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Retinoic acid induces nuclear FAK translocation and reduces breast cancer cell adhesion through Moesin, FAK, and Paxillin

Angel Matías Sanchez [a], Jorge Eduardo Shortrede [a], Laura María Vargas-Roig [a, b], Marina Inés Flamini [a, *]

[a] Institute of Medicine and Experimental Biology of Cuyo (IMBECU), National Research Council of Argentina, Mendoza, Argentina
[b] School of Medical Sciences, National University of Cuyo, Mendoza, Argentina

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ABSTRACT

Breast cancer is the most common malignancy in women, with metastases being the cause of death in 98%. In previous works we have demonstrated that retinoic acid (RA), the main retinoic acid receptor (RAR) ligand, is involved in the metastatic process by inhibiting migration through a reduced expression of the specific migration-related proteins Moesin, c-Src, and FAK. At present, our hypothesis is that RA also acts for short periods in a non-genomic action to cooperate with motility reduction and morphology of breast cancer cells. Here we identify that the administration of 10^{-6} M RA (10–20 min) induces the activation of the migration-related proteins Moesin, FAK, and Paxillin in T-47D breast cancer cells. The phosphorylation exerted by the selective agonists for RARα and RARβ, on Moesin, FAK, and Paxillin was comparable to the activation exerted by RA. The RARγ agonist only led to a weak activation, suggesting the involvement of RARα and RARβ in this pathway. We then treated the cells with different inhibitors that are involved in cell signaling to regulate the mechanisms of cell motility. RA failed to activate Moesin, FAK, and Paxillin in cells treated with Src inhibitor (PP2) and PI3K inhibitor (WM), suggesting the participation of Src-PI3K in this pathway. Treatment with 10^{-6} M RA for 20 min significantly decreased cell adhesion. However, when cells were treated with 10^{-6} M RA and FAK inhibitor, the RA did not significantly inhibit adhesion, suggesting a role of FAK in the adhesion inhibited by RA. By immunofluorescence and immunoblotting analysis we demonstrated that RA induced nuclear FAK translocation leading to a reduced cellular adhesion. These findings provide new information on the actions of RA for short periods. RA participates in cell adhesion and subsequent migration, modulating the relocation and activation of proteins involved in cell migration.

1. Introduction

Retinoic acid (RA), a pleiotropic signaling molecule derived from vitamin A, regulates critical genetic programs that control development, homeostasis, cell proliferation, and differentiation, as well as cell death or survival (Clagett-Dame and Knutson, 2011,Samarut and Rochette-Egly, 2012). This is the basis for the use of RA in cancer therapy (Altucci et al., 2007).

RA activity is primarily mediated by members of the retinoic acid receptor (RAR) subfamily, namely RARα, RARβ, and RARγ, which belong to the nuclear receptor superfamily. Classically, RARs function as ligand-inducible transcriptional regulators that heterodimerized with retinoid X receptors (RXRs). As such, they regulate the expression of subsets of target genes. Much of the research on the effects of retinoids is focused on the regulation of gene expression. Retinoids regulate the activity of a number of genes and proteins in many cell types, including growth factors, the extracellular matrix (ECM) proteins, and intracellular signaling molecules, such as protein kinase C and cyclic AMP-dependent protein kinases (Li et al., 2014,Scheibe et al., 1991, Sporn and Roberts, 1991,Zhang et al., 1996).

In addition to this scenario, recent studies highlighted a novel paradigm in which RA also induces the rapid activation of different signaling pathways (Al Tanoury et al., 2013). Studies from several laboratories have shown that RA rapidly and transiently activates several kinase cascades, which are exemplified by the mitogen

* Corresponding author. Tumor Biology Laboratory, Institute of Medicine and Experimental Biology of Cuyo (IMBECU), CCT-CONICET, National Research Council of Argentina, C.C.855, C.P. 5500, Mendoza, Argentina.
E-mail address: mflamini@mendoza-conicet.gob.ar (M.I. Flamini).

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activated kinase (MAPK) pathways. Indeed, RA activates p38 MAPK in fibroblasts, mouse embryo carcinoma cells, mammary breast tumor cells, and leukemia cells (Alsayed et al., 2001,Bruck et al., 2009,Gianni et al., 2002,Piskunov and Rochette-Egly, 2012). Most interestingly, the activation of p38 MAPK by RA occurs very rapidly (within minutes), suggesting a non-genomic action of RA and RARs, as described for steroid hormone receptors (Losel and Wehling, 2003).

The metastatic process requires the acquisition of invasive properties such as remodeling, adhesion, motility, and invasion of the ECM. Another important component of this process is the organization of cell adhesion sites that are directed by receptors, which physically link the ECM to the cytoplasmic actin cytoskeletal network and may transmit signals from the ECM to the cytoplasm.

The actin-binding protein Moesin, which belongs to the ezrin/radixin/Moesin (ERM) family, is a key regulator of ECM signals. Activated Moesin triggers the de-polymerization of actin fibers and the re-assembly of microfilaments toward the cell membrane edge, leading to the formation of cortical actin complexes and specialized cell membrane structures that are implicated in the generation of the cellular locomotive force (Louvet-Vallee, 2000).

The focal adhesion kinase (FAK) is another ECM controller. This non-receptor protein-tyrosine kinase is involved in cell attachment, migration, and invasion, which are crucial steps for cancer development and metastasis (Gabarra-Niecko et al., 2003,McLean et al., 2005). FAK is phosphorylated by c-Src, a non-receptor tyrosine kinase and then the recruitment of Src/FAK/PI3K complex occurred (Calalb et al., 1995). The activated Src/FAK/PI3K complex mediates the phosphorylation of multiple adhesion components involved in the dynamic regulation of cell motility. Considerable evidence suggests that enhanced protein tyrosine phosphorylation occurs during focal adhesion plaque formation. A specific tyrosine residue within another focal adhesion protein, Paxillin, has been identified as a primary target for phosphorylation by FAK (Parsons et al., 1994). FAK and Paxillin also show a high stoichiometry of tyrosine phosphorylation upon integrin activation (Bellis et al., 1995,Turner, 1994).

Retinoids have also been shown to regulate cell adhesion and migration. Some authors, including us, have demonstrated that RA and other biologically active retinoids inhibit cellular migration in several cell lines, such as human colon carcinoma cells (Woo and Jang, 2012) as well as human breast cancer MCF-7 and MDA-MB-231 cells (Dutta et al., 2009,Dutta et al., 2010). Our laboratory recently demonstrated that RA inhibits cellular migration by remodeling the actin cytoskeleton and down-regulating the expression of Moesin, Src, and FAK in T-47D and MCF7 breast cancer cells (Flamini et al., 2014).

At present, little is known about the possible mechanism by which retinoids affect breast cancer cell migration. We believe that retinoids act both non-genomically and genomically to regulate cellular processes. For this reason, in this work we aimed at deciphering the rapid non-genomic effects of RA on protein phosphorylation and actin cytoskeleton remodeling.

Recent studies revealed new aspects of FAK action in the nucleus where in a normal growth condition, the localization of FAK is prominent in focal adhesions and the cytosol. However, FAK mobilization occurred from integrin adhesion sites to the nucleus when cell de-adhesion occurred due to stress (Lim, 2013). In the nucleus, FAK interacts with tumor suppressor p53, resulting in p53 turnover to enhance cell survival (Lim, 2013).

We demonstrate that RA induces the activation of Moesin, FAK, and Paxillin through RARα and RARβ-selective retinoids in T-47D human breast cancer cells, which leads to nuclear FAK translocation and reduced cellular adhesion.

