

# Involvement of Protein Kinase C $\alpha$ and $\delta$ Activities on the Induction of the Retinoic Acid System in Mammary Cancer Cells

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It has been established that retinoids exert some of their effects on cell differentiation and malignant phenotype reversion through the interaction with different members of the protein kinase C (PKC) family. Till nowadays the nature and extension of this interaction is not well understood. Due to the cytostatic and differentiating effects of retinoids, in the present study we propose to evaluate whether the crosstalk between the retinoid system and the PKC pathway could become a possible target for breast cancer treatment. We could determine that ATRA (all-trans retinoic) treatment showed a significant growth inhibition due to (G1 or G2) cell cycle arrest both in LM3 and SKBR3, a murine and human mammary cell line respectively. ATRA also induced a remarkable increase in PKC $\alpha$  and PKC $\delta$  expression and activity. Interestingly, the pharmacological inhibition of these two PKC isoforms prevented the activation of retinoic acid receptors (RARs) by ATRA, indicating that both PKC isoforms are required for RARs activation. Moreover, PKC $\delta$  inhibition also impaired ATRA-induced RAR $\alpha$  translocation to the nucleus. In vivo assays revealed that a combined treatment using ATRA and PKC $\alpha$  inhibitors prevented lung metastatic dissemination in an additive way. Our results clearly indicate that ATRA modulates the expression and activity of different PKCs. Besides inducing cell arrest, the activity of both PKC is necessary for the induction of the retinoic acid system. The combined ATRA and PKC $\alpha$  inhibitors could be an option for the hormone-independent breast cancer treatment. © 2014 Wiley Periodicals, Inc.

Key words: mammary cancer; retinoic acid receptors; PKC

## INTRODUCTION

Breast cancer is the leading cause of cancer death in women and it is estimated that in 2012 near 250,000 women have been diagnosed with breast cancer and 39,000 have died [1,2].

Retinoic acid (RA) is a potent cell differentiator and growth arrest inducer, both in vitro and in vivo [3]. Modulation of cell growth by retinoids is thought to result from direct and indirect effects on gene expression. These effects are mediated by the nuclear retinoic acid receptors (RARs)  $\alpha$ ,  $\beta$ , and  $\gamma$  and retinoid X receptor (RXR)  $\alpha$ ,  $\beta$ , and  $\gamma$ , which are ligand-activated transcription factors [4–6]. RA binds to their receptors and induces the formation of RAR/RXR heterodimers, which associate with specific DNA-binding sequences present in the promoters of RA-responsive genes called retinoic acid responsive elements (RAREs) [4–6]. In addition to RAR/RXR complexes formation, RA induces a variety of effects involved in the induction of cell arrest in target cells including MAPKs members modulation and activation of the AP-1 protein [7,8] via a cAMP-responsive element-binding protein (CBP)-regulated mechanism [9,10].

It has been postulated that some retinoids exert their effects on cell differentiation and reversion of

the malignant phenotype through the interaction with  $\alpha$  and  $\delta$  isoforms of protein kinase C (PKC) [11,12].

PKC is a family of lipid-dependent serine/threonine kinases that play central roles in signal transduction pathways controlling proliferation, apoptosis, and malignant transformation [13,14]. On the basis of their structural similarities and biochemical

Abbreviations: RA, retinoic acid; RARs, retinoic acid receptors; RXR, retinoic X receptor; RAREs, retinoic acid responsive elements; MAPK, mitogen activated protein kinase; PKC, protein kinase C; DAG, diacylglycerol; Erk, extracellular signal-regulated kinase; ATRA, all trans retinoic acid; FBS, fetal bovine serum; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate buffered saline.

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properties, PKC isoforms have been grouped into three families [15,16]: classical ( $\alpha$ ,  $\beta$ I,  $\beta$ II, and  $\gamma$ ), which are activated by calcium and diacylglycerol (DAG); novel ( $\delta$ ,  $\epsilon$ ,  $\eta$ , and  $\theta$ ), which require DAG but are calcium-insensitive; and atypical ( $\zeta$  and  $\lambda/\iota$ ), which are not responsive to either DAG or calcium [13,17].

PKC $\alpha$  has long been recognized to have a role in regulating different aspects of tumor growth and progression [18]. Several groups demonstrated that PKC $\alpha$  is overexpressed in human breast cancer cell lines and in breast tumor samples [19,20]. Moreover, the human breast cancer cell line T47-D, which does not express detectable levels of PKC $\alpha$ , has a lower proliferative potential when compared with other PKC $\alpha$ -positive cell lines such as MCF-7, MDA-MB231, or MDA-MB468 [21]. In addition, the finding that a small molecule PKC $\alpha$  inhibitor markedly inhibited SKBR-3 cell line proliferation due to arrest in the G1 phase [22], further supports this proliferative role for PKC $\alpha$ .

The role of PKC $\delta$  in breast cancer remains ambiguous and little information is available regarding the expression levels of PKC $\delta$  in primary tumors. It has been described that PKC $\delta$  can promote proliferation [14] and metastasis development [23], and that its depletion is sufficient to drive murine mammary cancer cells into apoptosis [24]. On the other hand, several studies showed that PKC $\delta$  mediates antiproliferative responses. For example, PKC $\delta$  mediates the antimitogenic effect of inositol hexaphosphate in MCF-7 human breast cancer cells, which involves inhibition of Erk and Akt as well as pRb hypophosphorylation [25].

As mentioned above, it has been postulated that some retinoids can revert the malignant phenotype through their interaction with  $\alpha$  and  $\delta$  isoforms of protein kinase C. These findings led us to evaluate whether the crosstalk between the retinoid system and the PKC pathway could become a novel target for the treatment of breast cancer. We could demonstrate ATRA (all-trans retinoic) treatment showed a significant growth inhibition and an increase in PKC $\alpha$  and PKC $\delta$  expression both in LM3 and SKBR3, a murine and human mammary cell line respectively. Furthermore, both PKC isoforms were necessary for RARs activation. PKC $\delta$  inhibition also impaired ATRA-induced RAR $\alpha$  translocation to the nucleus. In vivo assays revealed that a combined treatment using retinoids and PKC $\alpha$  inhibitors induced an additive effect in the reduction of lung metastatic dissemination.

Altogether our results contribute to the elucidation of PKC-retinoids crosstalk and may allow in the future the rationale design of novel therapeutic agents that include a combination of retinoids and PKC inhibitors.

## MATERIALS AND METHODS

### Reagents and Antibodies

Medium for cell culture and agarose were obtained from Life Technologies Inc. (Rockville, MD). Fetal bovine serum (FBS) was from GEN (Buenos Aires, Argentina). Acrylamide and ATRA were from Sigma (St. Louis, MO). All other reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad (Richmond, CA). Monoclonal anti-PKC $\alpha$  and anti-PKC $\delta$  antibodies were purchased from BD Biosciences (San Diego, CA). Monoclonal antibodies for Erk and phospho-Erk (pErk), and polyclonal antibodies for E-cadherin, RAR $\alpha$ , actin, cyclin D1, and p27 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies for Akt and phospho-Akt (pAkt, Ser 473) were purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase conjugated anti-rabbit or anti-mouse antibodies and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma. Hybond-P membranes for blotting and chemiluminescence reagents (ECL) were from Amersham (Aylesbury, UK). The PKC $\delta$  inhibitor Rottlerin and the PKC $\alpha$  inhibitor Gö6976 were obtained from Calbiochem (Billerica, MA).

