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PKCδ Inhibition Impairs Mammary Cancer Proliferative Capacity But Selects Cancer Stem Cells, Involving Autophagy

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ABSTRACT

Protein kinase C (PKC) is a family of serine/threonine kinases that regulate diverse cellular functions including cell death, proliferation, and survival. Recent studies have reported that PKCδ, are involved in apoptosis or autophagy induction. In the present study we focused on how PKCδ regulates proliferation and cancer stem cell (CSC) properties of the hormone-independent mammary cancer cell line LM38-LP, using pharmacological and genetic approaches. We found that pharmacological inhibition of PKCδ, by Rottlerin treatment, impairs in vitro LM38-LP proliferation through cell cycle arrest, inducing the formation of cytoplasmic-vacuoles. Using immunofluorescence we confirmed that Rottlerin treatment induced the apparition of LC3 dots in cell cytoplasm, and increased autophagy flux. On the other side, the same treatment increased CSC growth rate and self-renewal. Furthermore, Rottlerin pre-treatment induced in CSC the development of a "grape-like" morphology when they are growing in 3D cultures (Matrigel), usually associated with a malignant phenotype, as well as an increase in the number of experimental lung metastasis when these cells were inoculated in vivo. The PKCδ knockdown, by RNA interference, induced autophagy and increased CSC number, indicating that these effects are indeed exerted through a PKCδ dependent pathway. Finally, the increase in the number of mammospheres could be reversed by a 3MA treatment, suggesting that autophagy mechanism is necessary for the increased of CSC self-renewal induced by PKCδ inhibition. Here we demonstrated that PKCδ activity exerts a dual role through the autophagy mechanism, decreasing proliferative capacity of mammary tumor cells but also regulating tumor stem cell self-renewal. J. Cell. Biochem. 117: 730–740, 2016.

KEY WORDS: PROTEIN KINASE Cδ; MAMMARY CANCER; CANCER STEM CELLS; AUTOPHAGY

Autophagy is a conserved lysosomal degradation pathway, in which cells self-digest its cytoplasmic components and intracellular materials [Mizushima, 2007]. Autophagic process is initiated by a membrane nucleation step which requires Beclin 1 and Atg5–Atg12 induction. Microtubule-associated protein 1 light chain 3-phosphatidylethanolamine (LC3) is necessary for elongation of the isolation double-membrane structure identified as autophagosome [Mizushima, 2011]. Autophagosome is then fused with lysosomes, forming finally the autolysosome where degradation occurs [Yorimitsu and Klionsky, 2005].

Autophagy can function both as a tumor suppressor process as well as a cell-survival mechanism against environmental stress, inducing resistance to chemotherapy [Berardi et al., 2011]. Interestingly, recent studies have demonstrated that autophagy

Abbreviations: 3-MA, 3-methyladenine; ATRA, all trans retinoic acid; BAF, bafilomycin A1; DAG, diacylglycerol; CSC, cancer stem cells; ERα, estrogen receptor-α; FBS, fetal bovine serum; LC3, microtubule-associated protein 1 light chain 3; LC3 II, microtubule-associated protein 1 light chain 3-phosphatidylethanolamine; PBS, phosphate buffered saline; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; RNA interference, RNAi; SDS, sodium dodecyl sulfate; TNBC, triple-negative breast cancers.

Alejandro J. Urtreger and Laura B. Todaro contributed to this work equally.

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pathway is critical for cancer stem cells (CSC) survival and tumorigenicity in breast cancer [Gong et al., 2013].

It has been reported that Protein kinase C (PKC) isoenzymes are involved in autophagy induction [Ozpolat et al., 2007; Sakaki et al., 2008] however, the underlying mechanism still remains unanswered. PKC is a lipid-dependent serine/threonine kinase family that plays a central role in signal transduction pathways controlling proliferation, apoptosis, and malignant transformation [Nakagawa et al., 2005; Diaz Bessone et al., 2011]. In particular PKCδ, a member of the novel family isoforms, has an ambiguous role in breast cancer progression. It was described that PKCδ inhibition impaired phorbol ester induced arrest in G1 cell cycle phase in SKRB-3 breast cancer cells [Fujii et al., 2005]. However, several studies including our own, have demonstrated a pro-tumorigenic role for PKCδ in mammary cancer cells thought the induction of anchorage-independent growth and survival pathways [Liu et al., 2002; Grossoni et al., 2007].

Taking into account the possible implication of PKC in the autophagy process, we have used the LM38-LP cell line, a hormone independent murine mammary adenocarcinoma model constituted by luminal, myoepithelial, and CSC [Bumaschny et al., 2004; Berardi et al., 2015].

