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Paxillin, a novel controller in the signaling of estrogen to FAK/N-WASP/Arp2/3 complex in breast cancer cells

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ABSTRACT

Breast cancer is the major cause of cancer-related death in women. Its treatment is particularly difficult when metastasis occurs. The ability of cancer cells to move and invade the surrounding environment is the basis of local and distant metastasis. Cancer cells are able to remodel the actin cytoskeleton, which requires the recruitment of numerous structural and regulatory proteins that modulate actin filaments dynamics, including Paxillin or the Neural Wiskott-Aldrich Syndrome Protein (N-WASP). We show that 17-β estradiol (E2) induces phosphorylation of Paxillin and its translocation toward membrane sites where focal adhesion complexes are assembled. This cascade is triggered by a Gαi1/Gβ protein-dependent signaling of estrogen receptor α (ERα) to c-Src, focal adhesion kinase (FAK) and Paxillin. Within this complex, activated Paxillin recruits the small GTPase Cdc42, which triggers N-WASP phosphorylation. This results in the redistribution of Arp2/3 complexes at sites where membrane structures related to cell movement are formed. Recruitment of Paxillin, Cdc42 and N-WASP is necessary for cell adhesion, migration and invasion induced by E2 in breast cancer cells. In parallel, we investigated whether Raloxifene (RAL), a selective estrogen receptor modulator (SERMs), could inhibit or revert the effects of E2 in breast cancer cell movement. We found that, in the presence of E2, RAL acts as an ER antagonist and displays an inhibitory effect on estrogen-promoted cell adhesion and migration via FAK/Paxillin/N-WASP. Our findings identify an original mechanism through which estrogen regulates breast cancer cell motility and invasion via Paxillin. These results may have clinical relevance for the development of new therapeutic strategies for cancer treatment.

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1. Introduction

Breast cancer is the primary cause of cancer-related death in women. About one out of eight women develops breast cancer at some stage of her life (Torre et al., 2015). Metastasis is a complex process characterized by detachment of cells from the primary tumor followed by their dissemination through the blood vessels to distant organs, extravasation into the parenchyma of tissues and formation of secondary tumors at distant sites. The latter is the principal cause of death in cancer patients, which highlights the need for new therapeutic strategies (Liang and Shang, 2013; Mezi et al., 2012).

Sex steroid hormones play a key role in breast cancer development, progression and metastasis (Liang and Shang, 2013; Folkerd and Dowsett, 2010). Prolonged treatment with estrogen, such as late menopausal or postmenopausal hormone replacement therapy, is associated with a greater risk of developing breast cancer (Horwitz, 2008; Ma et al., 2006; Verkooijen et al., 2009). However, the effects of estradiol on breast tumor cell motility or invasion are poorly understood.

Cell migration is required for cancer spread, invasion and metastasis. It is a complex multistep process that involves protrusion of the leading edge of the cell, formation of adhesion complexes, myosin/actin-mediated cell contraction, and the release of adhesions at the cell rear (Deakin and Turner, 2008; Vinzenz et al., 2012). We have recently shown that 17β-estradiol (E2) promotes the depolymerization of actin fibers and the reassembly toward the cell membrane edge, leading to the formation of cortical actin complexes implicated in the generation of the breast cancer cell

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locomotive force (Flamini et al., 2009; Sanchez et al., 2010; Sanchez et al., 2009; Simoncini et al., 2006). Dynamic adhesion involves the coordinated action of different proteins, including the proteins kinase c-Src, and focal adhesion kinase (FAK). The Src/FAK complex interacts with Paxillin and activates it by phosphorylation on Tyr^{31} and Tyr^{118} (Zaidel-Bar et al., 2007). Paxillin is a scaffolding protein that serves as a platform for the recruitment of several regulatory and structural proteins that modulate the dynamic changes in cell adhesion, mediate the control in the assembly and disassembly of the focal adhesion complexes, and as well as cytoskeletal reorganization promoting the cell migration and invasion process (Deramaudt et al., 2014).

The Neural Wiskott-Aldrich syndrome protein (N-WASP) is one of the many controllers of the actin cytoskeleton. N-WASP acts as a scaffolding complex, relaying signals from small GTPases such as Cdc42 to the Arp2/3 complex (Rohatgi et al., 1999). Actin nucleation by the Arp2/3-complex appears to be fundamental for the rapid formation of an actin network at the leading edge of the cell (Takenawa and Suetsugu, 2007). Recent studies have demonstrated that Paxillin may allow the recruitment of N-WASP and modulate the activation of the Arp2/3 complex (Lettau et al., 2010; Oda and Eto, 2013; Dummler et al., 2009; Jessick et al., 2013). This activation leads to branching and actin polymerization, allowing the formation of specialized membrane structures that promote cell movement (Padrick and Rosen, 2010; Frugtniet et al., 2015).

The aim of the present study was to identify the molecular basis of the actions of estrogen on breast cancer cell morphology. In particular, we wanted to identify whether these actions may require the regulation of the actin cytoskeleton via Paxillin to the N-WASP-Arp2/3 complex, and to characterize the intracellular cascades that may be recruited during this signaling.

2. Materials and methods

2.1. Cell cultures and treatments

MCF-7, T-47D and MDA-MB-231 Human breast cancer cells were obtained from the American Type Culture Collection. MCF-7 cells were cultured in minimal essential medium (MEM) with L-glutamine (2 mM) and 10% fetal bovine serum (FBS). T-47D cells were grown in RPMI 1640 supplemented with L-glutamine (2 mM), 10% fetal bovine serum. MDA-231 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 10% penicillin/streptomycin. Before treatments, breast cancer cells were kept for 24 h in medium containing steroid-deprived FBS. To investigate non-genomic effects, cancer cells remained in medium without FBS for 8 h. 17β-estradiol (E_2) and pertussis toxin (PTX) were purchased from Sigma-Aldrich (Saint-Louis, MO, USA); ICI 182,780 (ICI) was 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, sc-203950A) and Arp2/3 inhibitor (CK666, sc-361151) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Whenever an inhibitor was used, the compound was added 45 min before starting the active treatments.

2.2. Immunoblottings

Cell lysates were separated by SDS-PAGE. Antibodies used were: Paxillin (sc-31010), p-Paxillin (Tyr^{118}), p-FAK (Tyr^{397}), ERα (H-184), ERβ (N-19), Gαi1 (R4), Gβ1 (C-16), Cdc42 (B-8), actin (Santa Cruz Biotechnology, Santa Cruz, CA); Phospho-Rac1/Cdc42 (Ser^{71}) (Cell Signaling Technology); FAK, p-FAK (Y^{397}) (Transduction Laboratories, Lexington, KY, USA); N-WASP (ab32707), p-N-WASP ($Ser^{484/485}$) (Chemicon International, Millipore). Primary and secondary antibodies were incubated with the membranes using standard techniques. Immunodetection was carried out using enhanced chemiluminescence and was recorded with a quantitative digital imaging system (Chemidoc XRS with Image Lab, Bio-Rad, Hercules, CA, USA).

2.3. Cell immunofluorescence

Human breast cancer cells were grown on coverslips and exposed to different treatments. Cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton for 5 min. Blocking was performed with 3% bovine serum albumin for 30 min. Cells were incubated with antibodies against p-Paxillin (Tyr^{118}, A-5); N-WASP (ab32707) (Chemicon International, Millipore); Arp3 (Transduction Laboratories, Lexington, KY, USA) overnight at 4 °C. Phospho-Paxillin and Arp3 were incubated with a $fluorescein^{488}$ (FITC)-conjugated goat anti-rabbit/mouse secondary antibody (1:200; Vector Laboratories). N-WASP was incubated with anti-mouse-$Dylight^{594}$ (1/125) secondary antibody. Texas Red-phalloidin (Sigma-Aldrich) was added for 30 min. The nuclei were counterstained with 4-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Immunofluorescence was visualized using an Olympus BX41 microscope and recorded with a high-resolution DP70 Olympus digital camera.