### Cell Lines and Culture Conditions

We used the hormone-independent Her2 positive LM3 cell line, derived from a murine mammary adenocarcinoma, with tumorigenic and metastatic capacity in BALB/c mice [26,27], and the human mammary adenocarcinoma derived cell lines MDA-MB231 and SKBR3 cell lines [28]. LM3 cells were grown in minimum essential medium (MEM) supplemented with 10% FBS and 80  $\mu$ g/ml gentamycin. SKBR3 and MDA-MB231 cells were grown in DMEM-F12 with the same supplement. All cell lines were cultured at 37°C in a humidified air atmosphere with 5% CO<sub>2</sub>.

### Proliferation Assays

Proliferative potential was determined by assessing LM3, SKBR3 and MDA-MB231 cell number during the exponential growth phase of unsynchronized monolayer cultures. Briefly,  $4 \times 10^5$  cells were seeded onto 35 mm Petri dishes and treated with ATRA (1  $\mu$ M) and/or Rottlerin (1  $\mu$ M) or Gö6976 (5  $\mu$ M) once a day during 72 h ( $n = 3$ ) in culture media supplemented with 10% FBS and 80  $\mu$ g/ml gentamicin. At different times after seeding, cells from triplicate wells were washed twice with PBS, trypsinized, and counted using a hemocytometer and trypan blue exclusion.

### Analysis of Cell Cycle Distribution by Flow Cytometry

Cells monolayers were treated with ATRA (1  $\mu$ M), Gö6976 (5  $\mu$ M), their combination or vehicle alone for 72 h. Cells were detached and fixed with 70% ice-cold ethanol. After staining with propidium iodide

(100 µg/ml), samples were examined for DNA content by flow cytometry using an Epics Elite ESP coulter cytometer (Beckman coulter, Fullerton, CA).

#### Nucleus/Cytoplasm Separation

Nuclear and cytoplasmic fractions were separated using NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce Biotechnology, Rockford, IL). Briefly, subconfluent monolayers growing in 100 mm Petri dishes were treated or not for different times with ATRA (1 µM) and/or Rottlerin (1 µM) or Gö6976 (5 µM) and were trypsinized and centrifuged (500g, 5 min). The supernatant was discarded and the pellet was resuspended in the cytoplasmic extraction reagent I buffer (CERI). Then CERII buffer were added and the mixture was centrifuged (13,000g, 5 min). Supernatant corresponds to the cytoplasmic protein fraction, and the remaining pellet was resuspended in nuclear extraction reagent buffer (NER) in order to obtain the nuclear protein fraction. Protein content in each fraction was determined, and samples were aliquoted and stored at -80°C, and used only once after thawing.

#### Subcellular Fractionation

Subconfluent monolayers growing in 100 mm Petri dishes were treated or not for different times with ATRA (1 µM) and PMA (50 nM) and were washed twice with ice-cold PBS and then trypsinized and centrifuged (720g, 5 min). The supernatant was discarded and the pellet was resuspended in the subcellular fractionation buffer (250 mM Sucrose, 20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA and 1 mM EGTA) and the mixture was centrifuged (13,000g, 5 min). The pellet was discarded and the supernatant was centrifuged again at 10,000g. The new pellet was discarded once more and the supernatant was centrifuged again at 100,000g. The cytosolic (soluble) fraction was obtained by collecting the supernatant. The remaining pellet represents the membrane fraction.

#### Western Blot

Subconfluent monolayers were pretreated for different times with ATRA (1 µM) and/or Rottlerin (1 µM), Gö6976 (5 µM) or PMA (50 nM) and were washed twice with ice-cold PBS and then lysed with 1% Triton X-100 in PBS by scraping with a Teflon scraper. After protein determination, samples were denatured by boiling in sample buffer with 5% β-mercaptoethanol and run in 10% SDS-PAGE. Fifty microgram of protein from total lysates or 20 µg from nuclear fractions were loaded into each lane. Gels were blotted to Hybond-P membranes. After incubation for 1 h in blocking buffer containing 5% skim milk with 0.1% Tween-20 in PBS, membranes were incubated with the first antibody overnight at 4°C, and then for 1 h with a secondary antibody coupled to horseradish peroxidase. Detection was performed by

chemoluminescence. Bands were digitalized with a Photo/Analyst Express System (Fotodyne, Inc., Hartland, WI) and signal intensity was quantified with Gel-Pro Analyzer software. When both the phosphorylated and total forms of a protein were studied, the same membrane was blotted initially with the antibody against the phosphorylated form, subsequently stripped, and finally probed with the antibody against the total protein.

#### RT-PCR

Subconfluent cultures of each cell line were treated or not for 1 h with PMA (50 nM). RNA from cells was prepared using the Gentra Purescript RNA isolation kit (Qiagen, Valencia, CA). cDNA was prepared with the iScript cDNA synthesis kit (Bio Rad). PCR products were obtained by use of the primers h/m RARα1, h/m RARα2, mRARβ1/β3, mRARβ2, mRARγ1, and mRARγ2 from Zelent [29] h/m RARβ2 primer was designed at Dr. Farias' laboratory (RARβ2-S 5' ATGGA-GTTCGTGGACTTTTCTGTG 3': RARβ2-AS 5' CTCG-CAGGCACTGACGCCAT 3').

#### RARE-Dependent Gene Reporter Assay

LM3 and SKBR3 cells were transiently co-transfected with RARE-luciferase plasmid and *Renilla*-Luciferase Control Reporter Vector (pRL-CMV; Promega, Madison, WI) in a 10:1 ratio, using Fugene (Roche, Indianapolis, IN) and following standard protocols. Briefly: 6 × 10<sup>5</sup> cells were transfected in suspension and then seeded onto individual wells of 24-well plates. Cells were then treated with different combinations of ATRA and PKC inhibitors for 24 h as described above. Luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega) and normalized to the constitutive *Renilla* luciferase activity.

#### Immunoprecipitation

LM3 monolayers were treated with ATRA for 24 h and then scraped in lysis buffer (1% NP-40, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Sodium Deoxycholate and 1 mM EDTA and protease inhibitors (Roche) and incubated during 30 min on ice. The cell lysates were spun at 14,000g for 15 min at 4°C to pellet insoluble material. Ten microliter of anti-PKCδ or an unspecific IgG antibody were added to the lysate and allowed to incubate overnight at 4°C. Fifty microliter of A/G-agarose beads (Santa Cruz Biotechnology, Inc.) were added and incubated with gentle rocking for 1 h at 4°C. Beads were washed four times with lysis buffer, and centrifuged to remove the lysis buffer. The pellet was stored at -20°C until use.

