of cellular ERα. This membrane-initiated ERα-signaling usually occur rapidly, which can be detected within seconds or minutes [11–13]. E2 rapidly activates several protein kinases, such as mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinases (ERK), phosphatidylinositol 3-kinase (PI3K), and protein kinase A (PKA) and phosphatases, as well as the release of several cyclic adenosine monophosphate (cAMP) and calcium, which orchestrate cell proliferation, migration, and survival [14–17]. On the other hand, there are a lot of molecules that interact with ERα in cytoplasm [18–20]. Studies show that estrogen can rapidly trigger cytoplasmic complex(es) containing ERα, the tyrosine kinase Src, and the p85 subunit of PI-3 kinase (PI3K) [21–25], leading to the activation of key downstream signaling kinases such as MAPK, AKT, mTOR, and FAK. Furthermore, it has been described that ERα lo-

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calized in mitochondria [26, 27]. Via the signals to and from membrane and nucleus, the mitochondrial ERα regulates the gene expression, biogenesis, and various functions of mitochondria, which play a critical role in activation of the proliferation, survival, migration, and invasion of cancer cells [28–30].

To investigate how cytonuclear ERα plays its roles in different cellular activities, ERα-negative endometrial carcinoma cells (ERα-) in the present study were stably transfected with plasmid of human ERα carrying a substituted phenylalanine at position 445 with alanine (ERα-F445A). The phenylalanine at 445 in ERα is involved in its palmitoylation and the palmitoylation-dependent membraneous localization [10]. Cell proliferation and migration, including AKT, mTOR, and FAK were evaluated in ERα-F445A cellls.

MATERIALS AND METHODS

Cell culture

An ERα-negative (ERα-) cell line, Ishikawa (Heraklio) 02 ER- was obtained from the European Collection of Authenticated Cell Cultures (ECACC: 98032302). The cells were derived from an endometrial carcinoma patient. Cells were grown in DMEM/F12 medium supplemented with Antibiotic-Antimycotic, GlutaMAX (Gibco, NY, USA), and 10% FBS (Biosera, MO, USA), and were maintained at 37°C in a humidified atmosphere at 5% CO_2. Before experiments, cells were routinely cultured overnight in the phenol-free medium containing 4% charcoal/dextran-stripped serum (cFBS), unless indicated otherwise. In 3 hours after seeding for experiments, the medium were changed with those containing 40 nM 17β-estradiol (E2), 40 μM bazedoxifene (BDF), or the vehicle dimethylsulfoxide (DMSO, 1/2000). All reagents were from Sigma-Aldrich, Japan, unless indicated otherwise.

Plasmid and stable transfection

Human ERα cDNA in pCMV5-ERα (kindly provided by M. Muramatsu) was cut out and inserted into plasmid pIRES2-EGFP (#6029-1, Clontech, CA, USA) between SalI and BamHI. The plasmid was then used as template for creating mutation by polymerase chain reaction (PCR)-based, site-directed mutagenesis using QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Japan). The forward primer was GAACCTGCAGGGTGAAGAGGCT, and the backward, GATGGATTTGAGGCACACAGCCTCT. The primers contained sequences for amino acid substitution replacing phenylalanine (F) at position 445 to alanine (A). The plasmid ERα-F445A was sequenced for mutation verification.

To generate cells expressing ERα-F445A, FuGENE 6 (Promega, WI, USA) was used for stable transfection of the constructed plasmids into ERα- cells following the manufacturer's protocol. Forty-eight hours post-transfection, cells were harvested and replated with a 1:5 dilution ratio in selective medium containing 600 μg/ml G418 sulfate (Gibco, NY, USA). Four to five GFP-positive clones were collected for each plasmid using cloning cylinders. Three pairs of clones with similar fluorescent intensity for GFP, respectively, were then purified by fluorescence activated cell sorting (FACS) using MoFlo Astrios EQ/EQs (Beckman Coulter Japan). The expression and localization of ERα-F445A was confirmed using immunohistochemistry and Western blot. The selected clones were routinely cultured in the medium containing 400 μg/ml G418 sulfate.

Immunocytochemistry

Immunocytochemistry was carried out as previously reported with minor modifications [31, 32]. Cells were seeded at 1.25×10^4 cells/cm^2 on Nuc Lab-Tek II chamber slides with four wells (Thermo Fisher Scientific, Tokyo, Japan) in a phenol red-free formulation of DMEM/F-12 containing 10% charcoal/dextran-stripped FBS. After culture for 24 hours, cells were fixed in ice-cold 100% methanol for 10 minutes at -20°C, permeabilized with 0.1% of Triton X-100 in 0.01 mM phosphate-buffered saline (PBS), pH 7.4 at room temperature for 10 minutes. The primary anti ERα (F-10, sc-8002) antibodies (Santa Cruz Biotechnology, TX, USA) were used at recommended dilution (1:400), which were detected using a biotinylated universal antibody (1:200) included in a Vectastain Universal Quick Kit from Vector Laboratories (Burlingame, CA, USA). Sections were incubated with 0.5 μg/ml Alexa Fluor® 594 conjugated streptavidin from Thermo Fisher Scientific (Tokyo, Japan) for 30 minutes at room temperature, counterstained with 0.5 μg/ml 4', 6-diamidino-2-phenylindole (DAPI) in PBS for 15 min and mounted. Images were captured using LSM 510 Confocal Microscope from Zeiss (Tokyo, Japan). The staining was repeated three times, using one or two slides each time. Each staining was repeated four times with similar results from three different clones.