2. Materials and methods

2.1. Cell cultures and treatments

The human breast carcinoma cell line T-47D was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). T-47D cells were routinely grown in RPMI 1640 supplemented with L-glutamine (2 mM) and 10% fetal bovine serum. All-*trans*-retinoic acid (RA) was obtained from Sigma-Aldrich (St. Louis, MO). RA stock solution was dissolved in DMSO at a concentration of 10^{-2} M and maintained at −20 °C, protected from light, and in an inert atmosphere. We used a final concentration of 10^{-6} M of RA according to ours previous published results (Flamini et al., 2014). The synthetic agonist retinoids selective for RARα (BMS753), RARβ (BMS453), and RARγ (BMS961), and the synthetic antagonist retinoids selective for RARα (BMS195614) (Tocris Bioscience, USA) were kindly provided by Dr. Hinrich Gronemeyer (IGBMC, Illkirch, France). Agonist and antagonist retinoids were diluted in ethanol and added to the culture medium to give a final concentration of 10^{-6} M. The Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo(3,4-d) pyrimidine (PP2) was obtained from Calbiochem (EMD Biosciences, Germany); the PI3K inhibitor Wortmannin was from Sigma-Aldrich (Saint-Louis, MO); and the FAK inhibitor 14 (FAKi, CAS 4506-66-5, sc-203950A) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Whenever an inhibitor was used, the compound was added 30–45 min prior to starting the active treatments. The final concentration of the solvents was 1 μl of solvent per 1 ml of medium. In control cultures, the vehicle was added at the same final dilution. All experiments with retinoids were performed in reduced room light.

2.2. Immunoblotting

Cell lysates were separated by SDS-PAGE. In fractionation experiments, after treatments, the cells were washed in PBS and resuspended in lysis buffer for nuclear and cytoplasmic proteins extraction as reported by Andrews NC et al. (Andrews and Faller, 1991). The following antibodies were used: p-FAK Tyr^{397} and Moesin (BD Transduction Laboratories); Actin (C-11), p-FAK Tyr^{397} (sc-11765-R), p-Moesin Thr^{558} (sc-12895), Paxillin (sc-31010), p-Paxillin Tyr^{118} (sc-365020), RARα (sc-551), RARβ (sc-552) and p53 (sc-126) (Santa Cruz Biotechnology); HSP27 (also known as HSPB1), HSP72 (also known as HSPA1A) (Stressgen, USA); α-Tubulin (T9026) and HDAC1 (Sigma-Aldrich Laboratories). Primary and secondary antibodies were incubated with the membranes using standard techniques. Inmunodetection was accomplished using enhanced chemiluminescence. The images were captured using ChemiDoc™ XRS + System with Image Lab™ Software #170-8265 (Biorad, USA).

2.3. Transfection experiments

The synthetic small interfering RNA for RARβ (sc-29466) was from Santa Cruz Biotechnology. T-47D cells (60–70% confluent) were transfected with 50–75 nM of target siRNA using Lipofectamine (Invitrogen- Thermo Fisher Scientific, USA). The cells were treated 24 h after siRNA transfection. Transfection efficiency was checked for expression of RARβ by immunoblotting.

2.4. Plasmid construction

To obtain the RARα silencing construct, a custom-synthesized double-stranded DNA coding for a RARα-targeting shRNA (5′-GATCCGCGGGCACCTCAATGGGTACTTCCTGTCAGA-TACCCATTGAGGTGCCCGCTTTTTG-3', the underlined sequence

corresponds to nt 629–646 of the NM_001145301.2 sequence, Sigma-Aldrich) was introduced into the pGreenPuro plasmid as previously described by Centritto et al., 2015 (Centritto et al., 2015). The plasmid (10 μg) was transfected into T-47D cells using Lipofectamine (Invitrogen). Cells (60%–70% confluent) were treated 24 h after transfection. Transfection efficiency was checked for expression of RARα by immunoblotting.

2.5. Cell adhesion assay

Five hundred thousand cells per condition were exposed to different treatments and then were seeded into 6-well plates on coverslips previously coated with 1% sterile gelatin (Sigma). The cells were incubated at 37 °C (in a tissue culture incubator) for 1 h. The plates were then shaken for 1 min at 150 rpm and washed with phosphate-buffered saline (PBS) to remove any non-adherent cells. The attached cells were fixed with 4% formaldehyde and stained with Giemsa. Images of attached cells were taken using a Nikon Eclipse E200 microscope (Japan) coupled to a high-resolution 590CU 5.0 M CCD digital camera. The attached cells were counted in ten randomly chosen fields per well. Cell adhesion was calculated as a percentage of attached treated cells compared to untreated cells.

2.6. Immunofluorescence

T-47D cells 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. The blocking step was performed with 3% BSA solution for 30 min at room temperature. Cells were incubated with the first antibody against FAK, pFAK, HSP27 or pPaxillin overnight at 4 °C. After washing, the cells were incubated with Dylight 488 (DI1488, Vectors Labs), or Dylight 594 (DI2594, Vectors Labs) for 1 h at room temperature. The cells were then washed and stained with Texas Red-phalloidin (Sigma-Aldrich) to reveal actin, and the nuclei counterstained with 4′-6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). The coverslips cells were mounted with Vectashield mounting media (Vector Laboratories, Burlingame, CA). Immunofluorescence images were captured using a Nikon Eclipse E200 microscope coupled to a high-resolution 590CU 5.0 M CCD digital camera.

2.7. Statistical analysis

Statistical analysis of the data was performed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer Multiple-Comparisons Test (GraphPad PRISM program version 5.0, San Diego, USA). $p < 0.05$ was considered statistically significant. All values are expressed as mean ± SD.

3. Results

3.1. RA recruits Moesin, FAK, and Paxillin

Our previous studies showed a reduced expression of Moesin and FAK after treatment with RA for long periods, leading to the inhibition of cellular migration induced by RA in breast cancer cells (Flamini et al., 2014). Hence, here we investigated whether these proteins and Paxillin (a protein closely related to FAK) might be activated by administration of RA during short periods in breast cancer cells. The phosphorylation on Thr^{558} (corresponding to activation) of Moesin was enhanced by short incubations with RA 10^{-6} M (10–20 min) (Fig. 1A–B). In parallel, treatment with RA 10^{-6} M rapidly increased focal adhesion kinase (FAK) phosphorylation on Tyr^{397} and Paxillin on Tyr^{118} with a similar temporal pattern (Fig. 1A, C–D). These phenomena were time-dependent and transient; they were highest after 10–20 min and receded to baseline after 60 min. The total level of Moesin, FAK and Paxillin was determined to draw conclusion on the activation state of the molecule and we demonstrated that they did not change during the period analyzed. Furthermore, the total cell content of α-Tubulin did not change during this period (Fig. 1A).

3.2. The synthetic retinoid RARα and RARβ agonists trigger Moesin, FAK, and Paxillin activation in breast cancer cells

We tested the effects of selective synthetic retinoid agonists for RARα (BMS753), RARβ (BMS453), and RARγ (BMS961), the RARα-selective antagonist (BMS195614), and the specific siRNA against RARβ in order to determine which subtype of RAR is involved in RA-induced proteins phosphorylation (Fig. 2A).

The RARα and RARβ-selective agonists, and to a lesser degree RARγ, induced $Moesin^{thr558}$, FAK^{tyr397}, and $Paxillin^{tyr118}$ phosphorylation/activation at a level comparable to the activation exerted by RA, indicating that RARα and RARβ are the most important receptors involved in RA-induced Moesin, FAK, and Paxillin activation (Fig. 2A–D). Administration of the RARα-selective antagonist and/or silencing of RARβ with specific siRNA in combination with RA did not affect protein phosphorylation, suggesting that RARα and RARβ receptors are required for RA effects on protein activation (Fig. 2A–D).