#### Animal Studies

For the in vivo experiments, randomized inbred female BALB/c mice, 2-4 months old, obtained from the Animal Care Division of the Institute of Oncology "Angel H. Roffo" were employed. Mice were housed

5 per cage, kept under an automatic 12 h light/12 h darkness schedule, and provided with sterile pellets and tap water ad libitum. All animal studies were conducted in accordance with the standards of animal care as outlined in the NIH Guide for the Care and Use of Laboratory Animals and the Committee for the Use and Care of Laboratory Animals (CICUAL) from the School of Medicine of the University of Buenos Aires.

#### Orthotopic Tumor Growth and Spontaneous Metastatic Ability

Mice were inoculated orthotopically into the fat pad of the 4th mammary gland with  $2 \times 10^5$  LM3 cells. When tumors became palpable approximately 7 days post-inoculation, mice received a subcutaneous silastic pellet containing ATRA (10 mg) or an empty pellet as control.

Mice were monitored daily. Twice a week tumor diameters were measured with a sliding caliper and tumor volume was calculated ( $D \times d^2/2$ ) for assessment of growth rate. Forty-five days post-inoculation of tumor cells, mice were sacrificed and necropsied. To investigate the presence of spontaneous metastases, lungs were removed and the number of surface lung nodules was recorded.

#### Experimental Lung Metastasis Assay

The experimental lung metastasizing ability was performed essentially as described previously [30]. Briefly prior to cell inoculation, mice were subcutaneously implanted with a silastic pellet containing or not ATRA (10 mg). Then  $2 \times 10^5$  LM3 cells, pretreated or not only with Gö6976 (1  $\mu$ M) for 24 h, were injected into the tail vein of syngeneic mice using a 27-gauge needle. Cell viability was higher than 95% as determined by trypan blue exclusion test, and the order of injection of different groups was randomized to eliminate any difference that may bias the outcome. Mice were monitored daily and sacrificed 21 days later. Lungs were removed and the number of superficial lung nodules were recorded as mentioned above.

#### Statistical Analysis

All assays were performed in triplicate, and independent experiments repeated at least twice. Statistical differences between groups were calculated by applying Anova, Student's *t* or Kruskal–Wallis tests, as indicated. A value of  $P < 0.05$  was considered to be significant.

## RESULTS

#### Retinoids Induce Cell Growth Inhibition Through Cell Cycle Arrest and Erk/MAPK Downmodulation

In order to investigate whether ATRA treatment is able to modulate the proliferative potential of mammary tumor-derived cell lines, LM3, SKBR3,

and MDA-MB231 were treated during different times with ATRA. The analysis of cell proliferation revealed that, upon 72 h treatment, ATRA induced a marked decrease in LM3 and SKBR3 cell number as compared to control untreated cells, while no effect was observed in MDA-MB231 cells under the same culture conditions (Fig. 1a). Three days of ATRA treatment increased the number of LM3 cells in G0/G1 and subG1 phases along with a decreased number of cells in S phase as compared to control cells. Regarding the human SKBR3 cells, treatment enhanced the number of cells in G2 and subG1 phases (Fig. 1b).

To analyze some of the mechanisms involved in ATRA-induced growth inhibition we evaluated different molecules involved in proliferation, survival, and cell cycle regulation. An important reduction in phosphorylated (active) p42 Erk MAPK, a crucial component of the mitogenic signaling pathway, together with an increase in phosphorylated Akt levels could be detected after 15 min of ATRA treatment in LM3 cells. No modulation in these signaling pathways could be detected in the ATRA unresponsive MDA-MB231 cell line (Fig. 1c). Moreover, ATRA treatment induced increased expression of the cyclin-dependent kinase inhibitor p27 in the nucleus of LM3 cells without affecting Cyclin D1 levels. None of these effects could be detected in MDA-MB231 treated cells (Fig. 1d).

#### PKC Activation Modulates RARs Expression

We could determine that the studied mammary cell lines showed a different expression profile of RAR isotypes by RT-PCR. The murine mammary cell line LM3 expressed RAR $\alpha$ 1 and RAR $\beta$ 1/ $\beta$ 3 (Fig. 2) but it did not express RAR $\beta$ 2, one of the main retinoid receptors (data not shown). On the other side, the human mammary cell line MDA-MB231 did not express RAR $\beta$  (Fig. 2). Both mammary cell lines expressed RAR $\gamma$ 2. Upon PKC activation by PMA treatment, LM3 cells responded by reducing RAR $\alpha$ 1 and RAR $\gamma$ 2 expression levels while MDA-MB231 cells responded by increasing RAR $\gamma$ 2 expression without changes in RAR $\alpha$ 1 (Fig. 2).

#### Retinoids Modulate PKC $\alpha$ and PKC $\delta$ Expression and Subcellular Localization

As we determined that PKC activation modulated RARs expression, we next studied whether ATRA treatment is able to alter the expression of different PKC isoforms. Upon 6 h treatment with ATRA an important increase in PKC $\alpha$  and PKC $\delta$  expression levels could be detected in LM3 and SKBR3 cell lines. On the contrary ATRA reduced the expression levels of both PKCs in MDA-MB231 cells (Fig. 3a).

It has been reported that the translocation of PKC isoforms from cytosol to membrane is an important indicator of the activation of these kinases [31]. Based on this group finding we evaluate whether ATRA is