Cell growth assay using alamarBlue

Cells were diluted in medium to a density of 7.5×10^4 viable cells per ml and 100 μl (0.75×10^4 cells) added to each well of a 96-well tissue culture-treated plate. The medium was then changed with those containing 40 nM E2, 40 μM bazedoxifene, or DMSO. Cell viability was determined using alamarBlue Cell Viability Reagent (Thermo Fisher Scientific, Japan). In 24 hours, 10 μl of alamarBlue was added to each well. After further incubation for 60 minutes, the fluorescence intensity was measured at 544/590 nm using SpectraMax M5e (Molecular Device, CA, USA). Each experiment was repeated four times with similar results from three different clones.

Scratch assay and single cell track analysis

To minimize the effects of cell proliferation on analyses of cell migration, cells were cultured in the medium supplemented with 8% cFBS. Cells (1.5×10^6 cells per well) were seeded in 6-well plate and allowed to attach for 3 hours, which would reach 100% confluence. Cross-shaped cell wounds were made using a 100 μl pipette tip and photographed immediately. The medium was then changed with those containing 40 nM E2, 40 μM bazedoxifene, or DMSO (1/2000), and photographs were taken in 24 hours after scratching. The area of the cross-shaped wound was measured using ImageJ 1.52v [33]. As all wounds created were not the exact same size, the wound areas at the endpoints were calculated by normalizing it to the wound area at the startpoint and expressed as a percentage.

To record single cell position in real time, images of 200 magnification were collected every 60 min for 20 hours using IncuCyte Live-Cell Analysis System (Essen BioScience, MI, USA). Single cell migration distance in pixel was tracked using Fiji [34] in combination with CellTracker v1.0 [35]. The end-to-end displacement was defined as the straight distance. Each experiment was repeated four times with similar results from three different clones.

Western blot analysis

Western blot was carried out as previously described [36]. Cells were treated with 40 nM E2 or the DMSO (1/2000) for 24 hours. In brief, cells were sonicated using an ultra-homogenizer (VP-60S; Taitec, Japan) in 1xSDS sample buffer (0.0625 mol/L Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 0.005% bromophenol blue, 5% 2-mercaptoethanol). Equal amounts of proteins were loaded onto the gels, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto Immobilon-P membrane (Millipore, Billerica, MA, USA). The membranes were probed with the following primary antibodies: anti-ERα (F-10, sc-8002), and anti-β-actin (sc-47778) all from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); or probed with anti-AKT (#9272), anti-phospho-AKT (Ser473) (#4058), anti-mTOR (TC10, #2983), anti-phospho-mTOR (Ser2448) (#5536), anti-focal adhesion kinase (anti-FAK, #3285), and anti-phospho-FAK (Tyr397) (#3283) all from (Cell Signaling Technology, Inc., Danvers, MA, USA). Immunoreactive proteins were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) and the Immobilon Western Chemiluminescent HRP Substrate (Merck KGaA, Germany) on a WSE-6300 Luminograph III molecular imager (ATTO Corporation, Tokyo, Japan). The experiment was repeated three times with similar results from three different clones.

Statistical analysis

Experimental data were presented as the mean ± standard deviation (SD). The fluorescent intensity in cell proliferation, the percentage of healed cell wound, and the straight distance in the scratch assay were carried out using a one-way ANOVA followed by Bonferroni's multiple comparison test. All analyses were performed with SPSS 19.0 (SPSS Inc., USA). A value of $p < 0.05$ was considered to indicate statistical significance, and $p < 0.01$, highly significance.

RESULTS

ERα- endometrial carcinoma cells were stably transfected with human ERα-F445A plasmid. Western blot analysis showed that the expression of ERα-F445A could be detected in the cells at the end of the experiments (Fig. 1a). The expression of ERα-F445A was exhibited in cytoplasm as well as nucleus (Fig. 1b–g). It is interesting to note that in ERα-F445A cells treated with 40 nM E2 for 24 hours, the amount of ERα expression was decreased 2 times more than that without E2 (Fig. 1a).

ERα is known for its proliferative effect in normal endometrial and carcinoma cells. Although not significantly, it was found that the proliferation of ERα-F445A cells was slower than that of ERα- cells without treatment (Fig. 1h, i). A significant increase in proliferation was found in ERα-F445A cells stimulated with 40 nM E2 (Fig. 1h) than those without. No significant difference, however, was observed between ERα- and ERα-F445A cells. On the other hand, significant repression in cell proliferation was observed not only between ERα-F445A cells treated with 40 μM BDF or without, but also between the treated ERα-F445A and ERα- cells (Fig. 1i).