In order to guaranteed the specific dose of RARα (BMS753) and RARβ (BMS453) agonists we performed a dose response curve (10^{-8}–10^{-6} M) to demonstrate the activation exerted on FAK protein. We found that RARα (BMS753) and RARβ (BMS453) selective agonists induce the maximal FAK phosphorylation at 10^{-6} M suggesting that RARα and RARβ agonists have their maximum effect at the dose used (Supplementary Fig. 1).

In parallel, we silenced RARα and RARβ proteins in breast cancer cells with specific shRNA vs. RARα and siRNA vs. RARβ. This resulted in a reduction of RARα and RARβ proteins expression, along with a dramatic decrease in Moesin, FAK, and Paxillin phosphorylation during exposure to RA (20 min/10^{-6} M) confirmed the specific role of the RARα and RARβ isoform in Moesin, FAK and Paxillin activation (Fig. 2E).

3.3. RA signals to Moesin, FAK, and Paxillin through a c-Src and PI3K-dependent signaling pathway

In search for the signaling pathways through which RARα/RARβ lead to Moesin, FAK and Paxillin activation, we interfered with a number of signaling cascades that are involved in the activation of these proteins. The c-Src kinase inhibitor (PP2, 10 μM), Wortmannin (WM, 30 nM), an inhibitor of phosphatidylinositol 3-OH kinase (PI3K), and the specific FAK inhibitor (FAKi, 1 μM) significantly inhibited Moesin, FAK and Paxillin phosphorylation induced by RA (10^{-6}M/20 min), indicating that RA recruits c-Src and PI3K via RARα and RARβ to activate these proteins in breast cancer cells (Fig. 3A–D). We also tested the G protein inhibitor pertussis toxin (PTX) and PD98059, an inhibitor of the ERK 1/2 mitogen-activated protein kinase (MAPK) cascade. However, none of them influenced the effect of RA (data not shown).

3.4. RA reduces T-47D cell adhesion via RARα and RARβ

We recently determined that treatment with RA (10^{-6}M/72h) causes 50% inhibition of cell migration and adhesion to gelatin in T-47D cells (Flamini et al., 2014). Here, we tested the rapid effect of RA and found that treatment with RA 10^{-6} M for 20 min significantly reduced T-47D breast cancer cell adhesion (Fig. 4A–D). To

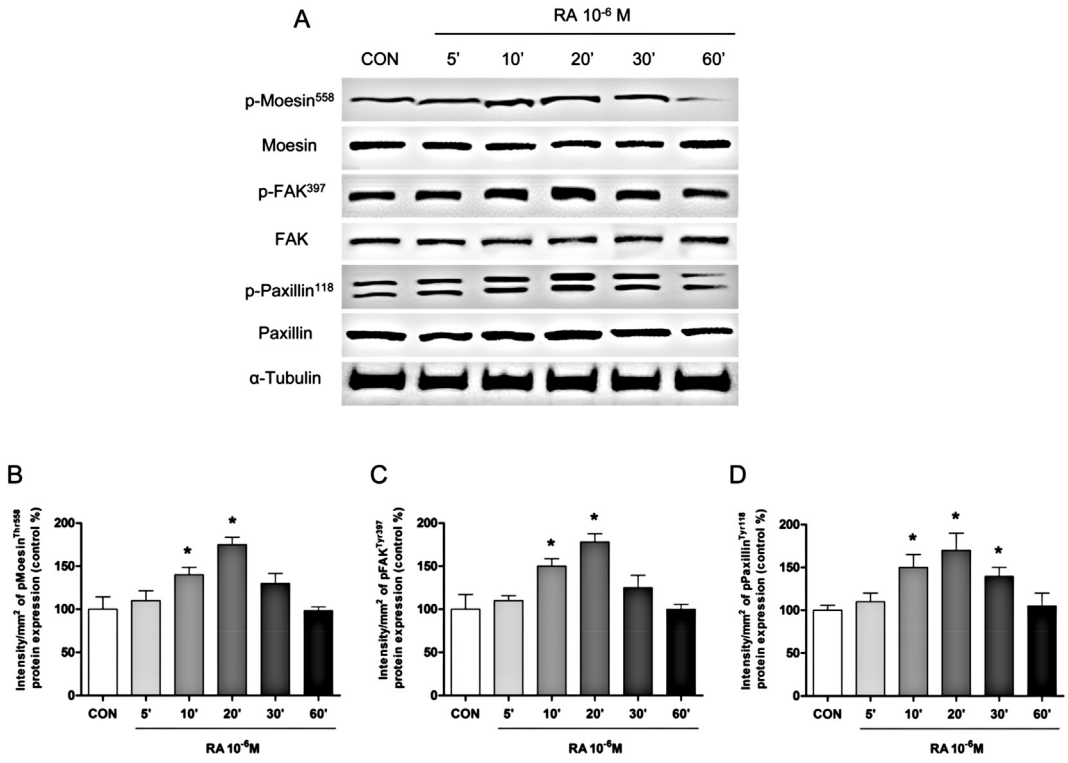

Fig. 1. RA controls Moesin, FAK, and Paxillin activation. A) T-47D cells were treated with RA 10^{-6} M for different periods of time (5, 10, 20, 30, and 60 min), and Western blot analyses for Moesin/pMoesin, FAK/pFAK, and Paxillin/pPaxillin proteins were performed. α-Tubulin expression is shown in the lower boxes as loading control. **B–D)** Phospho-Moesin^{558}, phospho-FAK^{397} and phospho-Paxillin^{118} densitometry values were adjusted to α-Tubulin intensity, then normalized to the control sample. * = $p < 0.05$ vs. corresponding control. Experiments were performed in triplicate. Representative images are shown.

determine which subtype of RAR is involved in RA-induced adhesion inhibition, we tested the effects of selective retinoid agonists for RARα, RARβ, and RARγ, the RARα-selective antagonist, and the silencing of RARβ with specific siRNA (Fig. 4A–B). The RARα and RARβ, but not RARγ-selective agonists, significantly reduced cell adhesion to levels comparable to the inhibition by RA, indicating that RARα and RARβ are involved in RA-inhibited cell adhesion (Fig. 4A–B). Furthermore, neither the RARα-selective antagonist nor silencing of RARβ with specific siRNA, in combination with RA, affected cell adhesion suggesting that RARα and RARβ receptors are required for RA effects on cell adhesion (Fig. 4A–B).

In order to ascertain the role of FAK in the cell adhesion assay, we examined the effects of administering the FAK inhibitor (FAKi, 1 μM) in combination with RA. FAKi significantly reduced the inhibition exerted by RA on attaching cells, indicating that RA recruits FAK to RA-induced adhesion inhibition in breast cancer cells (Fig. 4C–D).