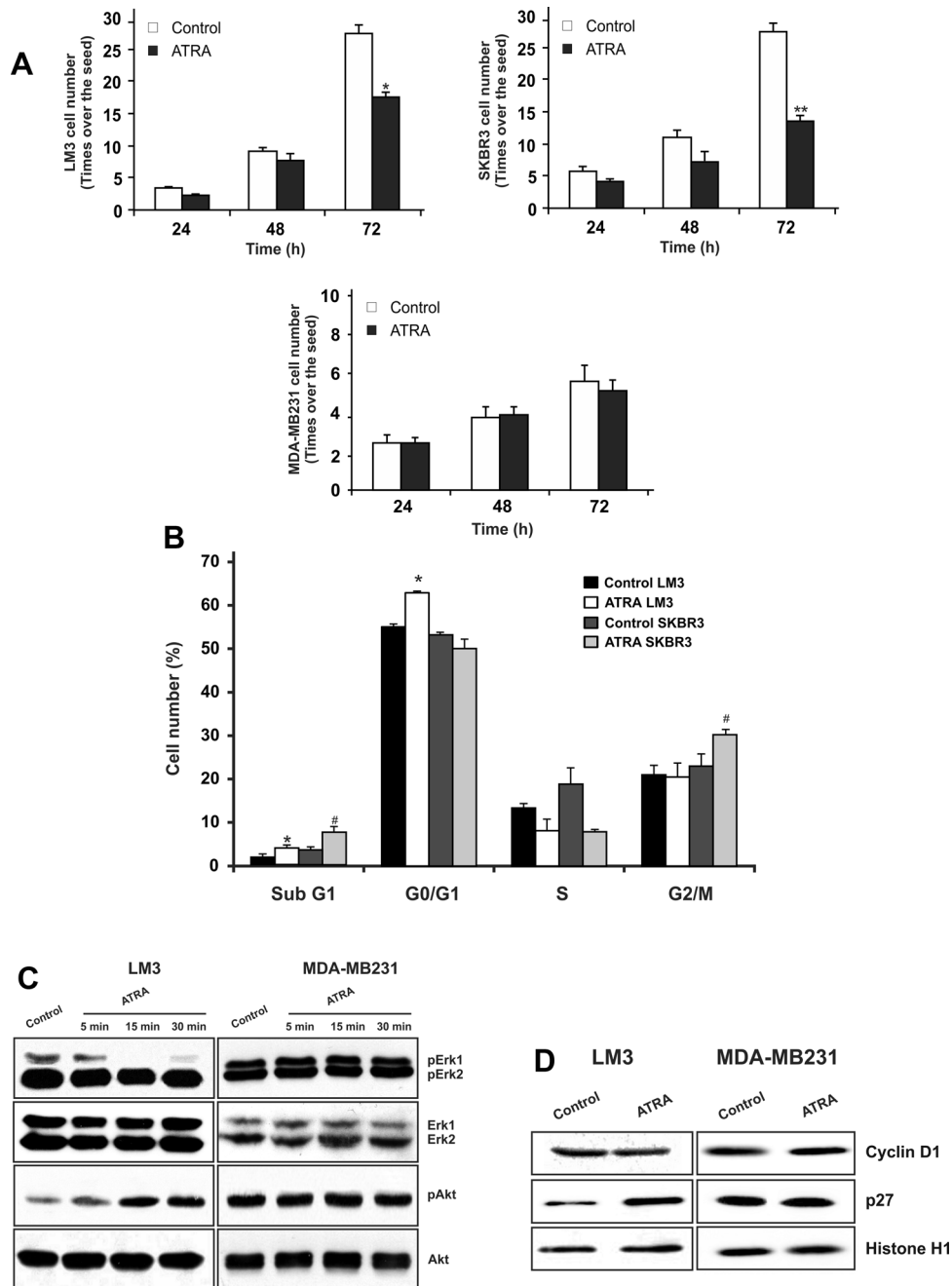


Figure 1. Effect of ATRA on in vitro cell proliferation. Panel a: LM3, SKBR3 and MDA-MB231 cell number was assessed at different times after the treatment with ATRA (1  $\mu$ M). Each data point represents the mean  $\pm$  SD of triplicate determinations, \* $P$  < 0.05 versus control (ANOVA). At least three independent experiments were performed with similar results. Panel b: LM3 and SKBR3 cell cycle analysis by flow cytometry after ATRA treatment (1  $\mu$ M). Each data point represents the mean  $\pm$  SD of triplicate determinations, \* $P$  < 0.05 versus control (ANOVA). At least three independent experiments were performed

with similar results. Panel c: Cell lysates prepared from LM3, and MDA-MB231 monolayers treated or not with ATRA for different times were subjected to Western blot analysis. Results are representative of three independent experiments. Panel d: Nuclear protein fractions of LM3 and MDA-MB231 cell monolayers treated or not with ATRA for 72 h were used in order to study the expression of Cyclin D1 and p27 by Western blot. Histone H1 expression levels was used as loading control. Results are representative of three independent experiments.

also involved in PKC activation using LM3 cells as model.

As expected, PKC activation by PMA induced the translocation of both PKC isoforms from cytosol to the membrane fraction within 2 h. ATRA induced a

similar effect for PKC $\alpha$ , but no evidences of PKC $\delta$  in the membrane fraction could be detected even after 6 h treatment (Fig. 3b). However, a more detailed subcellular localization assay revealed that ATRA induced PKC $\delta$  translocation to the cell nucleus,

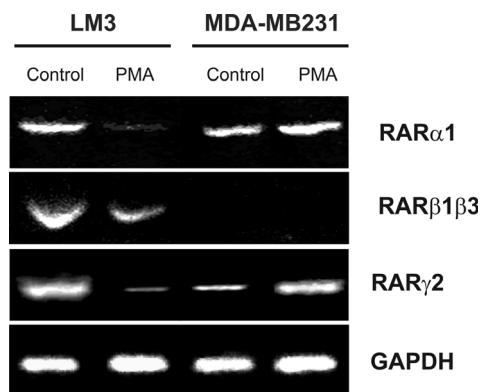


Figure 2. Modulation of RAR isotypes expression by PKC activation. RNA from LM3 and MDA-MB231 cells treated or not with PMA was isolated and expression of different RAR isotypes was analyzed by RT-PCR. Results are representative of three independent experiments.

phenomenon that could be detected after 24 h treatment (Fig. 3c). PKC $\alpha$  was not detected in the nuclear fraction (data not shown).

#### PKC $\alpha$ and PKC $\delta$ Activity Is Necessary to Modulate Retinoid Receptors Activation and Nuclear Translocation

To further analyze the crosstalk between the retinoid system and the PKC signaling pathway, we studied whether PKC $\alpha$  and  $\delta$  affected RAR transcriptional activity using a RARE-Luc reporter assay. For this, LM3 and SKBR3 cells received different treatments during 24 h, combining ATRA and PKC pharmacological inhibitors. We could determine that the pharmacological inhibition of both PKC $\alpha$  and  $\delta$  impaired the increase of luciferase activity induced by ATRA (Fig. 4a). In addition, RAR downstream targets such as E-cadherin and p27 were affected in the same way by both PKC inhibitors (Fig. 4b).

Next we analyzed whether the inhibition of PKC $\alpha$  or  $\delta$  activity altered the subcellular localization of the constitutive RAR $\alpha$ 1 receptor. Nuclear and cytoplasmic proteins were obtained after treating LM3 cells with ATRA and/or PKC inhibitors as mentioned above. Western blot assays showed that the pharmacological inhibition of PKC $\delta$  induced a significant reduction in cytoplasmic RAR $\alpha$ 1 while it completely abolished the nuclear translocation of this receptor (Fig. 4c). As expected, PKC $\delta$  expression, and nuclear translocation were also altered by Rottlerin treatment. On the other side, no changes in RAR $\alpha$  expression or localization were found following PKC $\alpha$  activity inhibition (Fig. 4c). Finally, we showed that PKC $\delta$  and RAR $\alpha$  interact in LM3 cells using an immunoprecipitation approach (Fig. 4d).