For colocalization assays, MCF-7 cells were grown on coverslips and exposed to different treatments. Cells were incubated with pPaxillin with a $fluorescein^{488}$ (FITC)-conjugated goat anti-mouse secondary antibody (1:200; Vector Laboratories), N-WASP with a $DyLight^{594}$-conjugated goat anti-rabbit secondary antibody (1:200; Vector Laboratories) and FAK with a $DyLight^{594}$-conjugated goat anti-rabbit secondary antibody (1:200; Vector Laboratories). The nuclei were counterstained with 4-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). The immunostainings were examined with a confocal microscope (FV 1000; Olympus) with a Paplon 60 × lens.

2.4. Quantitative colocalization analysis

The colocalization analysis was made using the JACoP plugin for Image J software according to Bolte and Cordelieres (Bolte and Cordelieres, 2006). Z-Stack images of MCF-7 cells stained for both pPaxillin/FAK and pPaxillin/N-WASP were deconvolved. The channel-specific point spread functions were generated and the signal:noise ratio was adjusted until deconvolved images were free of pixel noise. The colocalization analysis was made using two correlation coefficients, Pearson (PCC) and Manders (MCC). For PCC calculation, the dependency of pixels in dual-channel images (green and red channels for FAK/N-WASP and pPaxillin, respectively) was measured by plotting the pixel gray values of two images against each other. These values were displayed in a pixel-distribution diagram (scatter plot), and a linear equation of the relation between the intensities of the two images was calculated by linear regression. A cross-correlation function (CCF) was obtained by plotting the corresponding PCC for each pixel shift (delta x) of the green image in the x direction relative to the red image. The real value of the PCC is estimated at delta $x = 0$. This value can range from 1 to −1, with 1 standing for complete positive correlation, −1 for negative correlation, and 0 for no correlation. The MCC is based on the PCC, with average intensity values being taken out of a mathematical expression (Manders et al., 1992). Two coefficients were obtained, MCC-M1 and MCC-M2, for the fraction of FAK and/or N-WASP overlapping with pPaxillin and for the

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fraction of pPaxillin overlapping with FAK and N-WASP, respectively. These values varied from 0 to 1, corresponding to non-overlapping images or complete colocalization, respectively.

2.5. Quantitative analysis of cell membrane morphology and thickness and of actin fibers remodeling

The remodeling of actin fibers and the morphological changes of the membrane were quantified by assessing the intensity of actin fluorescence using the Leica QWin software (Leica Microsystems, Germany). This analysis was performed by randomly measuring fluorescence using boxes of a fixed size that included the extra- and intra-cellular space across the membrane, and recording the intensity of the signal in a spatial-related fashion. In other words, the program provides a graph in which the pixel fluorescence intensity is linearly recorded. By visually selecting the inner and outer parts of the membrane in each microphotograph, the thickness of the membrane as sampled in each box could be quantified. In addition, the mean of the mean fluorescent intensity in the areas identified as membrane space or intracellular space within the box was used to quantify the amount of cytosolic vs. membrane-associated actin. Five areas per cell, and 40 different cells per experimental condition, were sampled using these boxes.

2.6. Cell adhesion assays

Five hundred thousand cells per well were seeded into 6-well plates on coverslips previously coated with 1% sterile gelatin and exposed to different treatments. The cells were incubated at 37 °C for 2 h. Non-adherent MCF-7 cells were then removed by gently washing with PBS. The attached cells were fixed with 4% formaldehyde and stained with Giemsa. Images of attached cells were captured and the latter counted in ten randomly chosen fields per well using a Nikon Eclipse E200 microscope coupled to a high-resolution CCD digital camera, as previously described (Flamini et al., 2014).

2.7. Cell migration assays

MCF-7 cells were plated in 6-well plates and grown to 70–80% confluence. Wounds were made in the monolayers by scratching the surface with a pipette tip (1000 μl) as uniformly and straight as possible. Cells were washed with PBS, and 1.5 ml of RPMI 1640 containing 10% FBS were added. Cytosine b-D-arabinofuranoside hydrochloride (ARA-C) (Sigma-Aldrich, C6645) (10 mM), 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. At 24 h, fresh medium and treatment were replaced. Cells were digitally imaged with inverted Carl Zeiss microscopy and closed areas were quantified using imageJ software.

2.8. Cell invasion assays

Cell invasion was assayed using BD BioCoat™ Growth Factor Reduced (GFR) Matrigel™ Invasion Chambers (BD Bioscience, USA). In brief, after rehydrating the GFR Matrigel inserts, the test substance was added to the wells. An equal number of Control Inserts (no GFR Matrigel coating) were prepared as control. 0.5 mL of MCF-7 cell suspension (2.5×10^4 cells/mL) was added to the inside of the inserts. The chambers were incubated for 24 h at 37 °C, 5% CO_2 atmosphere. After incubation, non-invading cells were removed from the upper surface of the membrane using cotton-tipped swabs. The cells on the lower surface of the membrane were stained with Diff-Quick. Cells were counted in the central field of triplicate membranes.

2.9. Gene silencing with RNA interference

Synthetic small interfering RNAs targeting ERα (siRNAsSMARTpool ESR1), estrogen receptor β (siRNA SMARTpool ESR2) and control siRNAs (D-001810-01-05) were purchased from Dharmacon (Thermo Fisher Scientific Inc., USA); $G\beta_1$ siRNAs, Paxillin siRNAs, Cdc42 siRNAs, N-WASP siRNAs and control siRNAs were from Santa Cruz Biotechnology (Santa Cruz, CA). The siRNAs were used at the final concentration of 50 nM. Breast cancer cells were treated 48 h after siRNAs transfection. Efficacy of gene silencing was checked with western analysis and found to be optimal at 48 h.

2.10. Transfection experiments

Dominant negative constructs for Gαi1 (Gαi1 G202T) was from the Guthrie cDNA Resource Center (www.cdna.org). The inserts were cloned in pcDNA3.1+. The plasmids (10 μg) were transfected into MCF-7 cells using Lipofectamine (Invitrogen, Carlsbad, CA, USA). Parallel cells were transfected with empty pcDNA3.1 + plasmid. Cells (70% confluent) were treated 48 h after transfection.

2.11. Statistical analysis

All values are expressed as mean ± SD. Statistical analyses and graphs were done using InStat from GraphPad Prism Software. Statistical differences between mean values were determined by ANOVA, followed by the Fisher's protected least significance difference (PLSD) and by Tukey–Kramer Multiple-Comparisons Test.

3. Results

3.1. Estrogen rapidly activates and translocates Paxillin through ERα

As a first step to identify the effects of 17β-estradiol (E2) on breast cancer cell morphology we evaluated the actions of a rapid estrogen exposure on the actin cytoskeleton. Treatment with E2 (10 nM) resulted in a rapid change in actin organization, with a remodeling of the actin cytoskeleton toward the cell membrane resulting in a thickening of the membrane (Fig. 1A-C). This phenomenon was time-dependent and transient, with maximum levels occurring after 15–20 min and reversing to baseline after 60 min (Fig. 1A-C). In parallel, a rapid change of the spatial organization of actin fibers and Paxillin subcellular localization was found that followed the same time-course (Fig. 1A-C). Actin translocated from the cytoplasm toward the edge of the cell membrane, where it co-localized with phosphorylated Paxillin, forming typical focal adhesion complexes in association with specialized membrane structures, such as filopodia and membrane ruffles (Fig. 1A).