3.5. RA induces a rapid FAK and Paxillin nuclear localization

To understand the dynamics of breast cancer cell adhesion and the different behavior observed after treatment of cells with RA, we next investigated changes in the actin cytoskeleton reorganization induced by RA treatments. For this purpose, we examined the subcellular localization of pFAK^{tyr397} in the presence of RA (10^{-6} M, 20 min) mediate immunofluorescence assay (Fig. 5A–B). We performed short-term exposure to RA and/or selective synthetic retinoid agonists for RARα, RARβ, and RARγ, the RARα-selective antagonist, and/or silencing of RARβ with specific siRNA. In control cells, actin filaments arranged longitudinally along the major axis and pFAK showed a diffuse distribution throughout the cytoplasm (Fig. 5A). In general, when FAK was phosphorylated and consequently activated, it concentrated at sites of ongoing actin remodeling in the cell plasma membrane, thus increasing membrane thickness. Phosphorylated FAK therefore co-localized with other actin-binding proteins, such as Vinculin/Paxillin, to form focal adhesion complexes (FA). Surprisingly, treatment with RA seemed to change pFAK spatial organization, leading to a translocation of pFAK toward the nucleus. In this localization, it is impossible for pFAK to form FA or specialized cell membrane structures, which are present in the cell membrane to induce cell motility (Fig. 5A–B). We then used immunofluorescence images to quantify the percentage of cells with pFAK nuclear localization (Fig. 5B). We found that the RA treatment (10^{-6} M, 20 min) induced a twofold increase in pFAK nuclear localization and that the retinoid agonists for RARα and RARβ showed a similar behavior, with RARβ being the most potent inducer of pFAK nuclear translocation (Fig. 5A–B).

Consistent with the previous results, we think that it would be relevant to evaluate the subcellular localization of FA, as pFAK and pPaxillin in order to determine their distribution after RA treatment. In control conditions, we found the presence of pFAK and pPaxillin in the citoplasmatic and membrane localization (Fig. 5C–E). The treatment with RA (10^{-6} M, 20 min) modified the pFAK and pPaxillin localization, inducing their nuclear translocation (Fig. 5C–E). Moreover membrane localization of pFAK/pPaxillin decreased after RA treatment (Fig. 5E–D). In order to visualize the formation of focal adhesion complex via pFAK, we used 17-β estradiol (E2) (10^{-8} M, 20 min) a promoter of FA sites, previously determined by our group (Sanchez et al., 2010). The rapid treatment with E2 induces a quick membrane activation/redistribution and co-localization of pFAK/pPaxillin, with the consequent formation of focal adhesion sites (Fig. 5C). FAK-Paxillin

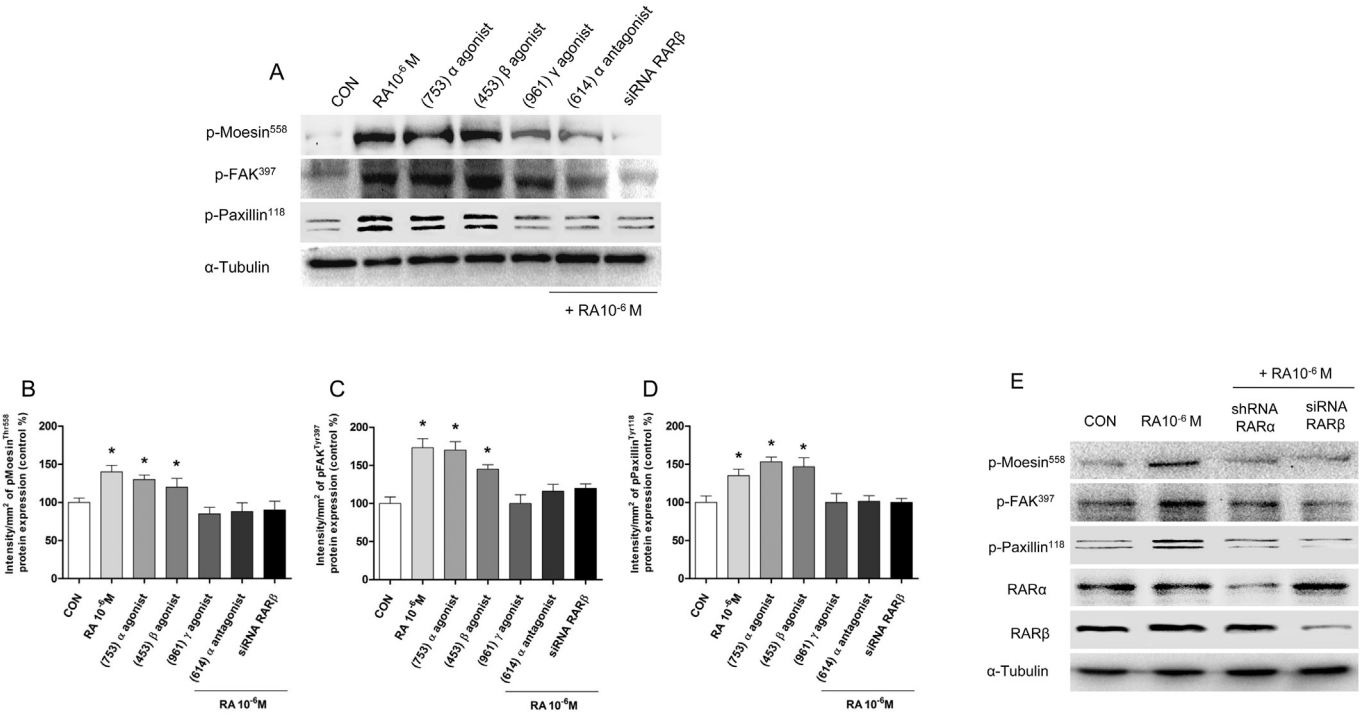

Fig. 2. The synthetic retinoid agonist of RARα and RARβ induces Moesin/FAK/Paxillin phosphorylation in breast cancer cells. A) T-47D cells were treated with or without RA (10^{-6} M, 20 min) and the synthetic retinoid agonists selective for RARα (BMS753), RARβ (BMS453), and RARγ (BMS961); the synthetic retinoid antagonist for RARα (BMS195614); and with a specific RARβ siRNA. All retinoids were incubated at 10^{-6} M for 20 min and Western blot analyses for p-Moesin, p-FAK, and p-Paxillin proteins were performed. α-Tubulin expression is shown in the lower boxes as loading control. **B**–**D)** p-Moesin, pFAK, and pPaxillin densitometry values were adjusted to α-Tubulin intensity, and were then normalized to the control sample. * = $p < 0.05$ vs. control. **E)** T-47D cells were treated with or without RA (10^{-6} M, 20 min) and with the specifics shRNA RARα and siRNA RARβ. Western blot analyses for p-Moesin, p-FAK, p-Paxillin, RARα and RARβ proteins were performed. α-Tubulin expression is shown in the lower boxes as loading control. Experiments were performed in triplicate. Representative images are shown.