Given the promotive effect of ERα in cell migration, ERα-F445A and ERα- cells were subjected to scratch assay (Fig. 2). Addition of 40 nM E2 made the wound in ERα-F445A cells (Fig. 2g, h) close significantly faster (Fig. 2i) than those without E2 and those of ERα- cells (Fig. 2a–f). While the repression in wound closure was not identified between ERα- cells (Fig. 2j–m, r) treated with BDF or without, the wound closure in ERα-F445A cells treated with BDF (Fig. 2n, o) was significantly decreased (Fig. 2r) compared with those without (Fig. 2p, q).

To observe how each cell migrate in scratch assay, 10 cells were tracked in real time for each one of treatments. When treated with 40 nM E2, ERα-F445A cells migrates significantly further in the straight distance than those without (Fig. 3). Significant repression in the straight distance, however, was only identified between ERα-F445A cells treated with 40 μM BDF (Fig. 4).

To elucidate the observed cellular activities, Western blot analysis (Fig. 5) was carried out to observe the alteration of molecules in ERα signal transduction pathways. In ERα- F445A cells irrespective addition of E2, while the expression of non-phosphorylated AKT decreased more than 1.5 times (Fig. 5b), the ratio of p-AKT (Ser473)/AKT increased more than 1.5 times than those in ERα- cells (Fig. 5c). As for the non-phosphorylated mTOR, no obvious difference was identified between ERα-F445A and ERα- cells (Fig. 5d), irrespective addition of E2. However, although the ratio of p-mTOR (Ser2448)/mTOR in ERα-F445A cells without E2 attenuated more than 2 times below that in ERα- cells, the treatment with 40 nM E2 elevated the level of p-mTOR (Ser2448) 2 times more above that ERα-F445A cells without E2 (Fig. 5e). The non-phosphorylated FAK in ERα-F445A cells is 3 times more below that in ERα- cells (Fig. 5f), irrespective addition of E2, but more than 1.5 times less amount of p-FAK (Tyr297) was detected in ERα-F445A cells treated with E2 (Fig. 5g).

DISCUSSION

In the present study, human ERα was engineered to carry a substituted phenylalanine at position 445 with alanine (ERα-F445A) and stably transfected ERα- endometrial carcinoma cells. The cytonuclear ERα-F445A induced both cell proliferation and migration following treatment with E2. Furthermore, the treatment induced disturbance in either the amount of the non-phosphorylated or phosphorylated AKT, FAK and mTOR. Our results suggest that the cytonuclear ERα-F445A regulates cell proliferation and migration through a complex mechanism in endometrial carcinoma cells.