MCF-7 human breast cancer cells express both Estrogen Receptor (ER) isoforms, ERα and ERβ (Al-Bader et al., 2011). To identify which isoform mediates the signaling of E2 to Paxillin, we evaluated the phosphorylation level of Paxillin in presence of ICI 182,780, a specific inhibitor of ERs (Fig. 1D-E). We observed a significant increase on Paxillin-Tyr^{118} phosphorylation during E2 exposure, while treatment of cells with ICI resulted in a clear reduction in Paxillin-Tyr^{118} during exposure of E2. In parallel, we used the breast cancer cell line MDA-MB-231 (ER-) to verify the specific control of estrogen on Paxillin phosphorylation/translocation via ERs (Supplemental File S1 A-C). E2 did not induce Paxillin phosphorylation and translocation in these cells (Supplemental File S1 A-D), confirming the specific action via ERs. Furthermore, we verified the effect of E2 on other ER + cell lines using T-47D breast cancer cells (ER +) to confirm the estrogen-dependent Paxillin phosphorylation

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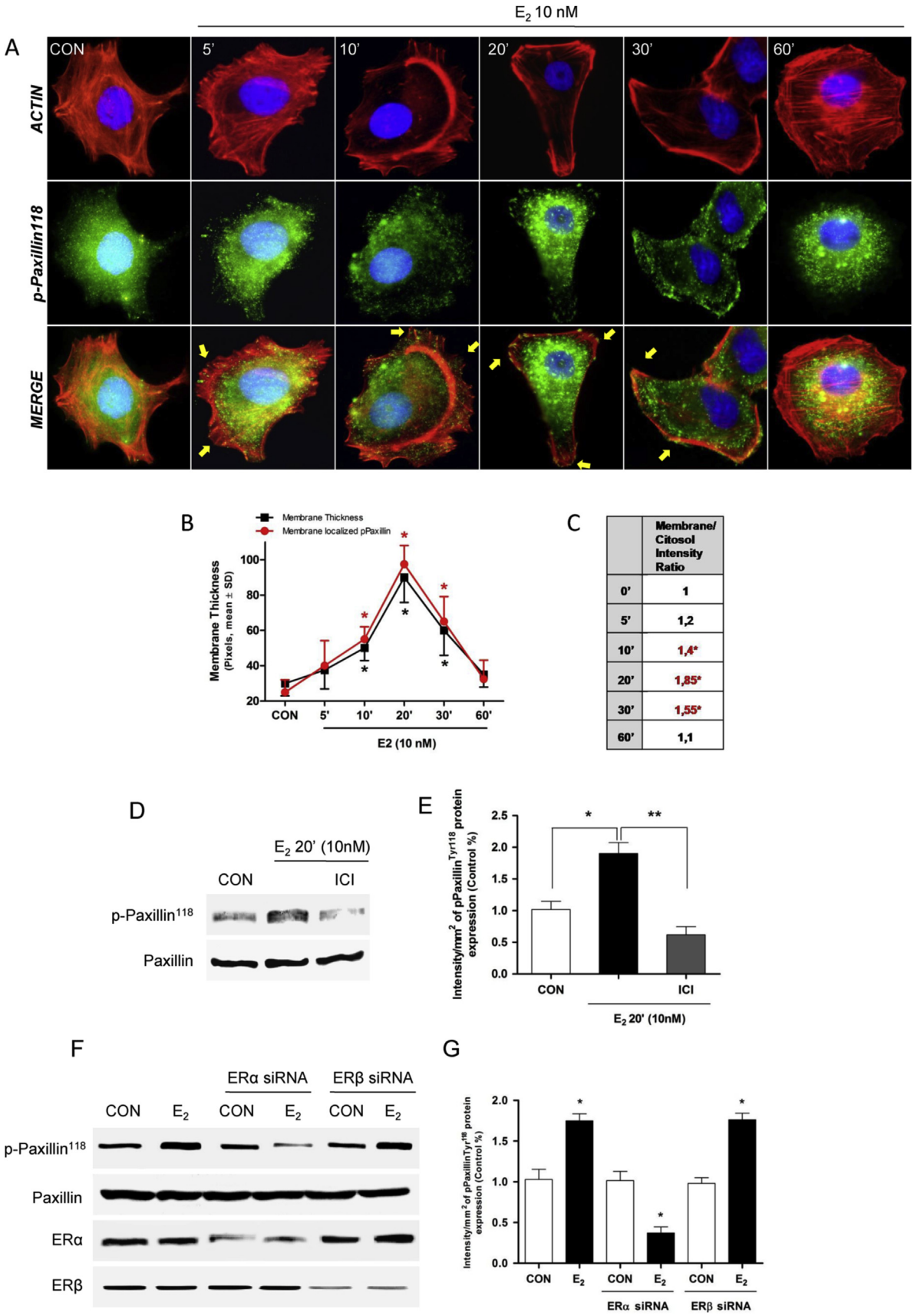

Fig. 1. Estrogen activates Paxillin and induces a dynamic actin cytoskeleton remodeling. A) MCF-7 breast cancer cells were treated with E2 (10 nM) for the indicated time. Cells were stained with anti-phospho-Paxillin-Tyr^{118} linked to fluorescein isothiocyanate (FITC), actin was stained with Texas Red phalloidin, and nuclei were counterstained with DAPI. Yellow arrows indicate membrane-localized pPaxillin-Tyr^{118}. **B-C)** Mean intensity ratio of actin staining in the membrane/cytoplasm in the same experiment. The results are derived from

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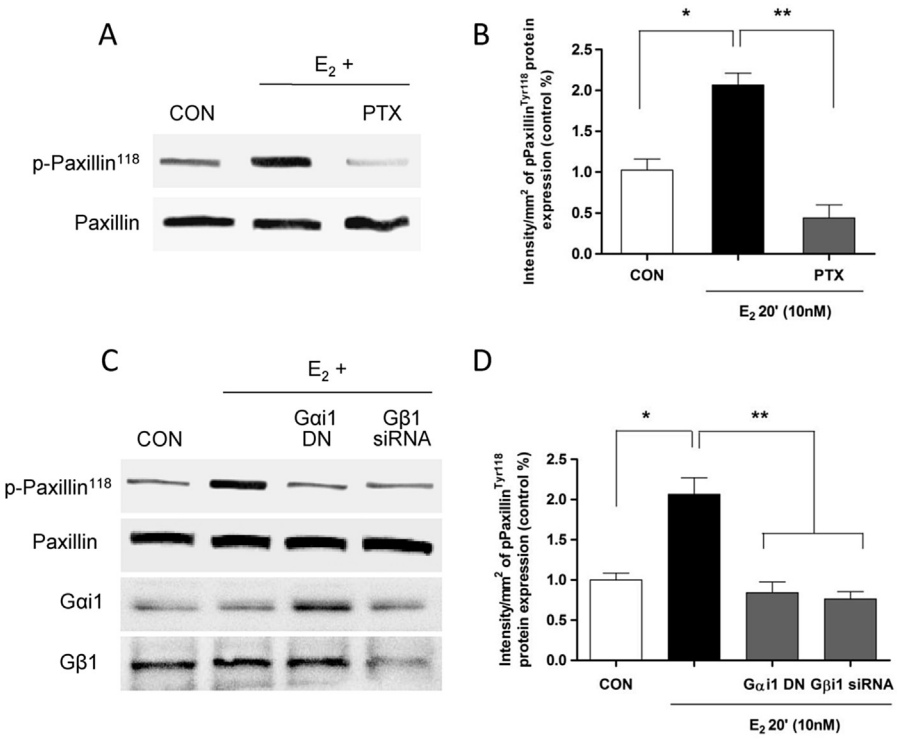

Fig. 2. ERα signal to Paxillin via $G\alpha_i$/Gβ proteins. A) Breast cancer cells were exposed to E2 (10 nM) for 20 min in the presence or absence of the G protein inhibitor PTX (100 ng/ml), and PaxillinTyr^{118}phosphorylation was assayed with western analysis. **B)** p-Paxillin densitometry values were adjusted to Paxillin intensity and normalized to the control. *< P 0.05 CON vs. E2, **< P 0.05 E2 vs. E2 + PTX. **C)** MCF-7 cells were treated with E2 after transfection with dominant-negative $G\alpha_i$ constructs or siRNAs vs. $G\beta_1$ and Paxillin, p-Paxillin Tyr^{118}, $G\alpha_i$ and $G\beta_1$ proteins were assayed with western blot analysis. **D)** The graph shows the quantitative analysis of the intensity of the bands in C, obtained as number of photons measured by the ChemiDoc digital imaging system and evaluated with the Quantity One Software (Bio-Rad, Hercules, CA). CON, Control. *< P 0.05 CON vs. E2, **< P 0.05 E2 + Gαi1 DN and E2 + Gβ1 siRNA.All experiments were performed three times and representative blots are presented.

and translocation into focal adhesion complexes (Supplemental File S1 B-D). These result confirms the fundamental role of ERs on Paxillin phosphorylation.