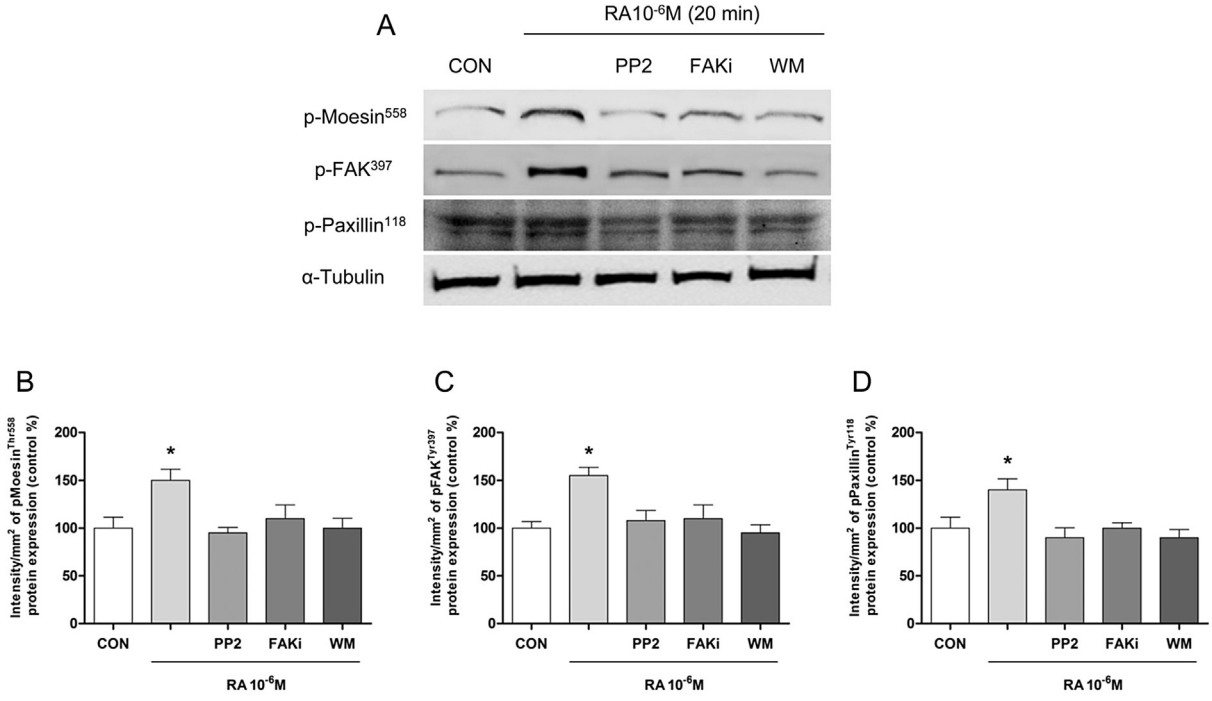

Fig. 3. RA controls Moesin, FAK, and Paxillin phosphorylation via c-Src, FAK, and PI3K signaling cascade. A) Breast cancer cells were exposed for 20 min to RA (10^{-6} M) in the presence or absence of the c-Src inhibitor (PP2), FAK inhibitor (FAKi), or PI3K inhibitor (Wortmannin, WM). Moesin, FAK, and Paxillin phosphorylation were assayed with Western Blot analyses. **B**–**D)** Phospho-protein densitometry values were adjusted to α-Tubulin intensity and then normalized to the control sample. * = $p < 0.05$ vs. control. Experiments were performed in triplicate. Representative images are shown.

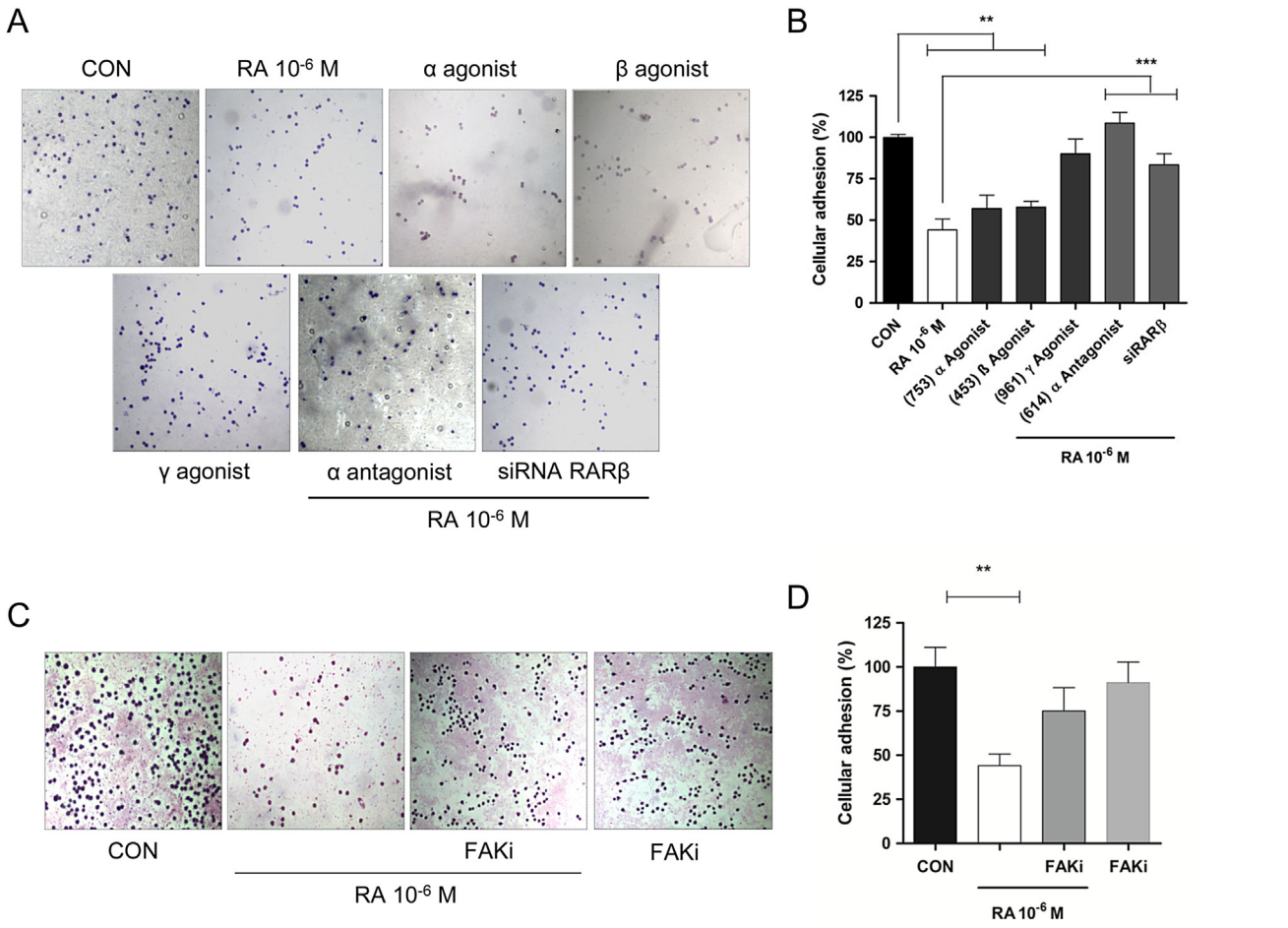

Fig. 4. RA reduces T-47D cell adhesion via RARα and RARβ. A–B) T-47D cells were treated with or without RA (10^{-6} M, 20 min) and the synthetic retinoid agonists selective for RARα (BMS753), RARβ (BMS453), and RARγ (BMS961); the synthetic retinoid antagonist for RARα (BMS195614); and with a specific siRNA vs. RARβ. **C–D)** T-47D cells were treated with or without RA (10^{-6} M, 20 min), in the presence or absence of FAK inhibitor (FAKi). All retinoids were incubated at 10^{-6} M for 20 min and cell adhesion assay was performed. The percentage of attached cells and representative images of T-47D cell adhesion to gelatin after treatment are shown. ** = $p < 0.05$ vs. control and *** = $p < 0.05$ vs. RA. Experiments were performed in triplicate.

interactions have being proved to be crucial for regulating adhesion, migration and invasion processes (Deramaudt et al., 2014).

3.6. RA control FAK/Paxillin and HSP27 nuclear redistribution

The unusual nuclear pFAK localization has been reported previously under stress cell conditions (Lim et al., 2008). Therefore, we decided to study whether RA induced molecular stress in T-47D cells evaluating the expression of the heat shock proteins HSP27 and HSP72, markers of cellular stress. RA treatment for increasing periods of time from 1 to 24 h was performed. The FAK and HSP27 localizations were homogeneous throughout the cytoplasm in control cells and after 1-h treatment with RA 10^{-6} M. Treatment with RA 10^{-6} M during 3–6 h induced, however, HSP27 and FAK nuclear localization (Fig. 6A).