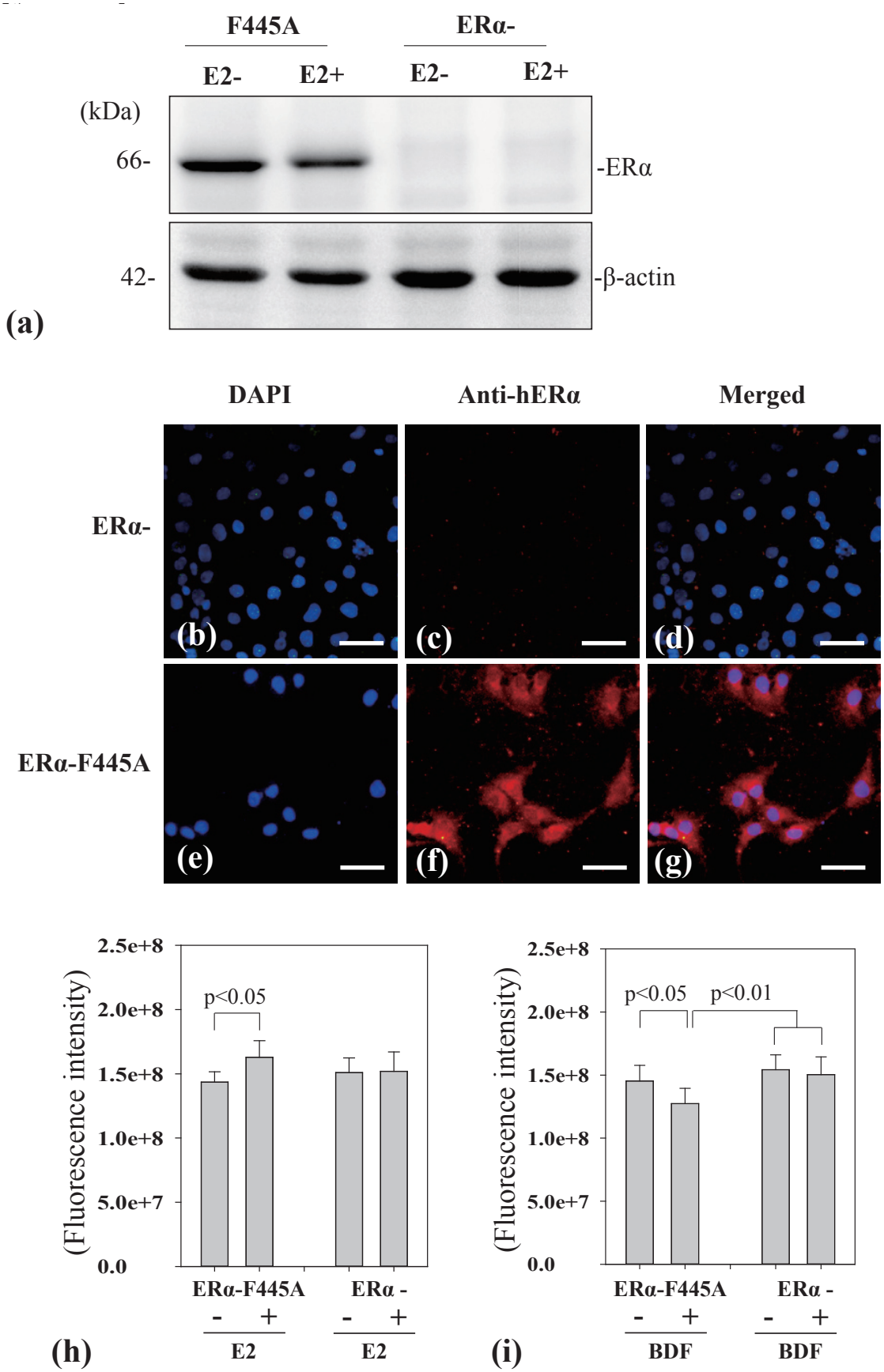

Fig. 1 Western blot analysis (a) of ERα-negative endometrial carcinoma (ERα-) cells transfected with human ERα carrying a substituted phenylalanine at position 445 with alanine (ERα-F445A). Cells were treated without or with 40 nM 17β-estradiol (E2- or +). ERα- (b, c, d) and ERα-F445A (e, f, g) cells were subjected to immunocytochemistry using anti-ERα antibody (red color). The nucleus was counter-stained (blue). Note the positive signals in cytoplasm and nuclei (f, g). Scale bars indicate 25 μm. The effects of ERα-F445A on cell proliferation were observed using alamarBlue without (-) or with (+) addition of 40 nM E2 (h) or 40 μM bazedoxifene (BDF) (i) to culture medium for 24 hours.