In order to clarify which isoform mediates the signaling of E2 to Paxillin, we silenced with specific siRNA to downregulate ERα and ERβ (Fig. 1F-G). Transfection of ERα siRNAs resulted in a clear reduction of ERα expression along with a dramatic decrease in Paxillin-Tyr^{118} phosphorylation during exposure to E2 (Fig. 1F-G) in the absence of modifications of Paxillin expression (Fig. 1F). On the contrary, silencing of ERβ did not influence Paxillin phosphorylation by E2 (Fig. 1F-G), suggesting a specific role of ERα in this signaling pathway.

3.2. ERα regulates Paxillin phosphorylation through a Gαi/Gβ-dependent signaling pathway

We used pertussis toxin (PTX), a specific inhibitor of G proteins, to evaluate the participation of G proteins on Paxillin phosphorylation/activation (Fig. 2A-B). We observed that Paxillin^{Y118} phosphorylation induced by E2 was significantly prevented by PTX, demonstrating the participation of G proteins on the Paxillin phosphorylation (Fig. 2A-B). To further evaluate the involvement of the specific G protein subunits Gαi and Gβ on the activation of Paxillin, we transfected MCF-7 cells with a dominant negative construct Gαi1DN and a specific Gβ1 siRNA. We found that treatment of cells with Gαi1DN, which encodes a nonfunctional protein, leads to a decrease in Paxillin-Tyr^{118} phosphorylation (Fig. 2C-D). Similarly, in Gβ1 siRNA-treated cells we observed a dramatic decrease in phosphorylation of Paxillin (Supplemental data S1 E), suggesting the involvement of the two subunits Gαi/β in the signaling of estrogen to Paxillin (Fig. 2C-D).

3.3. E2 triggers the phosphorylation of N-WASP through Src/FAK/Paxillin

We used different pharmacological inhibitors in cells exposed to estradiol to determine the signaling pathways through which ER leads to Paxillin phosphorylation. The pertussis toxin (PTX, 100 ng/ml), c-Src kinase inhibitor (PP2 10 μM) and the focal adhesion kinase inhibitor (FAKi 1 μM) all impaired Paxillin-Tyr^{118} phosphorylation by E2, but did not affect the silencing of Cdc42 and N-WASP with specific siRNAs on the Paxillin phosphorylation (Fig. 3A-B and Supplemental data S1 F and H).

N-WASP is a scaffolding protein that relays upstream signals

the sampling of five areas of the cell membrane of 40 different randomly selected cells. Areas of minimum and maximum cell membrane thickness were always included. **D**) Cells were exposed for 20 min to E2 (10 nM) in the presence or absence of the ER antagonist ICI 182,780 (ICI; 100 nM). Total cell amount of wild-type Paxillin and p-Paxillin Tyr^{118} are shown with western blot. **E)** The graph shows the quantitative analysis of the intensity of the bands in D. *<P 0.05 CON vs. E2, **<P 0.05 E2 vs. E2 + ICI. **F)** Breast cancer cells were transfected with siRNA vs. ERα (siRNA ERα) or ERβ (siRNA ERβ) or with vehicle, and protein analysis was performed on cell lysates after treatment for 20 min with E2. **G)** Optical densitometry of three independent experiments of F. The results are expressed as the mean ± SD of the measurements. *< P 0.05 vs. 0 min. CON, Control. All experiments were repeated three times with consistent results, and representative images are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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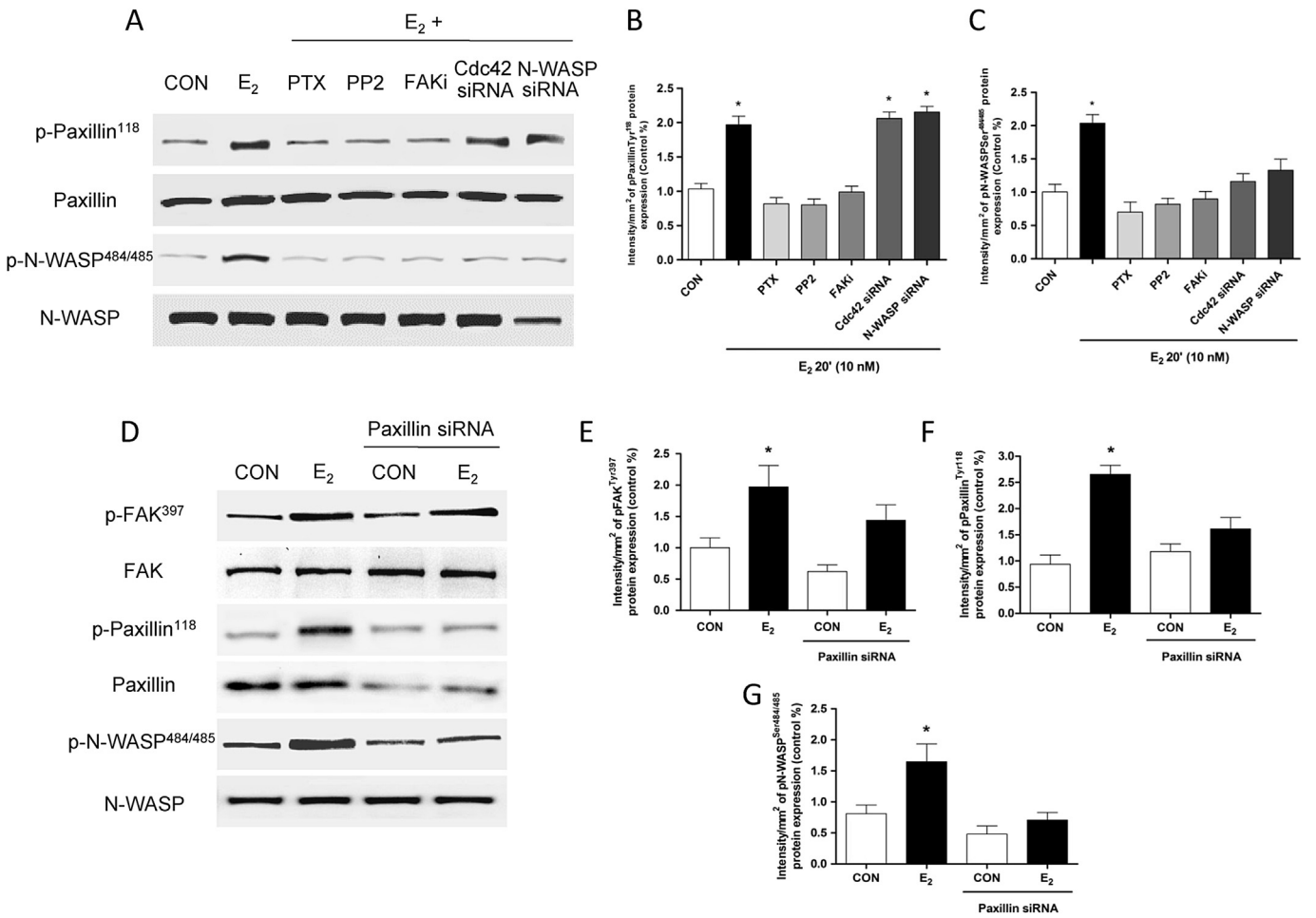