#### ATRA Induced Growth Impairment is Mediated by PKC

In order to determine the role of PKC $\alpha$  or PKC $\delta$  on ATRA growth inhibition, LM3 cells were treated combining ATRA and PKC pharmacological inhib-

itors for 72 h. PKC $\alpha$  inhibition induced an important reduction in LM3 proliferative capacity. The combination of PKC $\alpha$  inhibitors with ATRA showed a significant additive effect (Fig. 5). The inhibition of PKC $\delta$  had no direct effect on cell proliferation. However the pharmacological inhibition of PKC $\delta$  prevented ATRA induced growth inhibition in LM3 cells after 72 h treatment, suggesting a role for PKC $\delta$  as a mediator of ATRA effect on cell growth (Fig. 5).

#### Additive Effect of ATRA and PKC $\alpha$ Inhibition on Metastatic Dissemination

We further evaluated whether the above described *in vitro* results had a correlation with mammary cancer response to ATRA *in vivo*. For this, we performed an orthotopic assay evaluating LM3 tumor growth in the 4th mammary gland of female BALB/c bearing or not a slow-release ATRA-containing pellet. ATRA induced a significant inhibition in the growth of the primary LM3 tumor (Fig. 6a). However, ATRA did not significantly impair the development of spontaneous lung metastasis (data not shown). Thus, to assess whether the combination of ATRA with the PKC $\alpha$  inhibitor Gö6976 was able to affect the metastatic potential of LM3 cells, we performed an experimental metastatic assay. As shown in Figure 6b, ATRA treated mice presented a lower but non-significant number of lung nodules than the control group, treated with the empty pellet. In the absence of ATRA treatment (empty pellets), a significant reduction in the number of metastatic foci was observed when mice were inoculated with cells pretreated with the PKC $\alpha$  inhibitor as compared with control animals, injected with untreated cells. An additional significant reduction in the number of lung metastases was observed when mice that received Gö6976 pretreated cells were also bearing ATRA-pellets (Fig. 6b).

## DISCUSSION

The RA is currently being used successfully in acute promyelocytic leukemia treatment. Nonetheless, only circumstantial evidence has been obtained related to the retinoid signaling pathway impact in solid tumors metastatic spread [32]. Regarding solid tumors, retinoids are currently only being used with the purpose of preventing a recurrence. In fact, a phase III study showed significant differences in appearance timing of a second breast tumor in premenopausal patients [33].

However, the role of retinoids in malignant progression remains controversial, since some act by promoting transcription of tumor suppressor genes and others providing an increase in cell growth. Furthermore, it is known that retinoids can exert cytostatic and antiproliferative effects through non-genomic interactions [34].

Related to the interaction between PKC and RA pathway it has been proposed that retinoids may alter

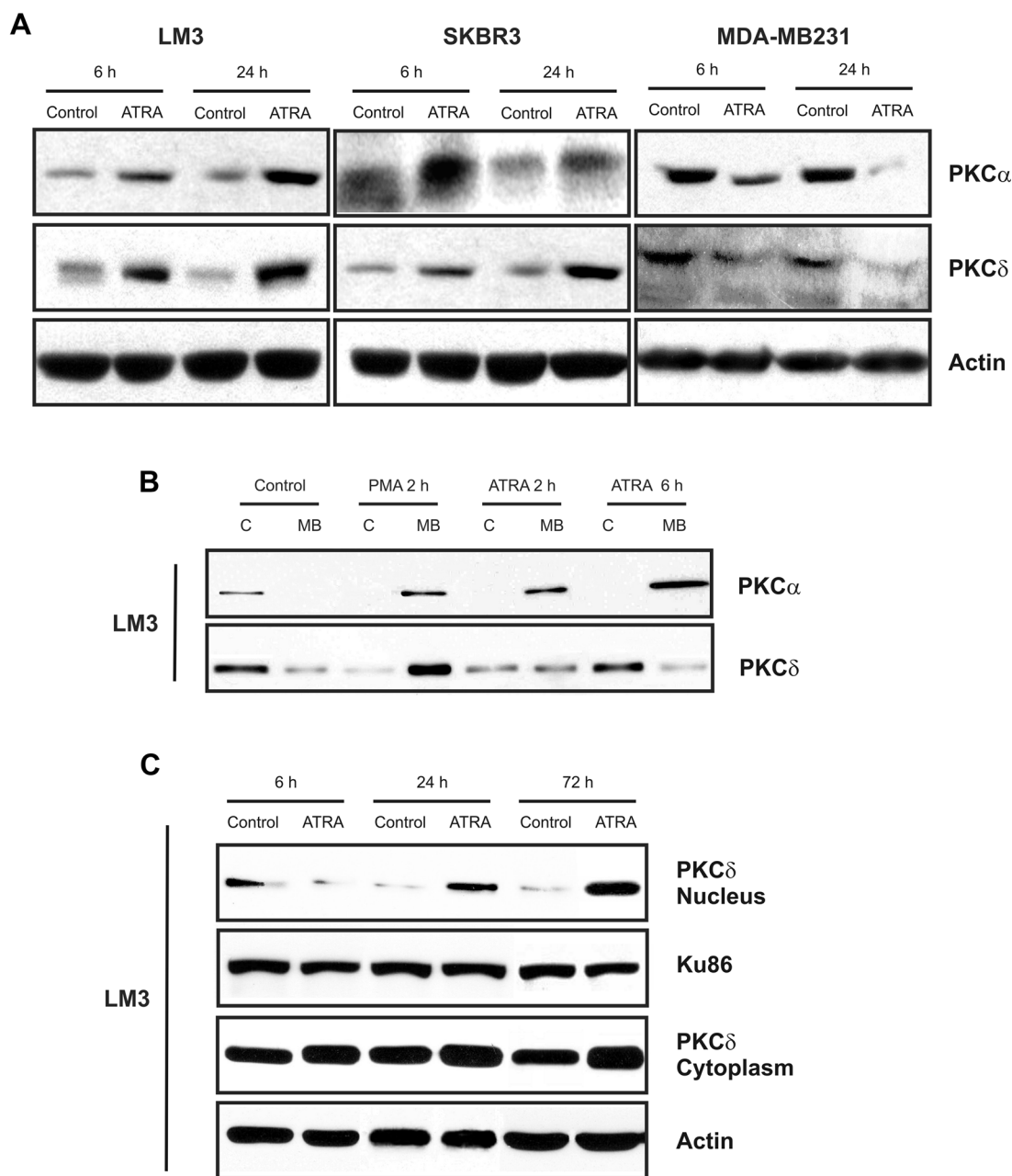


Figure 3. Modulation of PKC $\alpha$  and PKC $\delta$  expression and subcellular localization by retinoid treatment. Panel a: LM3, SKBR3 and MDA-MB231 cells were subjected to ATRA treatment and PKC $\alpha$  and  $\delta$  expression levels were assessed by Western blot. Panel b: Western blot analysis for the presence of PKC $\delta$  in soluble, cytosolic (C) or particulate, membrane (MB) fractions in LM3 cells, after treatment with PMA

or ATRA. A representative experiment is shown. Panel c: PKC $\delta$  detection by Western blot in nuclear and cytoplasmic protein fractions corresponding to LM3 cells subjected to ATRA treatment. Actin and Ku86 expression levels were used as loading control for cytoplasmic and nuclear fractions respectively. A representative experiment is shown.