Additionally, we performed cell fractionation experiments to confirm nuclear proteins translocation upon RA addition. In fact, treatment with RA 10^{-6} M induced FAK, Paxillin and HSP27 nuclear translocation specially at 3 and 6 h. Vinculin did not change their spatial organization. The HDAC1 was used as nuclear fraction control and actin as cytoplasmic fraction control (Fig. 6B).

3.7. RA regulates HSP27, HSP72, and p53 expression

Finally, we studied how increasing periods of RA treatment influenced the phosphorylation/expression of Moesin, FAK, Paxillin, HSP27, HSP72 and p53. RA treatment (10^{-6} M) from 1 to 24 h induced HSP27, HSP72, and p53 expression. It was highest from 3 to 24 h for HSP27; from 1 to 24 h for HSP72; and from 6 to 24 h for p53, suggesting the existence of cellular stress that increases expression levels of HSP27/72 and p53 after RA treatment (Fig. 7). In addition the phosphorylation of HSP27 increases from 1 h indicating that a stressful situation occurred and this protein has been activated. On the other hand both the phosphorylation and the expression of Moesin, FAK, and Paxillin decrease to almost undetectable levels after 24 h.

4. Discussion

Although at early stages breast cancer is a well curable disease, the prognosis is severe when metastasis occurs. Metastasis constitutes the final step of neoplastic progression and is the primary cause of death from solid tumors (Sporn, 1996). Detailed knowledge of molecular actions linked to the metastasis process is therefore critical for the development of novel therapeutic strategies in oncology.

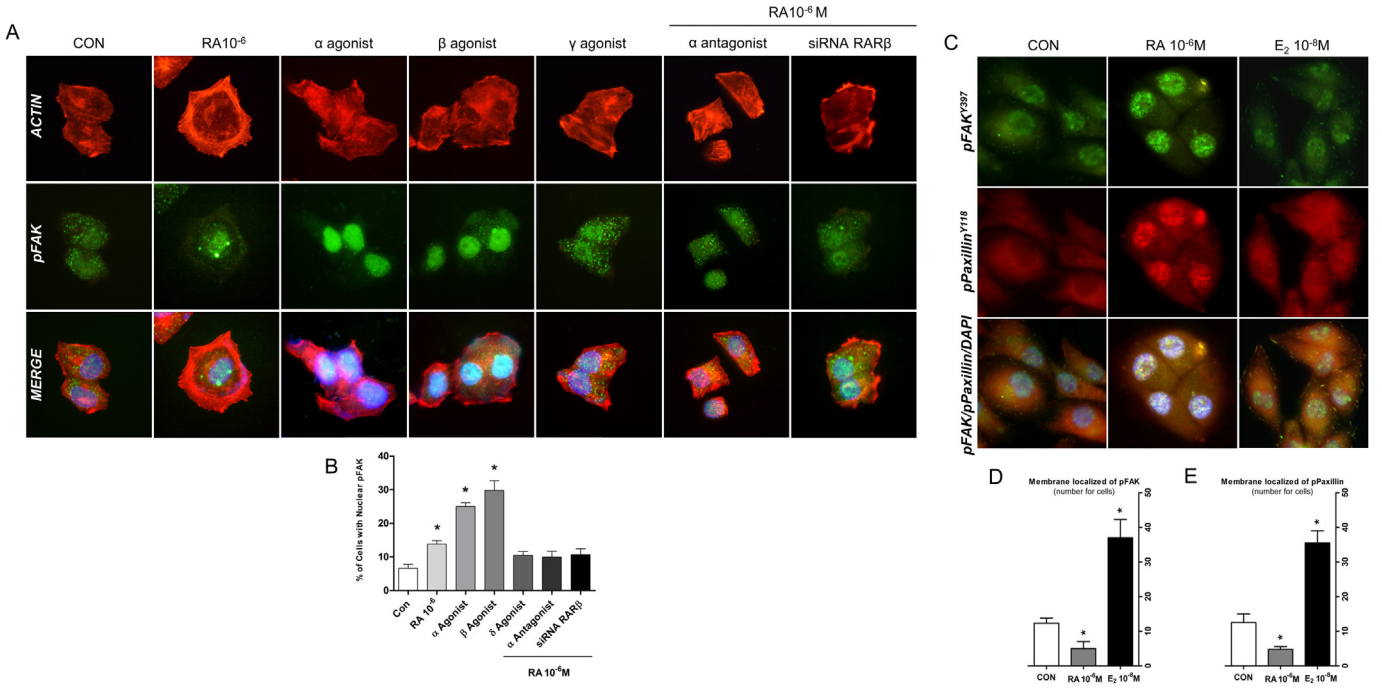

Fig. 5. RA induces a pFAK nuclear localization via RARα and RARβ A–B) T-47D cells were treated with or without RA (10^{-6} M, 20 min) and the synthetic retinoid agonists selective for RARα (BMS753), RARβ (BMS453), and RARγ (BMS961); the synthetic retinoid antagonist for RARα (BMS195614); and with a specific siRNA RARβ. Immunofluorescence assay was performed thereafter. The cells were stained with p-FAK Tyr^{397} linked to Dylight 488 (green); actin was stained with Texas Red Phalloidin (red); and nuclei were counterstained with DAPI (blue). All experiments were repeated three times with consistent results. Representative images and the % of cells with nuclear pFAK are shown. **C)** T-47D cells were treated with or without RA (10^{-6} M, 20 min), and estradiol (E2, 10^{-8} M) a strong inducer of focal adhesion complex formation in T-47D cells (positive control). The cells were stained with p-FAK Tyr^{397} linked to Dylight 488 (green), and p-Paxillin Tyr^{118} linked to Dylight 594 (red) and nuclei were counterstained with DAPI (blue). Representative images are shown. The microphotographs were taken with a 100× objective. **D–E)** Quantification of the membrane-localized pFAK and pPaxillin are shown. Results are expressed as number of cells vs. control cells (mean ± SD). Membrane-localized pFAK/pPaxillin were counted in 40 different cells. All experiments were performed in triplicate and representative images are shown. * = $p < 0.05$ 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.)

Several studies have reported that RA may inhibit invasion and metastasis in diverse types of cancer, such as in breast (Liu et al., 2003) and colon cancer cells (Adachi et al., 2001). The mechanism by which RA blocks the late stages of carcinogenesis is, however, largely unknown. Our laboratory and others have previously demonstrated that RA and other biologically active retinoids inhibit cellular migration in several cell lines, such as human colon carcinoma cells (Woo and Jang, 2012) and human breast cancer MCF7, T-47D, and MDA-MB-231 cells (Dutta et al., 2009, 2010, Flamini et al., 2014).

Alterations in the adhesion and motility properties of neoplastic cells may play a pivotal role in the development and progression of the malignant phenotype in various tumor types. Invasion is a multiphase process that consists of different coordinated interdependent steps which are controlled by cross-talk mechanisms between cells and the extracellular microenvironment (Alessandro and Kohn, 2002).

Cell movement is a complex and highly integrated process triggered by multiple signaling molecules and implemented by actin reorganization (Yamazaki et al., 2005). Rearrangement of actin fibers is critical to the formation of membrane protrusions at the leading edge. Cell adhesion to the ECM further requires the development of integrin-dependent adhesions to large extracellular proteins, which then mature into FA by recruiting proteins such as the integrin-binding proteins Paxillin, Vinculin, talin, and p130CAS, along with the tyrosine kinases c-Src and FAK (Yamazaki et al., 2005). Tyr^{397}-phosphorylation of FAK is also critical in this process (Parsons, 2003).