As an experimental approach we inhibited PKCδ through pharmacological and genetic procedures in order to study how this PKC isoform regulates proliferation and stemness properties of LM38-LP cell line. We found that PKCδ inhibition reduced tumor cell proliferation, increasing autophagy flux. Strikingly, PKCδ inhibition enriched CSC subpopulation, with high Nanog expression, through autophagy mechanism.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

Media for cell culture and agarose were obtained from Life Technologies (Rockville, MD). Fetal Bovine serum (FBS) was from GEN (Buenos Aires, Argentina). Acrylamide was obtained from Sigma (St. Louis, MO). All other reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad (Richmond, CA). Monoclonal anti-PKCδ antibodies were purchased from BD Biosciences (San Diego, CA). Polyclonal anti-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies for LC3I/II were purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase conjugated anti-rabbit or anti-mouse antibodies were obtained from Sigma (St. Louis, MO). Hybond-P membranes for blotting and chemiluminiscence reagents (ECL) were from Amersham (Aylesbury, UK). The PKCδ inhibitor Rottlerin was obtained from Calbiochem (Billerica, MA). 3-methyl adenine (3-MA) and Bafilomycin A1 were purchased from Santa Cruz Biotechnology.

CELL LINE AND CULTURE CONDITIONS

We used the LM38-LP cell line, derived from a murine mammary papillary adenocarcinoma with tumorigenic and metastatic capacity in BALB/c mice [Bumaschny et al., 2004]. LM38-LP cells were grown in DMEM-F12 medium supplemented with 10% fetal bovine serum (FBS) and 80 μg/ml gentamycin. LM38-LP cell line was cultured at 37°C in a humidified air atmosphere with 5% CO_2.

PROLIFERATION ASSAYS

Proliferative potential was determined by assessing LM38-LP cell number, during the exponential growth phase of unsynchronized monolayer cultures under different treatments. Briefly, 4×10^5 cells were seeded onto 35 mm Petri dishes and treated or not with Rottlerin (1 μM) and/or 3MA (1 mM) during 4 days. 96 h after seeding, cells from triplicate wells were washed twice with PBS, trypsinized, and counted using an hemocytometer and trypan blue exclusion.

ANALYSIS OF CELL CYCLE DISTRIBUTION BY FLOW CYTOMETRY

Cell monolayers treated Rottlerin (1 μM), or vehicle for 96 h were detached, pooled with their supernatant 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).

MAMMOSPHERE ASSAY

To obtain mammospheres, a suspension containing 10^4 cells was plated in low attachment 35 mm culture dishes in serum-free DMEM-F12 medium supplemented with B27 (1:50) (Life Technologies) and 20 ng/ml epidermal growth factor (BD Biosciences). To propagate the mammospheres in vitro, primary mammospheres were enzymatically dissociated with 0.05% trypsin for 15 min at 37°C to obtain a single-cell suspension, and then cultured in suspension as described above, to produce the next generation of mammospheres. In both cases sphere formation was assessed 5 days after seeding and the number and size of mammospheres were recorded.

WESTERN BLOT (WB)

Subconfluent monolayers were pretreated or not with Rottlerin (1 μM) for 48 h and/or BafA1 (2 nM) for the last 1 h. Mammospheres were only pretreated or not with Rottlerin as mentioned above. Then cells were washed twice with ice-cold PBS, scraped with a Teflon scraper and finally lysed with 1% Triton ×-100 in PBS. After protein determination, samples were denatured by boiling in sample buffer with 5% β-mercaptoethanol and run in 10% SDS-PAGE. 50 μg protein were loaded into each lane. Gels were blotted to Hybond-P membranes. After the incubation for 1 h in blocking buffer containing 5% skim milk and 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 chemoluminiscence. Bands were digitalized with a Photo/Analyst Express System (Fotodyne Inc. Hartland, WI) and signal intensity was quantified with Gel-Pro Analyzer software.

REAL-TIME PCR (QPCR) DETERMINATIONS

RNA was prepared using the Gentra Purescript RNA isolation kit from Qiagen. (Valencia, CA). cDNA was prepared with the iScript cDNA synthesis kit from Bio-Rad. qPCR was performed in a CFX96 qPCR thermocycler (Bio-Rad). Reactions were run for 35 cycles under the following conditions: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 20 s, and 72°C for 30 s. The amplification of unique products in each reaction was verified by melting curve. Expression level of each gene was normalized to

GAPDH expression level using delta Ct method and specific primers. Means and standard deviation (SD) from at least three experiments were calculated and shown as fold induction with respect to the control. PCR products were obtained using Nanog, Sox2 and PKCδ primers [Gavrielides et al., 2006; Zhang et al., 2011].