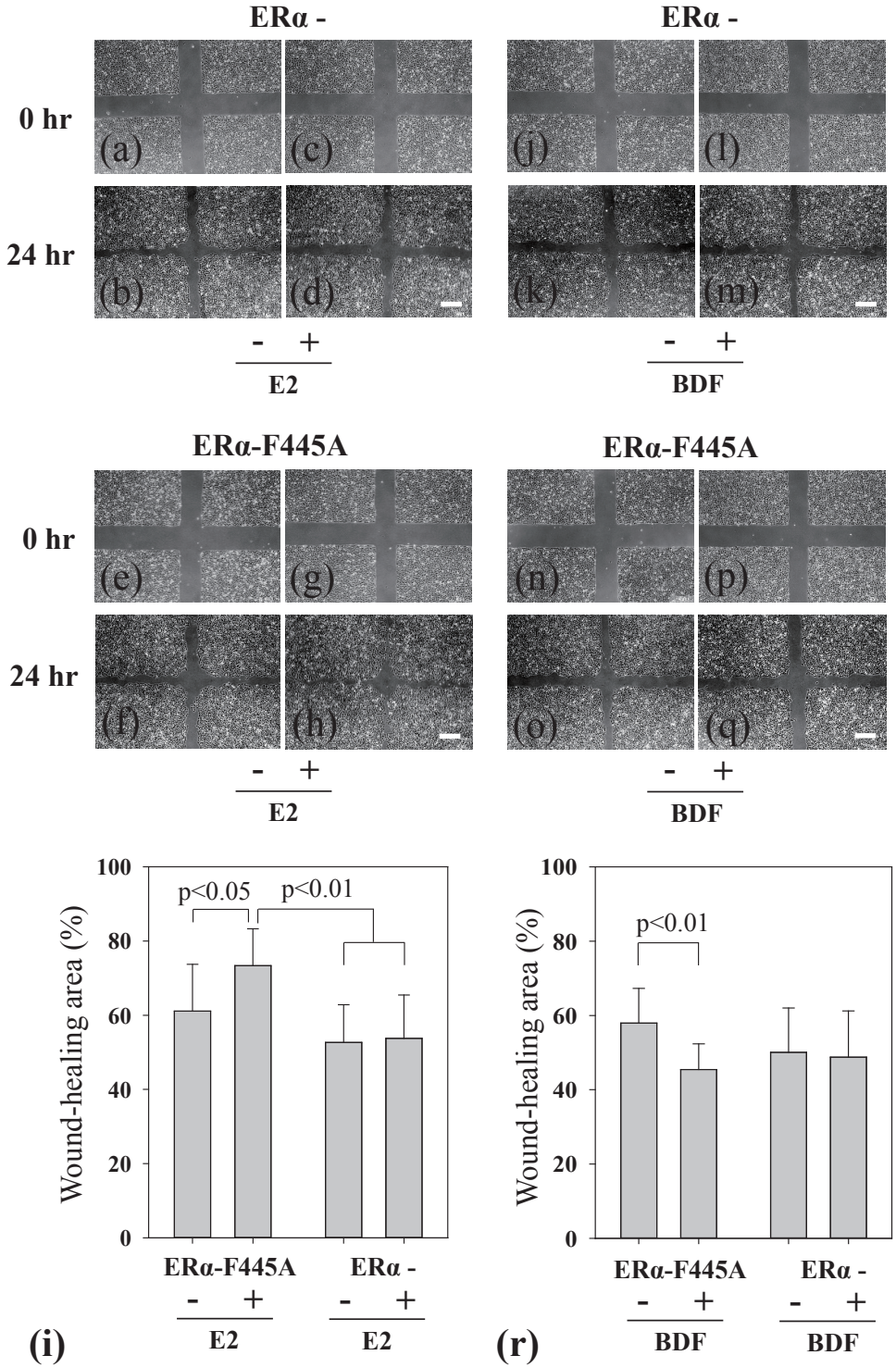

Fig. 2 Scratch assay of ERα-negative endometrial carcinoma (ERα-) cells transfected with plasmid of human ERα carrying a substituted phenylalanine at position 445 with alanine (ERα-F445A). The wound-healing effects in ERα- (a–d, j–m) and ERα-F445A (e–h, n–q) were observed for 24 hours (hr) without (-) or with (+) or addition of 40 nM 17 β-estradiol (E2- or +) or 40 μM bazedoxifene (BDF- or +) to culture medium. The percentages of wound-healing area in 24 hr against basal area at 0 hr are presented for ERα-F445A and ERα- cells treated without or with E2 (i) or BDF (r). Magnification x100; Scale bars indicate 40 μm.