Fig. 3. ERα signals to Paxillin and N-WASP via Gαi/Gβ, Src and FAK. A) Breast cancer cells were exposed for 20 min to E2 (10 nM) in the presence or absence of the G protein inhibitor (PTX, 100 ng/ml), the Src kinase inhibitor (PP2, 10 μM), FAK kinase inhibitor (FAKi, 1 μM) and transfected with specific siRNAs vs. Cdc42, N-WASP. Paxillin, p-PaxillinTyr^{118}, N-WASP and pN-WASP$^{Ser484/485}$ were assayed with western analysis. **B-C)** Optical densitometry of three independent experiments of A. **D)** Cells were treated with E_2, in presence or absence of Paxillin siRNA, and FAK, Paxillin and N-WASP phosphorylation were analyzed through western blot assay. **E-G)** Phospho-FAK-Tyr^{397}, p-Paxillin-Tyr^{118} and p-N-WASP$^{Ser484/485}$ densitometry values were adjusted to FAK, Paxillin and N-WASP intensity and normalized to the control. CON, Control. All experiments were performed in triplicate with consistent results; representative images are shown. * = $P < 0.05$ vs. corresponding control.

from small GTPases, such as Cdc42, to the activation of the Arp2/3 complex, leading to actin branching at the cell membrane and lamellipodia/filopodia protrusion (Takenawa and Suetsugu, 2007). Blockade with the specific inhibitors PTX, PP2 and FAKi and Cdc42 and N-WASP with siRNAs completely abolished E2-dependent N-WASP phosphorylation (Fig. 3A and C).

In order to evaluate where Paxillin exerts its action in the signaling to N-WASP after E2 treatment, we silenced Paxillin with small interfering RNAs. Paxillin expression was significantly reduced when MCF-7 cells were transfected for 24 h with Paxillin siRNA (Fig. 4D and Supplemental data S1 G). In Paxillin-silenced cells, phosphorylation of Paxillin and N-WASP, but not of FAK-Tyr^{397}, was significantly lower after E2 treatment, suggesting that Paxillin acts as a central pivoter between FAK and N-WASP, regulating the signal downstream to FAK and upstream to N-WASP (Fig. 3D-G).

3.4. Estrogen induces N-WASP phosphorylation/translocation by FAK/Paxillin via Cdc42 in breast cancer cells

To identify the effects of estrogen on human breast cancer cell morphology, we studied the actions of a rapid estrogen exposure on the actin cytoskeleton to visualize the dynamics of actin filaments and the subcellular translocation of phospho-Paxillin^{Y118} (Fig. 4A). We examined the sub-cellular localization of phospho-Paxillin^{Y118} with immunofluorescence in the presence of E2. Breast cancer cells treated with E2 displayed membrane phosphorylation/translocation of Paxillin (Fig. 4A-B) to the formation of focal adhesion complexes, which is consistent with actin cytoskeleton remodeling and consequent thickening of the membrane (Figs. 1 and 4A and Supplemental data S1 D). Blockade of G protein (PTX), Src (PP2) and FAK (FAKi) with specific inhibitors abrogated the estrogen-induced membrane translocation of Paxillin and the formation of focal adhesion complexes. The estrogen treatment led to a rapid change of the spatial organization of actin fibers and Paxillin subcellular localization (Fig. 4A-B), suggesting the fundamental role of Gαi/β, Src, FAK and Paxillin in focal adhesion complex formation.

We therefore examined the sub-cellular localization of N-WASP in the presence of E2. MCF-7 cells treated with E2 displayed a membrane translocation of N-WASP and Arp-3 that was time-consistent with the activation/translocation of Paxillin (Fig. 4C–E and Supplemental data S1 I). Silencing of Paxillin, Cdc42 and N-WASP with specific siRNAs resulted in decreased N-WASP translocation during exposure to estrogen (Fig. 4C and Supplemental data S1 I). Parallely, blockade of Paxillin and Cdc42 with specific siRNAs, and Arp2/3 complex, with the specific inhibitor CK-666,

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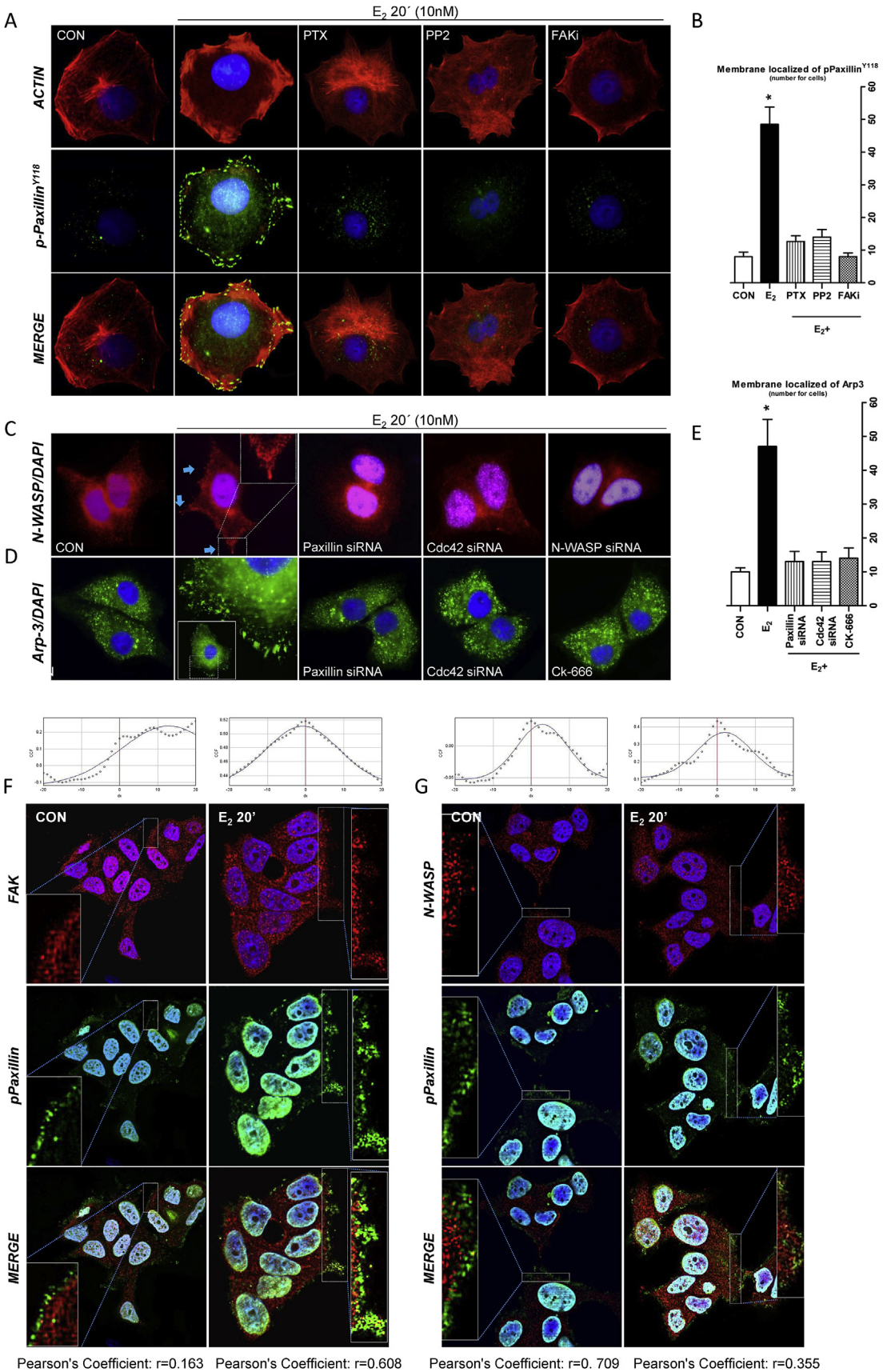

Fig. 4. Paxillin and Cdc42 regulates the activation of N-WASP to the Arp2/3 complex. A) Breast cancer cells were exposed to E2 (10 nM) for 20 min in presence or absence of the G protein inhibitor (PTX), the Src kinase inhibitor (PP2) and FAK kinase inhibitor (FAKi). Cells were stained with p-Paxillin^{Tyr118} (green), actin filaments were stained with phalloidin Texas Red (red) and nuclei were counterstained with DAPI (blue). **B)** Quantification of the membrane-localized Paxillin in the different conditions. **C)** Cells were stained with anti-N-

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blocked the estrogen-induced membrane translocation of Arp-3 (Fig. 4D-E), supporting the concept that Paxillin modulates the redistribution of N-WASP and Arp2/3 complex, and consequently their activity, thus promoting actin nucleation. Based on these experiments, Paxillin is suggested to represent a central pivot protein controlling different steps of the actin reorganization recruited by ER, favoring the formation of focal adhesion complex via Src/FAK and the regulation of the actin nucleation through the Cdc42, N-WASP and Arp2/3 complex.