PKC $\alpha$  localization, inducing its inactivation and favoring tumor cells differentiation [35,11]. It has also been described that PKC $\delta$  plays an important role in the regulation of RA dependent gene-expression since this PKC isoform could form complexes with RARs favoring the binding to RAREs sites [36].

As till nowadays breast cancer treatment with retinoids as single drug has not been successful [37], a combined treatment using retinoids and PKC

modulators could become a powerful tool in order to reverse the malignant phenotype of solid tumors [38].

In this scenario, our aim was to study the molecules involved in the interaction between the retinoic acid system and the PKC family in order to identify novel target/s for an effective treatment of breast cancer, using the hormone-independent breast cancer cell lines LM3 (murine) [26], SKBR3 and MDA-MB231

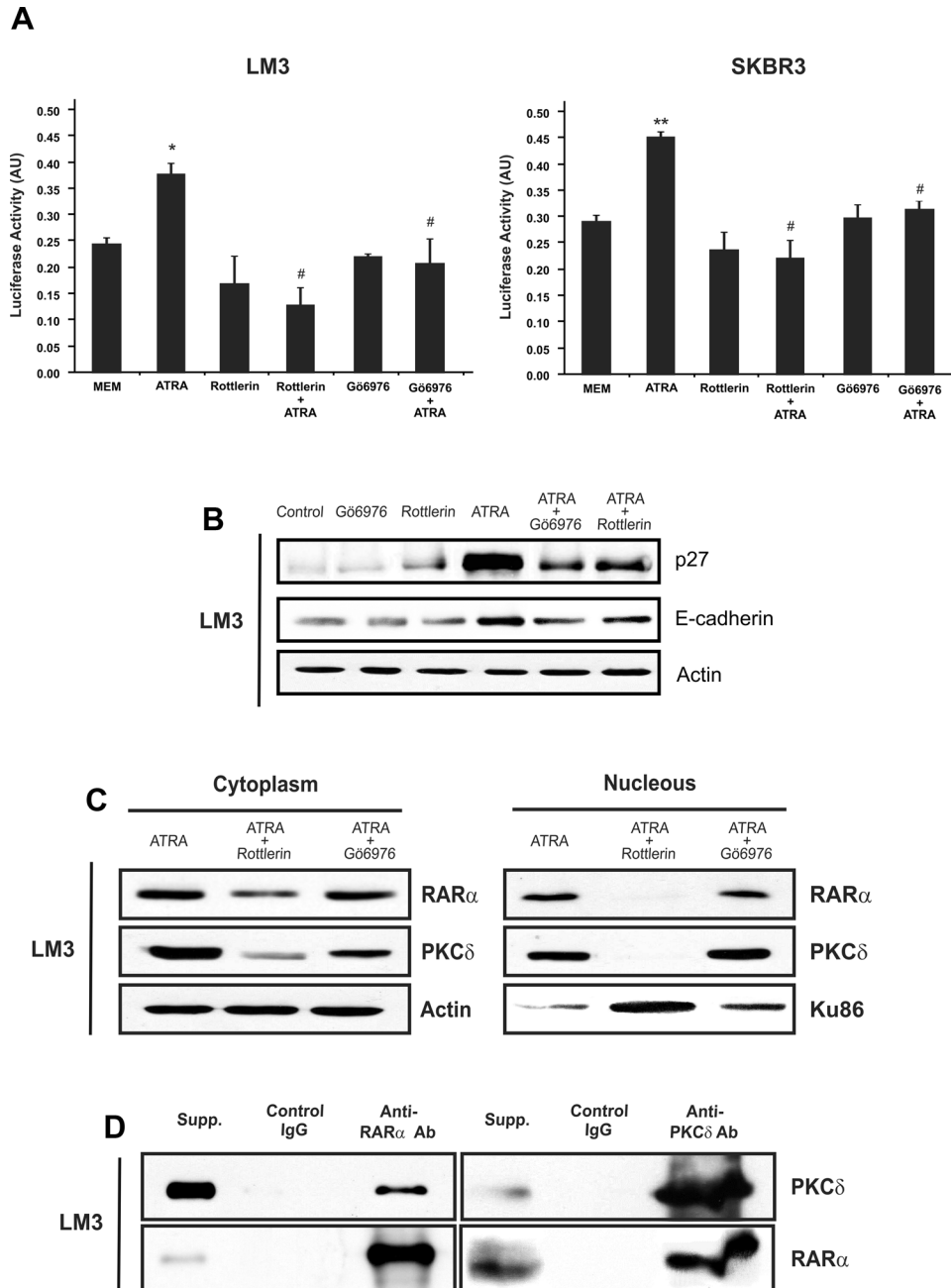


Figure 4. Retinoid receptors activation and nuclear translocation of RAR $\alpha$  and PKCs. Panel a: Luciferase activity of LM3 and SKBR3 cells co-transfected with RARE-Luc reporter plasmid and a Renilla luciferase vector as control. Data expressed as the mean  $\pm$  SD ( $n=4$ ) are representative of three independent experiments. \* $P < 0.05$  versus MEM and # $P < 0.05$  versus ATRA (ANOVA). Panel b: p27 or E-cadherin expression in LM3 cells treated for 24 h with PKC inhibitors and/or ATRA. Actin expression levels were used as loading control. A

representative experiment is shown. Panel c: PKC $\alpha$  or PKC $\delta$  expression in nuclear and cytoplasmic fractions of LM3 cells treated for 24 h with ATRA or ATRA plus PKC inhibitors. Actin and Ku86 expression levels were used as loading control for cytoplasmic and nuclear fractions respectively. A representative experiment is shown. Panel d: Co-immunoprecipitations were performed using anti-PKC $\delta$  and RAR $\alpha$  antibodies or normal mouse IgG as control.

(human) as models. Hormone-independent breast cancers have the worst prognosis, so that a new therapy for this group would be of great impact.

We could establish that none of these cell mammary cancer lines expressed detectable levels of RAR $\beta$ 2, which is considered a tumor suppressor [39]. How-

ever, despite the absence of RAR $\beta$ 2, the murine mammary LM3 cell line and the human SKBR3 cell line responded to ATRA treatment with significant growth inhibition after 72 h treatment, while the human mammary MDA-MB231 cell line did not respond at all [40]. Since ATRA treatment was able



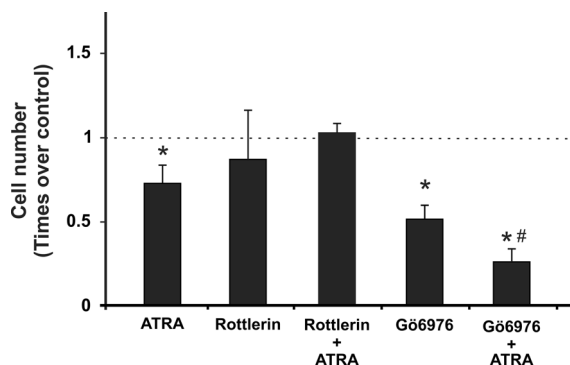


Figure 5. Modulation of cell growth capacity by ATRA and PKCs inhibitors combined treatment. LM3 cell number was assessed at different times after treating the cells with ATRA and/or PKC inhibitors. Each data point represents the mean  $\pm$  SD of triplicate determinations. At least three independent experiments were performed with similar results. Data were normalized to control cell number without treatment (dotted line) \* $P < 0.05$  versus control, # $P < 0.05$  versus Gö6976 and versus ATRA after 72 h for treatment (ANOVA).