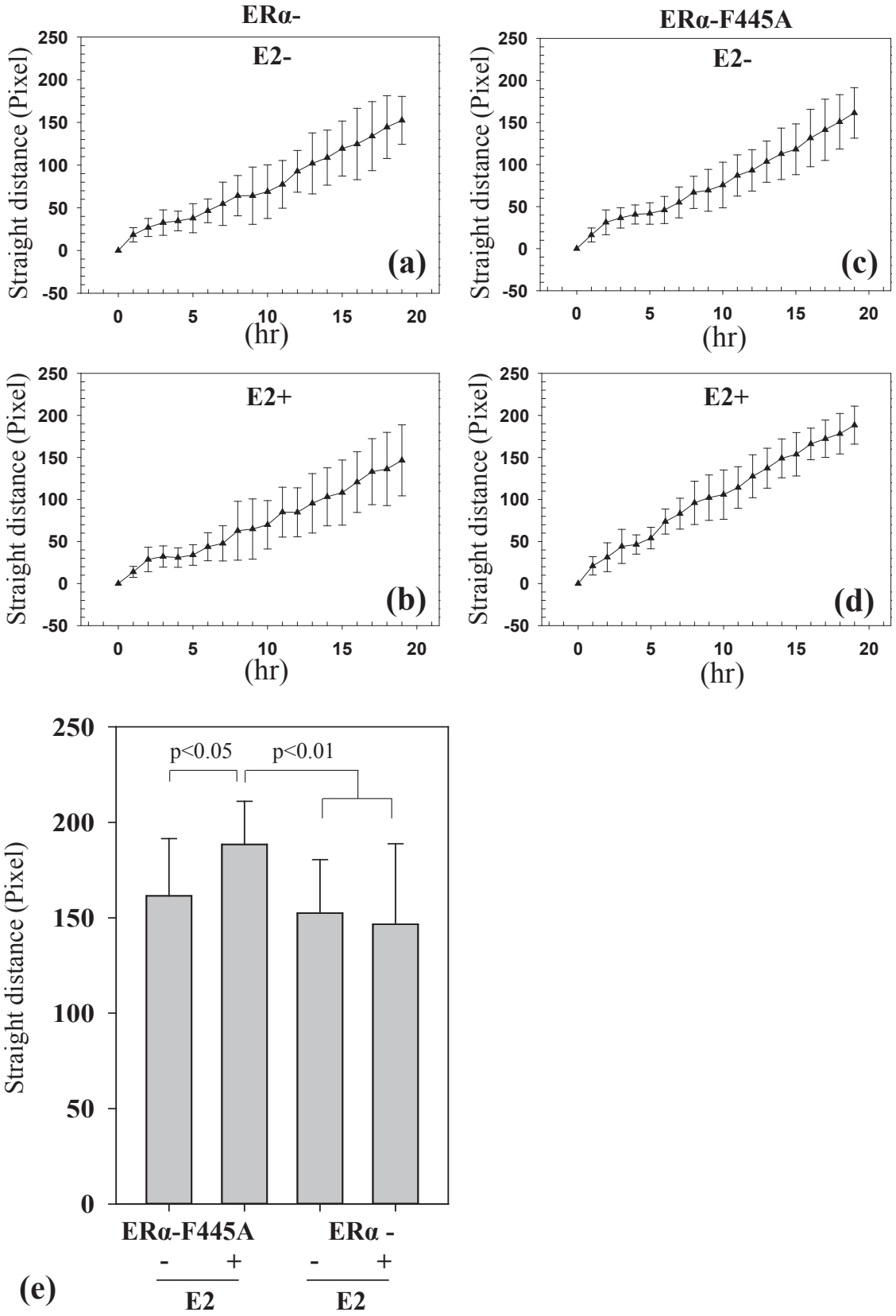

Fig. 3 Analysis of single cell migration in ERα-negative endometrial carcinoma (ERα-) cells transfected with plasmid of human ERα carrying a substituted phenylalanine at position 445 with alanine (ERα-F445A). The straight distances showing cell migration are shown for ERα- (a, b) and ERα-F445A (c, d) cells treated without (-) or with (+) 40 nM 17β-estradiol (E2- or +) for 19 hours. The final straight distances between ERα-F445A and ERα- cells were compared and results (e) are shown.

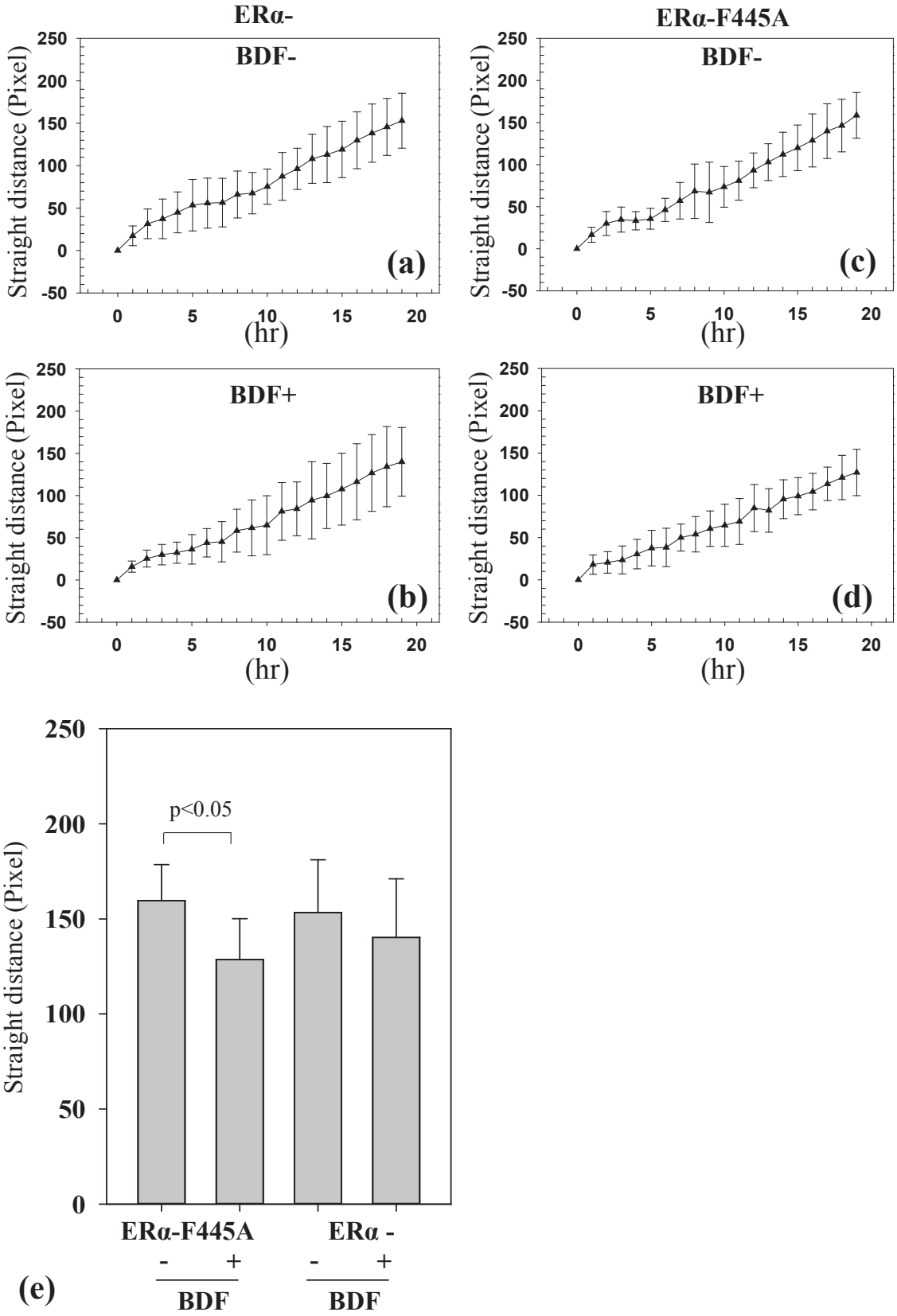

Fig. 4 Analysis of single cell migration in ERα-negative endometrial carcinoma (ERα-) cells transfected with plasmid of human ERα carrying a substituted phenylalanine at position 445 with alanine (ERα-F445A). The straight distances showing cell migration are shown for ERα- (a, b) and ERα-F445A (c, d) cells treated without (-) or with (+) 40 μM bazedoxifene (BDF- or +) for 19 hours. The final straight distances between ERα-F445A and ERα- cells were compared and results (e) are shown.