In parallel, E2 20 min (10 nM) induced a quick membrane co-localization of pPaxillin with FAK but not pPaxillin with N-WASP, suggesting that FAK colocalized with Paxillin, and Paxillin signal via cdc42 GTPase to N-WASP/Arp2/3 complex (Fig. 4F-G). To determine the degree of pPaxillin-FAK colocalization after treatment with E2, we used PCC, MCC-M1, and MCC-M2. The value of PCC was 0.608, respect to 0.163 (CON), indicating that the scatter plot distribution corresponds to partial colocalization (Fig. 4G). In addition, the shape of the CCF curve indicated a nonrandom partial colocalization (Fig. 4G). These values near to 1.0 indicate that the colocalization of pPaxillin and FAK was almost complete.

3.5. Paxillin activation modulates cell adhesion and migration

We have previously demonstrated that E2 activates and stimulates the redistribution of Paxillin to the cell periphery, induces the formation of focal adhesion complexes and controls the cellular adhesion and migration process (Supplemental data S1 J-M). In order to investigate whether Paxillin could modulate cellular processes related with metastasis, such as cell adhesion, migration and invasion, we performed cell adhesion on gelatin matrix, scratch-wound healing and invasion assays. When cells were treated with E2, we found a two-fold increase in the cell number attached to the gelatin matrix relative to control treatment (Fig. 5A-B). In cells pretreated with Src, FAK and Arp2/3 inhibitors, and siRNAs versus Paxillin and N-WASP, we observed a dramatic reduction of the number of cells attached to the matrix compared to the basal condition (Fig. 5 A–B, and Supplemental data S1 J-K).

We then studied whether the signaling actions of E2 to cytoskeletal regulation translate into functional modulation of breast cancer cell motility. In order to distinguish cell migration from proliferation, we pretreated breast cancer cells with cytosine b-D-arabinofuranoside hydrochloride (ARA-C, 10 mM), a selective inhibitor of DNA synthesis that prevents cell division but does not inhibit RNA synthesis. This treatment allowed dissecting the actions of estrogen on movement from those on cell proliferation. We conducted a scratch-wound healing assay. Treatment of cells with E2 (10 nM) significantly increased the number of MCF-7 human breast cancer cells that migrated through the starting line, as well as the mean length of migration compared to control (Fig. 5 C-D). This was blocked by the inhibition of ER, G protein, c-Src, FAK, Paxillin, cdc42, N-WASP and Arp-2/3 (Fig. 5 C–D, and Supplemental data S1 L-M), demonstrating that ERs/G proteins, Src, FAK, Paxillin, Cdc42, N-WASP and Arp2/3 complex are involved in breast cancer cellular migration induced by E2.

To test the action of the E2-dependent signaling to Paxillin/N-WASP/Arp2-3 complex on breast cancer cell invasion, we performed three-dimensional invasion assays using Matrigel. Ara-C-pretreated cells showed enhanced invasion of the matrix in the presence of E2 (Fig. 5 E-F). Inactivation of c-Src, FAK, Paxillin, Cdc42, N-WASP and Arp2/3 all blocked the effect of E2 (Fig. 5 E-F).

3.6. Raloxifene reduces cell adhesion and migration by antagonizing FAK, Paxillin and N-WASP phosphorylation by estradiol

Finally, we investigated the effects of short-term treatments with Raloxifene (RAL 10 nM), a selective estrogen receptor modulator (SERMs) that binds to the estrogen receptor and exhibits selective agonistic or antagonistic effects depending on the target tissue (Gambacciani, 2013). We tested the effect of RAL (20 min) on FAK, Paxillin and N-WASP phosphorylation in presence or absence of E2 (Fig. 6 A). Cells treated with RAL showed no significant change in FAK^{Y397}, $Paxillin^{Y118}$ and $N\text{-}WASP^{S484/485}$ phosphorylation compared to untreated cells (CON). Moreover, in cells treated with E2 plus RAL, we observed a slight reduction of FAK, Paxillin and N-WASP phosphorylation compared to E2 treatment alone (Fig. 6 A). These results suggest an antagonistic effect of RAL on the phosphorylation of FAK, Paxillin and N-WASP proteins mediated by E2. To further support our hypothesis, we explored the effects of RAL on cell adhesion and migration in presence or absence of E2 action. We found that RAL has no effect on the cell number adhered to the gelatin matrix. The enhancement of cancer cell adhesion induced by E2 was notably reduced by the use of the selective estrogen receptor modulator (Fig. 6 B-C).

As we previously demonstrated with T-47D breast cancer cells (Flamini et al., 2009), treatment with E2 (10 nM) significantly increased the mean length of migration (Fig. 6 D-E), but we observed a significant reduction of cellular migration in MCF-7 cells simultaneously treated with RAL and E2 (Fig. 6 D-E). On the other hand, RAL alone has no effect on cellular migration (Fig. 6 D-E).

4. Discussion

A better understanding of the molecular changes that occur inside cells due to different stimuli, such as estrogens is vital to identify new molecular markers and novel therapeutic targets. In addition, this knowledge is necessary to improve treatments against breast cancer.

The use of multidomain scaffold proteins by cells is a conserved mechanism that allows the cell to regulate and integrate multiple extracellular signals, leading to a set of related biological responses. Paxillin is a clear example of how these proteins work (Zaidel-Bar et al., 2007). The elevated binding motif present in the structure of Paxillin allows it to recruit several intermediary proteins, which allows a suitable activation of multiple pathways. FAK-Paxillin has been reported to be involved in many aspects of the metastatic process, including adhesion, migration, secretion of MMPs (matrix metalloproteinases) and invasion (Brown and Turner, 2004; McLean et al., 2005; Qin et al., 2015). Indeed, numerous reports have described overexpression, hyperphosphorylation and/or elevated activity of FAK in a variety of human cancers, including sarcomas, astrocytomas and carcinomas of the breast, colon, thyroid, prostate, oral cavity, liver, stomach and ovary (McLean et al., 2005). These observations highlight a possible key role of FAK-