The major finding of this study is the identification of a novel rapid signaling avenue of RA to Moesin/FAK/Paxillin in a time-dependent manner, which may reduce the ability of breast cancer cells to adhere and invade the surrounding environment. Here, we have found that RA rapidly activates Moesin, FAK, and Paxillin. A similar study, which used a different breast cancer cell line, demonstrated the ability of high doses of RA to phosphorylate FAK and Paxillin. However in this study FAK phosphorylation led to an increase in cell adhesion contrarily to the decrease observed here. It is important to mention that in this study the cell adhesion assay was performed with different conditions since the cells were treated with RA for long periods while we used it only minutes, and then the attachment was allowed to occur in a culture clusters precoated with fibronectin over a period of 1–5 h (Zhu et al., 1999). In any case FAK regulates cell migration via modulation of focal adhesion turnover (Owen et al., 1999). The cellular migration process required the continuous cellular attach/de-attachment to the substrate. The role of FAK in the generation of adhesive forces remains poorly understood as cell migration is a multistep, dynamic process that exhibits complex dependencies on adhesion/de-adhesion strength and focal adhesion and cytoskeletal dynamics (Gupton and Waterman-Storer, 2006).

FAK has been established as a central controller of cell migration, particularly during tumor metastasis. Numerous studies evidence that FAK could be an important player in signaling cascades associated with cancer progression and metastasis (van Nimwegen and van de Water, 2007,Hao et al., 2009). Overall, these findings highlight the relevance of the activity of FAK for cancer progression. The identification of FAK regulation by RA may thus offer important mechanistic insights that allow a better understanding of the role of

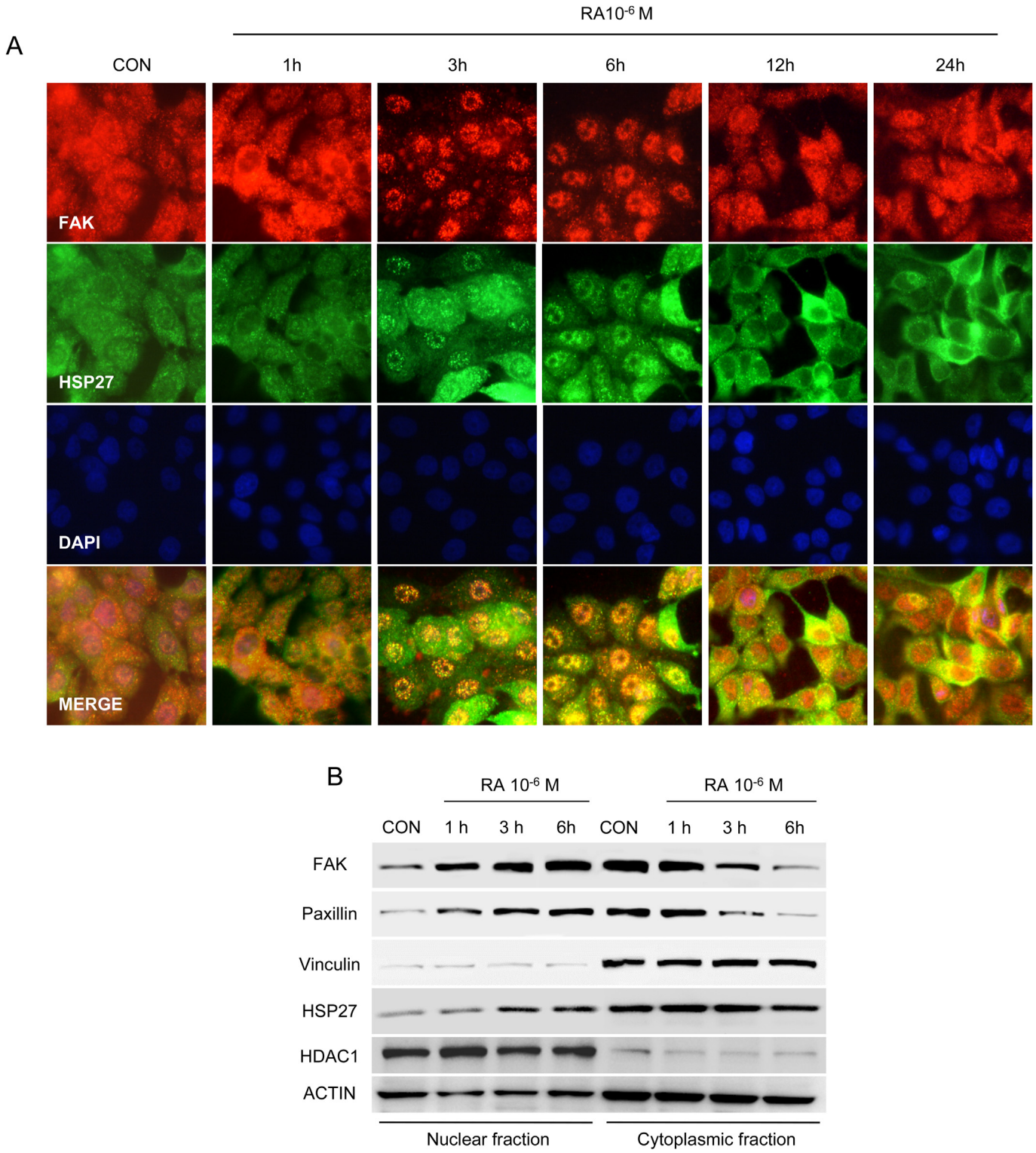

Fig. 6. RA induces a FAK, Paxillin and HSP27 nuclear localization. A) T-47D cells were treated with or without RA 10^{-6} M for 1, 3, 6, 12, or 24 h. Immunofluorescence assay was performed thereafter. The cells were stained with HSP27 linked to Dylight 488 (green); and FAK linked to DyLight 594 (red). Nuclei were counterstained with DAPI (blue). All experiments were repeated three times with consistent results. Representative images with nuclear pFAK are shown. The microphotographs were taken with a 100× objective. **B)** T-47D cells were treated with or without RA 10^{-6} M for 1, 3, 6 h. Cells fractionation experiments and Western blot analyses for FAK, Paxillin, Vinculin and HSP27 proteins were performed. As loading standard for cytoplasmic proteins we used ACTIN and for nuclear proteins HDAC1. Data are representative of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

this retinoid in breast cancer metastasis.