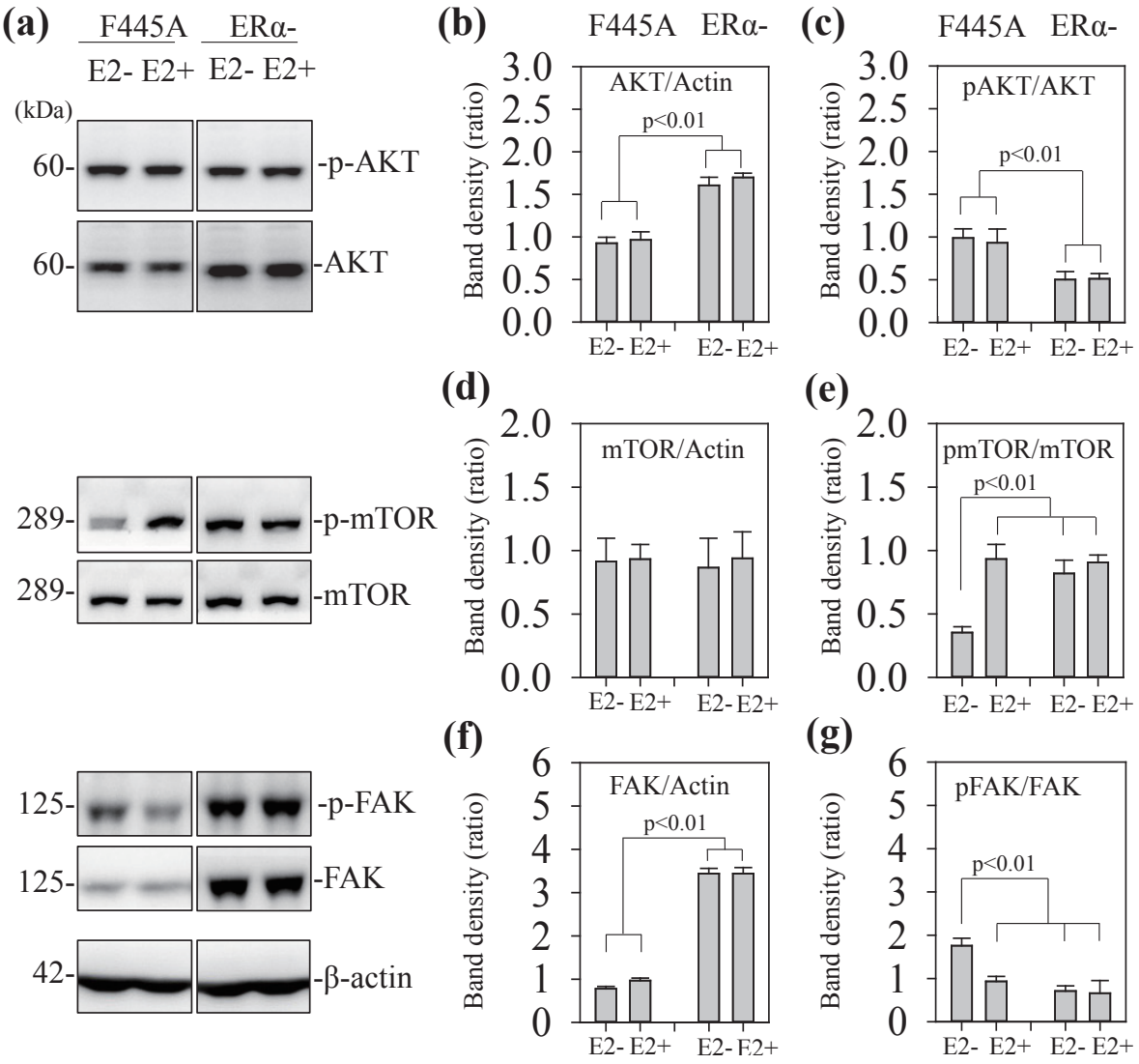

Fig. 5 Western blot analysis (a) of ERα-negative endometrial carcinoma (ERα-) cells transfected with plasmid of human ERα substituted phenylalanine at position 445 with alanine (ERα-F445A). ERα-F445A and ERα- cells were treated without (-) or with (+) 40 nM 17β-estradiol (E2- or +) for 24 hours. The amount of expression is shown in the relative density of immunoblot bands for the nonphosphorylated AKT (b), mTOR (d), and FAK (f) normalized to actin, respectively. The amount of the phosphorylated molecule was normalized to that of its non-phosphorylated one: p-AKT (c), for AKT phosphorylated at serine 473 (Ser473); p-mTOR (e), mTOR phosphorylated at serine 2448 (Ser2448); and p-FAK (g), FAK phosphorylated at tyrosine 397 (Tyr397).

The phenylalanine at 445 in ERα is involved in its palmitoylation and the palmitoylation-dependent membrane localization [10]. When ERα was engineered to carry a substituted phenylalanine at position 445 with alanine (ERα-F445A), the expression of ERα-F445A was localized to the cytoplasm and nucleus (Fig. 1), being consistence with other report [10]. However, the effects of ERα-F445A on cell proliferation and migration have not been thoroughly examined in endometrial carcinoma cells.