WASP linked to Texas Red (red) and **D)** anti-Arp3 linked to FITC (green). Nuclei were counterstained with DAPI. **E)** Quantification of the membrane-localized Arp3 in the different conditions. Results are expressed as percent vs. control cells (mean ± SD). Membrane-localized Paxillin, N-WASP and Arp3 were counted in 40 different cells. **F-G)** Colocalization of pPaxillin with FAK and N-WASP in MCF-7 cells. MCF-7 Breast cancer cells observed by confocal microscopy. **F)** Colocalization analysis with JACoP between pPaxillin and FAK. **G)** Colocalization analysis with JACoP between pPaxillin and N-WASP. Pearson Coefficient of colocalization event for pPaxillin (Ch2) FAK (Ch1) **(F)**; and pPaxillin (Ch2) N-WASP (Ch1) **(G)**. Cross-correlation function: PCCs versus pixel shift (dx). All experiments were performed in triplicate and representative images are shown. CON, Control. * = $P < 0.05$ vs. corresponding control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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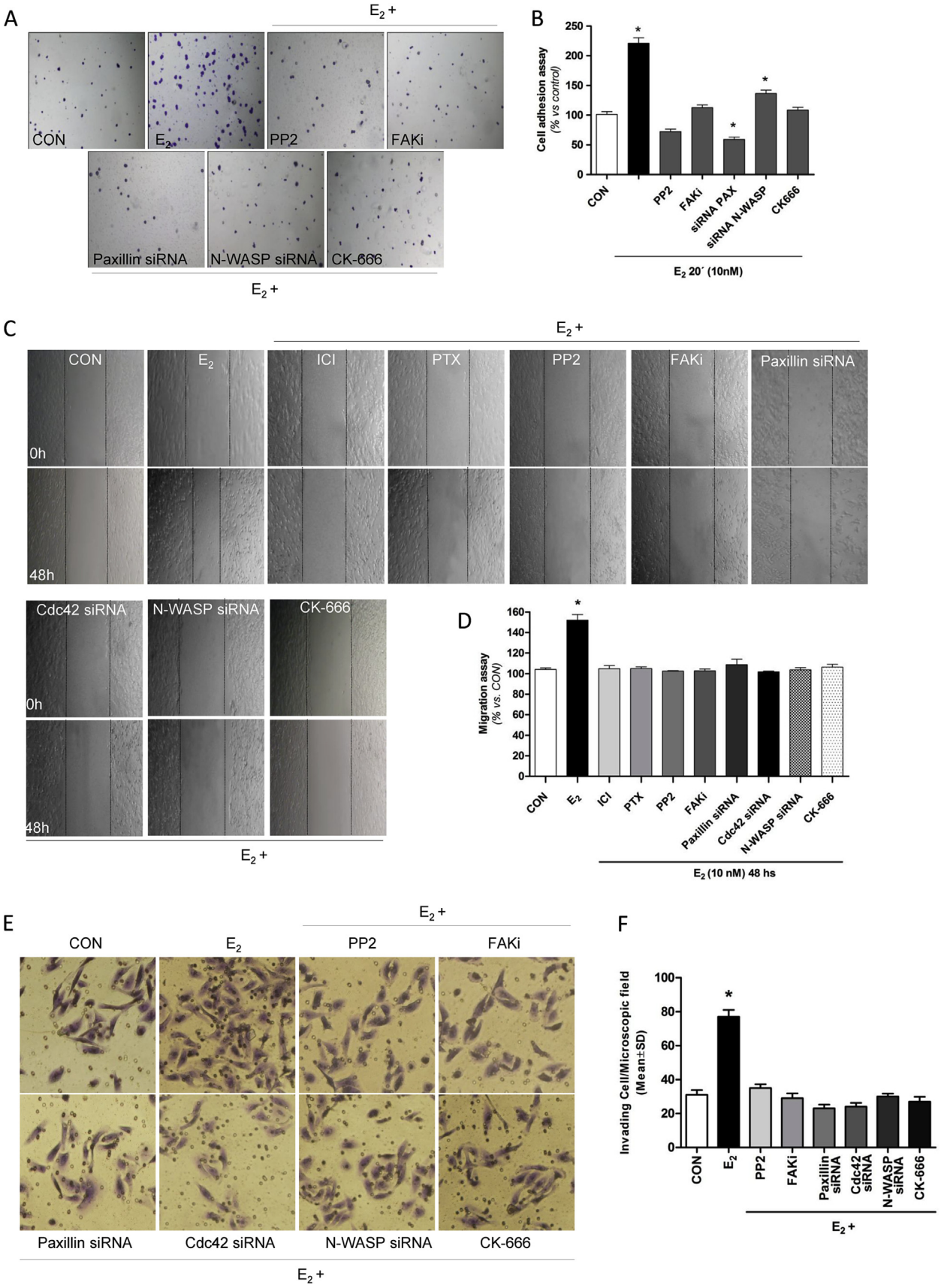

Fig. 5. Paxillin modulates cell adhesion, migration and invasion in breast cancer cells. A) Cells were treated with E2 in the presence or absence of the specific inhibitors to Src (PP2), FAK (FAKi), Arp2/3 (CK666) and transfected with siRNA to Paxillin and N-WASP. Representative images of MCF-7 cell adhesion to gelatin (E2 - 10 nM, 2 h) are shown. **B)** Quantitative adhesion graph of three independent experiments. * = $P \leq 0.05$ vs. Control. **C)** Cells were pretreated with inhibitors to ER (ICI), G proteins (PTX), Src (PP2), FAK (FAKi),

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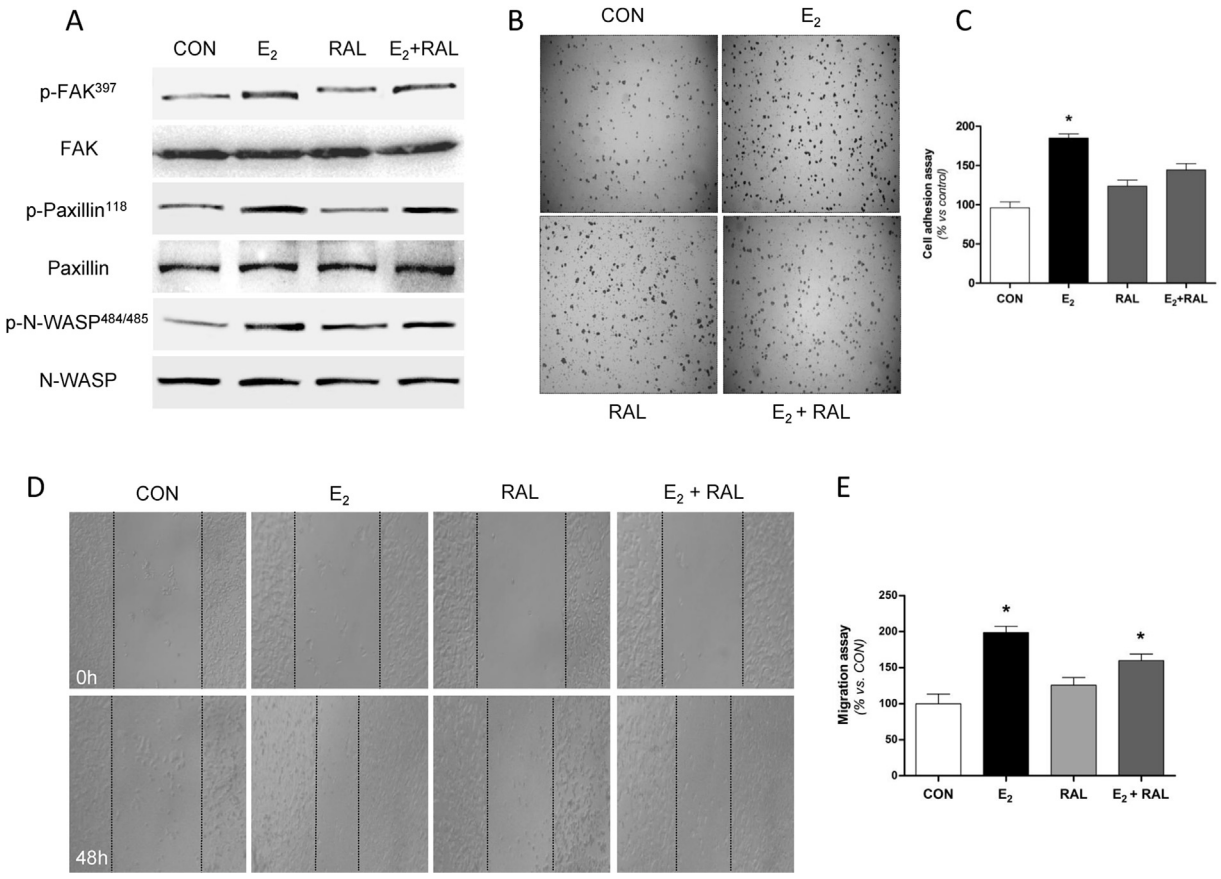

Fig. 6. Raloxifen antagonizes the effects of estradiol in cellular adhesion and migration via FAK/Paxillin/N-WASP. A) MCF-7 breast cancer cells were treated for 20 min with E2 (10 nM), RAL (10 nM) or E2 plus RAL. Phospho-FAK-Tyr^{397}, pPaxillin-Tyr^{118} and pN-WASP$^{Ser484/485}$ phosphorylation are shown in the lower boxes. Wild-type, un-phosphorylated FAK/Paxillin/N-WASP expression are shown in the upper boxes as loading control. **B-C)** Cell adhesion assay and quantification graph. **D-E)** Wound healing assay and quantification graph. Measures are from three independent experiments. * = $P \leq 0.05$. Cell migration assay (wound healing assay) and quantification graph were measured as the mean migration distance from the starting line. The quantitative analysis in terms of mean migration length is shown. Data are expressed as the mean ± S.D. of three separate experiments. * = $P < 0.05$ vs. control.