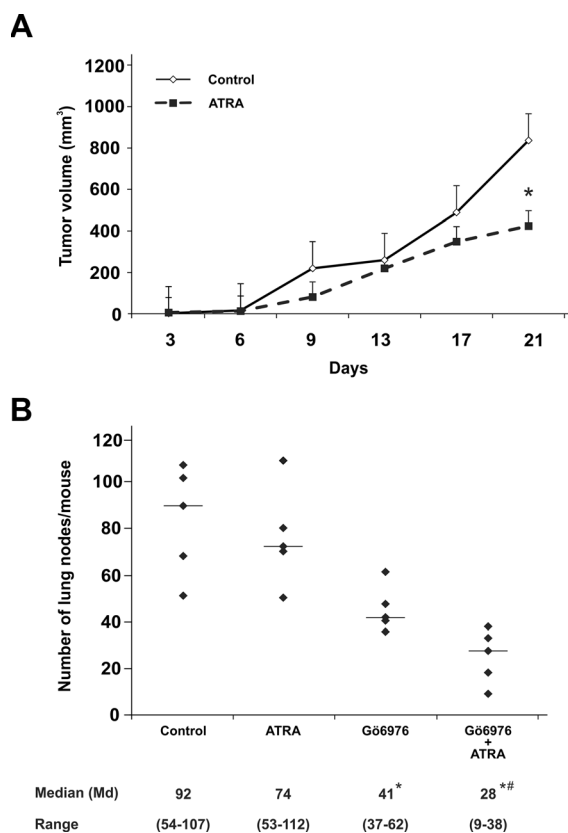


Figure 6. Tumor growth and experimental lung metastasis assays. Panel a: Evaluation of LM3 tumor growth under ATRA treatment. Tumor diameters were measured twice a week and volume was calculated for growth rate assessment. \* $P < 0.05$  versus control (2-way ANOVA). Panel b: LM3 cells were pretreated in vitro during 5 days with ATRA, Gö6976 or ATRA plus Gö6976 and then cells were i.v. inoculated into BALB/c mice. Twenty-one days post-inoculation, mice were sacrificed and the number of lung foci was recorded. The figure shows the results of one experiment representative of three independent assays \* $P < 0.001$  versus control, # $P < 0.05$  versus Gö6976 and versus ATRA (Kruskal-Wallis test).

to inhibit LM3 and SKBR3 cells growth, we performed a flow cytometry assay in order to determine which phases of the cell cycle were affected. We could determine that ATRA treatment induced the arrest of LM3 cells in the G0/G1 phase of the cell cycle. On the other hand, the same treatment induced accumulation of SKBR3 cells in the G2 phase. Both the murine and the human cell lines showed an enhanced subG1. Next, we analyzed cell cycle regulators and molecules involved in cell survival and proliferation to mechanistically define the biological effect we reported herein. We could observe that growth inhibition correlated with reduced levels of P-Erk1 and increased levels of P-Akt in LM3 cells. In addition we found an increased expression of the cell cycle inhibitor p27, particularly in the nuclear compartment. Recent studies revealed that activation of the PI3K/Akt signaling pathway by ATRA is essential for the differentiation of leukemia cells [41]. Therefore it is conceivable that increased levels of P-Akt after the treatment for short periods with ATRA could be leading LM3 cancer cells to a less malignant phenotype, which would involve cell cycle arrest.

Related to ATRA effects on the expression of PKC $\alpha$  and PKC $\delta$  in the LM3 and SKBR3 cell lines, we observed that this treatment increased the protein levels of both PKC isoforms. This result suggests an association between the growth inhibition induced by ATRA and rising levels of both PKCs. The opposite effect was observed in the human tumor MDA-MB231 cell line, unresponsive to retinoid treatment. Although our results do not allow us to exclude the modulation of other PKCs by ATRA treatment, it is noteworthy that  $\alpha$  and  $\delta$  isoforms are the most implicated in breast cancer. To further analyze the specific involvement of PKC $\alpha$  and PKC $\delta$ , we have evaluated whether ATRA is able to induce changes in their subcellular localization. Other authors have also reported, in endometrial adenocarcinoma cells, that retinoids alter PKC $\alpha$  cellular localization, inducing its inactivation and the differentiation of these cells [35,11]. We found that ATRA differentially changed the subcellular localization of PKCs, as PKC $\alpha$  is translocated to the cell membrane and PKC $\delta$  translocates to the cell nucleus, being these events compatible with the activated forms of these PKCs.

From these results rises the idea that both PKC isoforms could be part of the RA genomic or pre-genomic pathways, suggesting further studies about the role of PKCs on the activation of RARs. Through a classical gene reporter assay involving the RARE sequence, we demonstrated that the activity of both PKC isoforms would be necessary to induce RARs activation by ATRA. However, since PKC $\alpha$  did not translocate to the cell nucleus, the mechanism through which it participates in RARs activation must be different from that of PKC $\delta$ .

As mentioned above, ATRA increased PKC $\alpha$  protein levels. Since the PKC $\alpha$  gene promoter contains a RARE

site [42], this response could be considered an indirect way of demonstrating the activation of RARs.

An exhaustive analysis of PKCs sequence revealed that ATRA can bind to the C2 domain pocket present in classical PKC isoforms [43–45,38]. Furthermore, retinoids may regulate the activity of PKCs, and in turn ATRA activated PKC can bind to receptors and stimulate RARs transcriptional activity [44]. Notably, PKC $\alpha$  can phosphorylate RAR $\alpha$  on Ser157, in the extended DNA-binding domain [46]. Moreover, several authors have confirmed that the transcriptional activity of RAR $\alpha$  and RAR $\gamma$  can be positively modulated by phosphorylation by other kinases in response to multiple signals. In fact, phosphorylation at serine 369 between H9 and H10 by PKA, upregulates RAR $\alpha$  transcriptional activity through recruitment of co-activators or inducing heterodimer RAR/RXR formation or RAR/RXR binding to DNA [47].