Overnight treatment with E2 induced reduction in cellular ERα-F445A content (Fig. 1a), which was reported in the breast cancer cell lines MCF-7 [37] and the human endometrial cancer cell line Hec 1A and Hec 1B [38]. The lack of palmitoylation may render ERα-F445A more susceptible to E2-dependent degradation [39]. Studies show that estrogen can rapidly trigger cytoplasmic complex(es) containing ERα, leading to the activation of signaling kinases such as MAPK and PI3K-AKT [22, 24, 25], which in return phosphorylate ERα at serine 118 and 167 [40]. In the present study, E2 induced enhancement in cell proliferation (Fig. 1h) and migration (Fig. 2a–i; Fig. 3). ERα-F445A may exert its effects through cross-talk with PI3K-AKT and the signal pathways discussed later. The repressive effects of bazedoxifene on cell proliferation (Fig. 1i) and migration (Fig. 2j–r; Fig. 4) indicate that the binding and cellular responsiveness to both E2 and bazedoxifene are not disturbed, which are in agreement with other report [10].

FAK is a cytoplasmic tyrosine kinase. Cell migration is achieved through the development of the focal contact complexes, of which FAK plays key roles in promotion of cell migration and invasion [41, 42]. In Hec cells treated with E2, FAK and ERK are rapidly and dose-dependently activated with no change in the non-phosphorylated FAK [38, 43]. In the present study, however, ERα-F445A induced reduction in the non-phosphorylated FAK as compared with ERα- cells (Fig. 5f), which was not responsive to E2. Furthermore,

when ERα-F445A cells were treated with E2, the decrease, rather than the increase in amount was detected in p-FAK (Try397) (Fig. 5g). It is worth to note that the absence of FAK in breast cancer cells results in reduction of AKT and downstream mTOR [44]. Although FAK has been shown to activate the PI3K-Akt-mTOR pathway [45], the present study suggests that the expression of total FAK and p-FAK (Try397) is not associated with the E2 activated proliferation and migration in our ERα-F445A cells, rather than that for mTOR.

Studies show that mTOR is a central regulator of cell proliferation, tumor cell motility, invasion and cancer metastasis [46]. Moreover, it is reported that the overexpression of ERα enhanced cell migration, invasion and proliferation through activation of the PI3K/AKT/mTOR transduction cascade [47]. Furthermore, studies show that mTOR phosphorylates ERα at serine 104/106 to promote its activities [48, 49]. In the present study, no obvious alteration in the amount of the non-phosphorylated mTOR was identified between ERα-F445A and ERα- cells (Fig. 5d). However, despite the amount of p-mTOR (Ser2448) in ERα-F445A cells was attenuated more than 2 times below that in ERα- cells, the treatment with 40 nM E2 elevated the level of p-mTOR (Ser2448) 2 times more above that without E2 in ERα-F445A cells (Fig. 5e). Our results indicate that mTOR in ERα-F445A cells play a dominant role in regulation of proliferation and migration.

In Chinese hamster ovary cells (CHO) expressing same amount of ERα-F445A and the wild type ERα (wtERα), although the basal level of the non-phosphorylated AKT was kept constant in both the transfected and non-transfected cells, robust activation of p-AKT (Ser473) was observed only in wtERα and ERα-F445A cells treated with E2 [10]. In ERα- endometrial carcinoma cells, however, ERα-F445A induced reduction in the amount of the non-phosphorylated AKT (Fig. 5b). Furthermore, both the non-phosphorylated AKT and pAKT was not responsive to E2, although the basal level of p-AKT (Ser473) in ERα-F445A cells was raised high above that in ERα- cells (Fig. 5b). It is now clear that ERα membrane association is required for the E2-dependent activation of rapid ERK/MAPK and PI3K/AKT signalling pathways [9, 10]. The phenylalanine at position 445 of ERα may be related to the regulation of amount of AKT and p-AKT (Ser473) in endometrial carcinoma cells, which requires the membraneous localized ERα for E2-responsiveness.

In the present study, there are two limitations that have strong impacts on our conclusions. The first is that to clarify the effect of ERα-F445A, it is necessary to observe the cellular and molecular effects of wild-type ERα under the same experimental conditions. The second is that additional experiments such as examination into the effects of mTOR inhibitor rapamycin or dominant-negative mTOR are necessary to prove our conclusions.

CONCLUSIONS

In the present study, ERα-F445A that localized in cytoplasm and nucleus induced disturbance in the non-phosphorylated and phosphorylated of AKT, FAK and mTOR, which suggest that the cytonuclear ERα-F445A regulates cell proliferation and migration through a complex mechanism in endometrial carcinoma cells. Cell proliferation and migration is critical to cancer cell invasion and metastasis. Much research effort has been devoted to understanding how estrogen controls cell proliferation and motility through the rapid, direct activation of cytoplasmic signaling cascades. Our results suggest study on cytonuclear ERα will help distinguish the specific but integrated functions of ERα at different subcellular localizations.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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