Paxillin in tumorigenesis.

Several small molecule inhibitors targeting the catalytic activity of kinases have recently been developed by pharmaceutical companies and are being tested in clinical trials (Hanahan and Weinberg, 2011; Baselga, 2006). Our work therefore aimed at addressing whether E2 could modulate cell motility through actin cytoskeleton remodeling by FAK/Paxillin/Cdc42/N-WASP-Arp2/3 complex pathway. As a consequence, we consider that interfering this pathway could represent an efficient alternative strategy to inhibit breast cancer cell migration and invasion.

It is well known that extranuclear ER signaling in breast cancer cells promotes cytoskeletal reorganization and cell migration (Sanchez et al., 2010; Hammes and Levin, 2011; Sanchez and Simoncini, 2010). Paxillin is one of the many regulators of the actin cytoskeleton. This focal adhesion-associated signaling molecule functions as an adaptor protein to recruit diverse cytoskeleton and signaling proteins into a complex, presumably to coordinate the transmission of down-stream signals (Schaller, 2001). One of the most interesting roles of Paxillin is the modulation of focal adhesion and actinic cytoskeleton remodeling by its specific phosphorylation on Tyr^{118}. The latter is considered an integrator region that ultimately could regulate cell migration (Deakin and Turner, 2008; Brown and Turner, 2004).

In this work we have clearly demonstrated that E2 exerts its action through ERα and G protein Gαi/βγ subunit on Paxillin-Tyr^{118} phosphorylation in MCF-7 human breast cancer cells. In addition, we provide evidence that Src and FAK are implicated in the phosphorylation of Paxillin induced by E2. This is in agreement with previous works that have shown that E2 promotes the phosphorylation of Src and FAK kinases in T-47D cells (Sanchez et al., 2010; Li et al., 2010).

The dynamic rearrangement of the actin cytoskeleton is a necessary step for cell migration. This event allows the formation of protrusions on the cell membrane to generate the translocation of the cell body towards the leading edge, an essential step for migration to occur (Yamaguchi and Condeelis, 2007). Focal adhesions are linkers between the extracellular matrix and the actin cytoskeleton through a large number of structural and regulatory

Arp2/3 (CK666) and transfected with specific siRNAs to Paxillin, Cdc42 and N-WASP and stimulated with E2. **D)** Quantitative migration graph of three independent experiments. * = $P \leq 0.05$ vs. Control. **E)** Breast cancer cells were seeded on top of a Matrigel invasion chamber and then treated with cytosine β-D-arabinofuranoside hydrochloride (10 μM) to arrest cell proliferation. The cells were then treated for 24 h with E2 (10 nM) in the presence or absence of the specific inhibitors and siRNAs. Invading cells were photographed at 100 × magnification and counted in the central field of triplicate membranes. **E)** Shows sample images of invasion in the different conditions. **F)** Indicates the mean number of invading cells ± SD from three separate experiments. Dates are expressed as the mean ± SD of three separate experiments and representative images are shown. * = $P \leq 0.05$ vs. control.

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proteins (Schlaepfer and Mitra, 2004; Ilic et al., 1997). The regulation of focal adhesions is led by the activation of Src and FAK kinases (Dumbauld et al., 2010) and followed by the Paxillin phosphorylation. Our results are consistent with the findings of Deramaudt, T.B. et al., who suggest that Src/FAK interaction is essential for Paxillin activity (Deramaudt et al., 2014).

Polymerization of actin filaments underneath the plasma membrane is the main driving force for protrusions on the leading edge. One of the key regulators in this process is the Actin-related proteins 2 and 3 (Arp2/3) complex. Arp2/3 complex nucleates new actin filaments from preexisting filaments, leading to membrane protrusions and lamellipodia or filopodia formation in the direction of cell movement (Insall and Machesky, 2009; Li et al., 2015). The main activators of the Arp2/3 complex are the Wiskott-Aldrich syndrome proteins (WASP). WASP is a large family of proteins that includes WASp, N-WASP and WAVEs. They are responsible for transmitting signals between small GTPases, such as Cdc42, to the actin cytoskeleton through activation of the Arp2/3 complex, which promotes actin polymerization (Takenawa and Suetsugu, 2007; Frugtniet et al., 2015). Activation of N-WASP is controlled by a phosphorylation and de-phosphorylation process, and several kinases such as Src, FAK, Abelson (Ab1) and casein kinase 2 (CK2) may activate N-WASP (Rotty et al., 2013).

We also demonstrated that the phosphorylation of Paxillin in Tyr^{118} residue could be essential for N-WASP activation and localization. For this, we have shown by immunofluorescence analysis that Paxillin silencing inhibits N-WASP and Arp2/3 redistribution to the cell membrane. It is interesting how the down-regulation of Paxillin may affect the translocation of N-WASP and Arp2/3 complex to actin branching sites. This reduces the formation of lamellipodia/filopodia in the direction of cell movement, eventually altering cell adhesion, migration and invasion processes (Sanchez et al., 2010).

Moreover, we determined that E2 modulates cell adhesion, migration and invasion of breast cancer MCF-7 cells via ERα/Gαi1-β1/Src/FAK/Paxillin/Cdc42/N-WASP and Arp2/3 complex. Deramaudt, T.B., et al. have shown that altering FAK-Paxillin interactions reduces adhesion, migration and invasion processes in mouse embryonic fibroblasts. These authors conclude that blocking this pathway could be an efficient strategy to reduce FAK signaling and may thus represent a target for the development of new FAK inhibitors (Deramaudt et al., 2014).

At present, knowledge about the different signaling pathways that may be activated or inhibited by selective estrogen receptor modulators (SERMS), such as raloxifene, is poor. This study shows for the first time that in the presence of E2, raloxifene inhibits the phosphorylation levels of p-FAK^{Y397}, p-$Paxillin^{Y118}$ and p-$N\text{-}WASP^{S484/485}$, reducing the cell adhesion and migration process. SERMs block the action of estrogen in the breast and other tissues by occupying ERs and inducing conformational changes that prevent the interaction of the receptors with co-activators (Riggs and Hartmann, 2003). The current understanding of the mechanism of action of these drugs is that, given the differences in the expression of the co-activators or co-repressors of ERs in different cells and tissues, each SERM will lead to turn into a specific spectrum of body effects. However, when E2 and RAL are administered simultaneously, RAL inhibits the E2-dependent recruitment of FAK, Paxillin and N-WASP. Taken together, our data suggest that the SERM raloxifene is able to alter ER + breast cancer cell adhesion and motility behavior by regulating the actin cytoskeleton through FAK, Paxillin and N-WASP proteins. Through this mechanism of action, RAL appears to function as an antagonist against breast cancer cell movement induced by E2. The precise mechanism of how raloxifene modulates the focal adhesion complex assembly remains, however, unclear.

In conclusion, this paper highlights original mechanistic insights into the effects of estrogen via Src/FAK/Paxillin/Cdc42/N-WASP/Arp2/3 complex on breast cancer progression, and may in future be helpful to develop new drugs against endocrine-sensitive breast cancers.

Disclosure statement

The authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2016.04.007.

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