Regarding PKC $\delta$  function, it has been described that its subcellular distribution is involved in the activation of different signaling pathways altering the final cell fate [48]. As we observed that PKC $\delta$  translocates to the cell nucleus after ATRA treatment, we wanted to analyze whether this isoform also interacts with RAR $\alpha$ 1, since this receptor is key in the response started by retinoids [39,6]. We found that the pharmacological inhibition of PKC $\delta$  in the presence of ATRA completely blocked RAR $\alpha$  translocation to the cell nucleus. Moreover, we could determine that both molecules co-immunoprecipitated suggesting that PKC $\delta$  could be a member of the transcriptional complex of RA dependent genes. In fact, Kambhampati and colleagues showed that RA can activate PKC $\delta$  in MCF-7 cells and that the overexpression of PKC $\delta$  increased RARE transcriptional activity in the same cell model [36]. On the other hand, it was reported that PKC $\delta$  inhibition also blocks activation of the p38 MAPK [49]. The p38/MSK1 pathway is the main signaling cascade involved in the phosphorylation and regulation of RARs activity and degradation. RA induces the rapid activation of the p38MAPK/MSK1 pathway, with characteristic downstream consequences on the phosphorylation of RARs and the expression of their target genes [50], so it could be possible that pharmacological inhibition of PKC $\delta$ , blocked RAR $\alpha$  translocation through the inhibition of p38MAPK/MSK1 pathway.

From the evaluation of the proliferative capacity on *in vitro* treated LM3 cells, we could observe that PKC $\delta$  pharmacological inhibition prevented growth inhibition exerted by retinoids. Furthermore, this modulation correlates with the implication of PKC $\delta$  in the activation of the retinoid system described in the present work for both RA responder cell lines. So, we can conclude that PKC $\delta$  plays a critical role in this biological effect of RA.

On the other hand, the pharmacological blockade of PKC $\alpha$  caused a significant inhibition of cell proliferation. It also showed an additive effect on

growth inhibition when LM3 cells received the PKC $\alpha$  inhibitor together with ATRA. All data together reinforce the idea that PKC $\alpha$  would be acting in a different way than PKC $\delta$  regarding their interaction with the retinoid system. While PKC $\delta$  activation would be helping to the effect exerted by retinoids on growth inhibition, PKC $\alpha$  would be attenuating retinoids effect on proliferation.

Other authors have demonstrated that the modulation of PKC $\alpha$  expression levels correlate with the proliferative and migratory potential *in vitro* of mammary cancer cells, two parameters highly related to the metastatic spreading capacity [21]. A subsequent analysis revealed that PKC $\alpha$  catalytic activity is critical for breast tumor cells proliferation, even under serum free culture conditions [21]. Additional studies performed in breast cancer patients showed that PKC $\alpha$  expression is highly associated with a worse prognosis of the disease [21].

Thus, in view of the data demonstrating PKC $\alpha$  activity exerts a key role in the progression of breast cancer, and our results indicating an additive effect using the pharmacological inhibitor of PKC $\alpha$  in combination with ATRA on LM3 cells proliferation *in vitro*, we studied the ability of pre-treated LM3 cells with the pharmacological inhibitor of PKC $\alpha$  to colonize the metastasis target organ *in vivo*, through an experimental metastasis assay. This is a useful method to analyze later stages of the metastatic cascade in a short period of time (21 days). In this assay we injected cells, pre-treated for 24 h with the pharmacological inhibitor of PKC $\alpha$ , into mice bearing subcutaneous pellets, containing or not ATRA. We observed that the inhibition of PKC $\alpha$  activity in combination with ATRA treatment was able to significantly decrease the total number of lung metastases.

Regarding LM3 tumor progression *in vivo*, ATRA could not inhibit the development of spontaneous metastasis, probably due to RAR $\beta$ 2 absence in the murine model. However, it is interesting to note that ATRA exerted remarkable growth inhibition of the primary LM3 tumor inoculated into the mammary fat pad.

Our results allow us to hypothesize a model, summarizing the participation of PKC $\alpha$  and  $\delta$  in the retinoid system. We could describe a pre-genomic retinoid pathway that includes the PKC $\alpha$  and PKC $\delta$  activation by ATRA which in turn lead to RAR $\alpha$  activation. This activation induces long-term effects, which include the modulation of numerous RA-response-genes that might lead to differentiation and/or to proliferation inhibition (Fig. 7).

PKC $\alpha$  could be involved in RAR $\alpha$  phosphorylation, allowing its correct heterodimerization with RXR receptors. On the other hand, active PKC $\delta$  translocates to the cell nucleus and induces RAR $\alpha$  translocation, where these molecules interact and would participate in the assembly of transcriptional

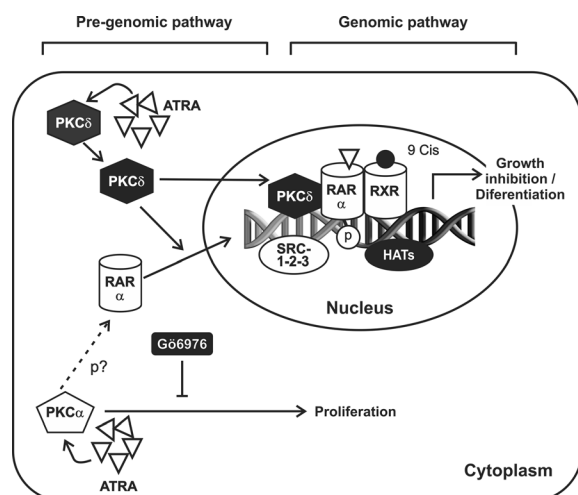


Figure 7. Schematic representation of PKC-RA crosstalk. A possible mechanism linking the PKC pathway with the retinoids system in mammary tumor derived cells is shown. PKC $\alpha$  activation by ATRA induces RAR $\alpha$  activation possibly by phosphorylation. Active PKC $\delta$  by ATRA induces RAR $\alpha$  translocation to cell nucleus, there PKC $\delta$  would bind to activated RAR $\alpha$  and together interact with several genes in the nucleus, allowing differentiation and growth inhibition. On the other hand, pharmacological inhibition of PKC $\alpha$  would lead to a reduction in the proliferation status.

complexes that regulate RA-responsive-genes. Then, the genomic retinoid pathway would be activated as a late response. The presence of a RARE sequence in the PKC $\alpha$  gene promoter would suggest that its transcription might be encompassed within this process. In contrast, the increased PKC $\delta$  protein levels observed after ATRA treatment would be mediated by a RARE-independent mechanism (Fig. 7).

The increase in expression and activation of both isoforms would lead, through multiple signaling pathways such as the activation of the PI3K/Akt pathway and the inhibition of the EGF pathway [41,34], to the differentiation and/or inhibition of proliferation.

Finally, we propose that the additive effect obtained by the pharmacological inhibitor of PKC $\alpha$  in combination with ATRA treatment is due to a balance between the inhibition of PKC $\alpha$ , which promotes cell proliferation, and the activation of PKC $\delta$  by ATRA, which would lead to the differentiation and / or inhibition of proliferation (Fig. 7).

These effects as a whole, would be leading to a less malignant phenotype.

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