Interestingly, phosphorylation of Moesin/FAK/Paxillin induced by RA is achieved via extranuclear signaling cascades. Such mechanisms of actions should be enacted through a variety of

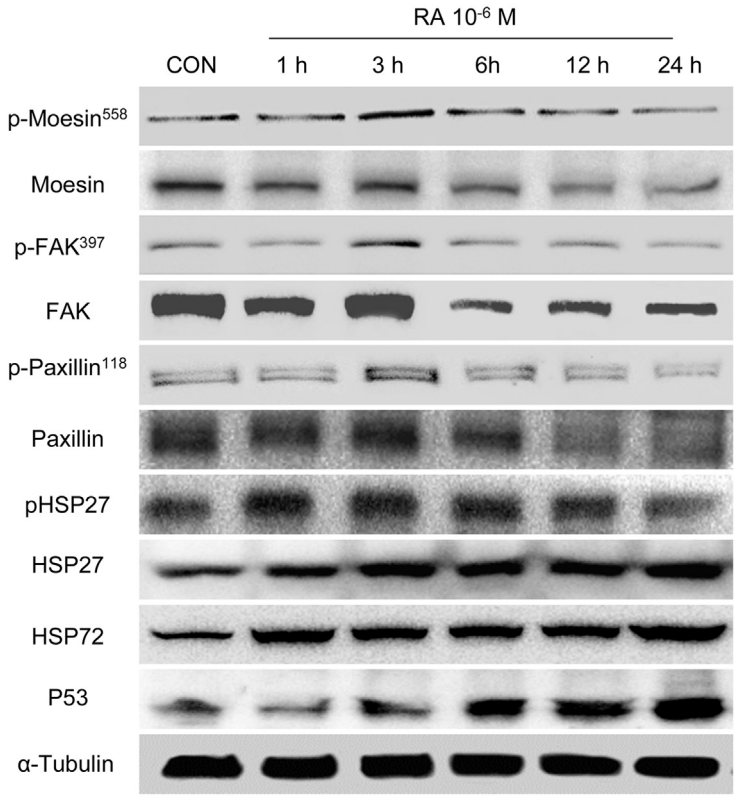

Fig. 7. RA induces HSP27, HSP72, and p53 expression. A) T-47D cells were treated with or without RA 10^{-6} M for 1, 3, 6, 12, or 24 h. Western blot analyses for pMoesin, Moesin, pFAK, FAK, pPaxillin, Paxillin, pHSP27, HSP27, HSP72, and p53 proteins were performed. α-Tubulin expression is shown in the lower boxes as loading control. These experiments were performed in triplicate. Representative images are shown.

protein–protein interactions of RAR with kinases and other signaling intermediates that take place within the cell or at the cell membrane. Although it lies beyond the scope of this manuscript to define the exact intracellular site where the signaling process of RAR begins, it is possible that a membrane-initiated process might be activated in this setting. Indeed, the existence of membrane-localized RARs has recently been reported in various mammalian epithelial and fibroblastic cells (Piskunov and Rochette-Egly, 2012). Moreover, membrane-associated RARα forms complexes with Gαq to generate rapid p38 MAPK activation in response to RA. Hence, this RAR is required for the activation of the rapid signaling pathway that occurs in response to RA (Piskunov and Rochette-Egly, 2012).

We also identified that c-Src and PI3K are involved in RA signaling, and we showed that this step is required for Moesin/FAK/Paxillin activation. This is consistent with previous reports that showed that the administration of RA resulted in activation of PI3K signaling through a mechanism involving a stable complex of RARα and p85α (the regulatory subunit of PI3K) (Donini et al., 2012,Lopez-Carballo et al., 2002,Masia et al., 2007). Moreover, we confirmed that RARα and RARβ are the most important receptors implicated in this pathway.

We have previously demonstrated that 72 h of RA incubation led to a static phenotype in MCF-7 and T-47D cells, with actin fibers being longitudinally arranged through the major axis of the cell surface. A statistically significant reduction in the number of cells with a migratory phenotype was observed (Flamini et al., 2014). Here we found by immunofluorescence staining that RA (10^{-6}M/20 min) induces a rapid activation of FAK. The latter did not translocate to the plasmatic membrane, which is what usually occurs. In general, when FAK is phosphorylated and consequently activated, it concentrates at sites of ongoing actin remodeling in the cell plasma membrane, thus increasing membrane thickness. Phosphorylated FAK therefore co-localizes with other actin-binding proteins, such as pPaxillin, to form FA. Surprisingly, treatment with RA seemed to change pFAK/pPaxillin spatial organization, which was reflected in a translocation of pFAK/pPaxillin towards the nucleus. There was a twofold increase of nuclear FAK under RA treatment, and a threefold increase when cells were treated with RARβ-selective agonists.

Lim and co-workers have proposed a working model for FAK shuttling between the cytosol and the nucleus. They described that under normal growth conditions, FAK is prominently localized in focal adhesions and the cytosol. However, cell de-adhesion from the substrate or stress signals such as a chemical or oxidative stress promote FAK mobilization from integrin adhesion sites to the nucleus via the FERM nuclear localization sequence. In the nucleus, FAK interacts with tumor suppressor p53, resulting in p53 turnover to enhance cell survival (Lim, 2013). This could be just our case, as 50% of the cells were detached after they were treated with RA 10^{-6} M for 20 min. Despite reports of FAK in the nucleus, the mechanism(s) that promote or regulate FAK nuclear accumulation and the biological role of nuclear FAK remain unknown. We found that time-dependent RA 10^{-6} M treatment for 1–24 h induces the expression of HSP27 and HSP72, which are markers of cellular stress. In addition, we demonstrated a translocation of FAK/Paxillin and HSP27 to the nucleus during 3–6 h of RA treatment. As a consequence, we speculate that RA treatment induces a cellular stress mechanism by which pFAK/pPaxillin detaches from FA to translocates toward the nucleus, which in turn inhibits migration of the cells. FAK-Paxillin interactions have being proved to be crucial for regulating adhesion, migration and invasion processes

(Deramaudt et al., 2014). Moreover, several reports have shown that HSP27 and HSP72 can translocate to the nucleus and accumulate there under stressful conditions, and that they are probably involved in either protection or recovery of DNA damage (Arrigo et al., 1988, McClaren and Isseroff, 1994,Sottile et al., 2015).

Nuclear FAK accumulation was associated with a loss of FAK at focal contacts, which supports the idea that there is a cytoplasmic pool of FAK that is available to shuttle in and out of the nucleus. Lim and colleagues proposed a model whereby, under conditions of cellular stress or reduced integrin signaling, the cytoplasmic pool of FAK is elevated, leading to increased FAK nuclear accumulation, which acts to enhance cell survival by facilitating p53 turnover (Lim et al., 2008). In fact, in our experiments we observed an increase in p53 expression from 6 h and until 24 h of treatment.

In addition to FAK other FA proteins, such as zyxin and Paxillin, also translocate to the nucleus (Lim, 2013). As none of these proteins directly bind to DNA, they may function as co-receptors for certain transcriptional factors in the nucleus (Wang and Gilmore, 2003). Although FAK shuttles from the cytoplasm to the nucleus, a few physiological stimuli have been documented. Overall, nuclear FAK could control various transcription factors, resulting in alteration of gene regulation in a kinase-independent manner.

It is therefore clear that non-genomic and genomic pathways cooperate to modify different cellular processes. In fact, in our previous work we have shown that long RA treatment (10^{-6}M/72 h s) reduces Moesin, Src, and FAK expression, which mediate the inhibition of cell motility in breast cancer cells. However, we are far from understanding how the non-genomic and genomic effects of RA coordinate gene expression and development. Increasing evidence suggests an intimate cross-talk between the “non-genomic” and “genomic” effects, which is just beginning to be understood.

5. Conclusion

We have shown that rapid treatment with RA reduces breast cancer cell adhesion by inhibiting the formation of FA complexes. RARα and RARβ activate c-Src/PI3K in the presence of RA, leading to Moesin/FAK/Paxillin activation. Once phosphorylated, FAK/Paxillin translocate to the nucleus and causes detachment of the cell and induction of cellular stress proteins. Our results provide original mechanistic insights into the effects of RA on breast cancer progression. They may be helpful to develop new drugs against endocrine-sensitive breast cancers. It is key to elucidate the molecular pathways activated by retinoids to develop rational retinoid-based therapy for breast cancer.

Conflict of interest statement

The authors declare that they have no competing interests.

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.2016.04.021.

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