PKCδ RNA INTERFERENCE (RNAI)

Small interference RNA (siRNA) duplexes against PKCδ were transfected into LM38-LP cells following the manufacturer's instructions. The following sequence was used: 5′AACCAC GAGTT-TATCGCCACC 3′. A scramble siRNA duplex: 5′AAGGUAUUGAC AGGGAUCUGA3′ was used as control. Twenty-four hours after transfection, cells were cultured in mammosphere conditions as described above.

IMMUNOFLUORESCENCE MICROSCOPY

Four thousand cells were seeded onto multiwell culture slides (Labteks, BD Biosciencies, Franklin Lakes, NJ). 24 h after seeding, cells were treated with Rottlerin or vehicle for 48 h and then fixed with paraformaldehyde, permeabilized with Triton ×-100, rinsed with PBS-glycine, and incubated for 1 h with blocking buffer (10 ml Triton ×-100, 2 ml Tween 20 in 500 ml PBS plus 10% FBS). Then, cells were incubated overnight at 4°C with a primary antibody for LC3 in blocking buffer. After washing, a secondary Alexafluor-conjugated antibody (Molecular Probes, Eugene, Or) was added for 1 h at room temperature. Finally cells were incubated with DAPI, and mounted with Prolong anti-fade (Molecular Probes). Photographs were taken with an inverted phase contrast microscope (Eclipse E400 Nikon, Tokyo, Japan).

MATRIGEL 3D CULTURES

Five thousand mammosphere derived cells, treated or not with Rottlerin, were resuspended in 400 μl of assay medium (DMEM-F12 added with 2% FBS and 2% Matrigel (BD Biosciences). The monocellular suspension was seeded onto multiwell culture slides (Labteks, BD Biosciencies) containing 40 μl of Matrigel. The assay medium was changed every 4 days. After 12 days in culture, cells were fixed with paraformaldehyde and used for direct observation in an inverted phase contrast microscope (Eclipse E400 Nikon).

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.

EXPERIMENTAL LUNG METASTASIS ASSAY

The experimental lung metastasizing ability was performed as described previously [Grossoni et al., 2009]. Two hundred thousand mammoesphere derived cells pretreated or not with Rottlerin (1 μM) for 96 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. 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 was recorded.

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 tests or Krustal-Wallis, as indicated in each case. A value of $P < 0.05$ was considered to be significant.

RESULTS

PKCδ IS EXPRESSED DIFFERENTIALLY IN THE LM38-LP MONOLAYER RESPECT TO CSC

With the aim to compare PKCδ expression levels between LM38-LP monolayers and CSC we performed WB and qPCR assays. In order to evaluate whether the CSC component present in LM38-LP cell line expresses the PKCδ isoform, we applied a mammospheres methodology, where mammary stem/progenitor cells can be enriched and propagated in culture as floating spherical colonies. Interestingly, we observed that mammosphere express higher PKCδ levels than LM38-LP cell line (Fig. 1A). In order to quantify PKCδ expression, we performed a qPCR analysis. This study revealed a two-fold increment in PKCδ levels in mammospheres as compared to LM38-LP monolayers (Fig. 1B).

PHARMACOLOGICAL INHIBITION OF PKCδ IMPAIRS IN VITRO LM38-LP PROLIFERATION THROUGH CELL CYCLE ARREST

In order to investigate the involvement PKCδ in the proliferative potential of LM38-LP cells, monolayers were treated with Rottlerin, a highly employed PKCδ activity inhibitor [Gschwendt et al., 1994]. Cell proliferation analysis revealed that, upon PKCδ inhibition, a marked decrease in the number of viable LM38-LP cells was observed as compared to control untreated cells (Fig. 2A). Moreover, the determination of cell cycle distribution revealed that PKCδ activity inhibition led to the accumulation of cells in the G0/G1 phase, with the concomitant reduction in S phase (Fig. 2B). No cell accumulation was observed in sub G1 phase, thus no apoptosis could be inferred (Fig. 2B).

PHARMACOLOGICAL INHIBITION OF PKCδ INCREASED AUTOPHAGY FLUX IN LM38-LP CELL LINE

Next, we analyzed the mechanism involved in the inhibition of cell proliferation induced by Rottlerin. We found that, pharmacological inhibition of PKCδ triggered the accumulation of vacuolated LM38-LP cells in the monolayers (Fig. 2C). Considering that autophagy is characterized by accumulation of cytoplasmic vesicles and autophagolysosomes formation are recognized by the presence of the membrane-bound microtubule-associated protein light chain 3 (LC3), we performed an immunofluorescence assay in order to detect

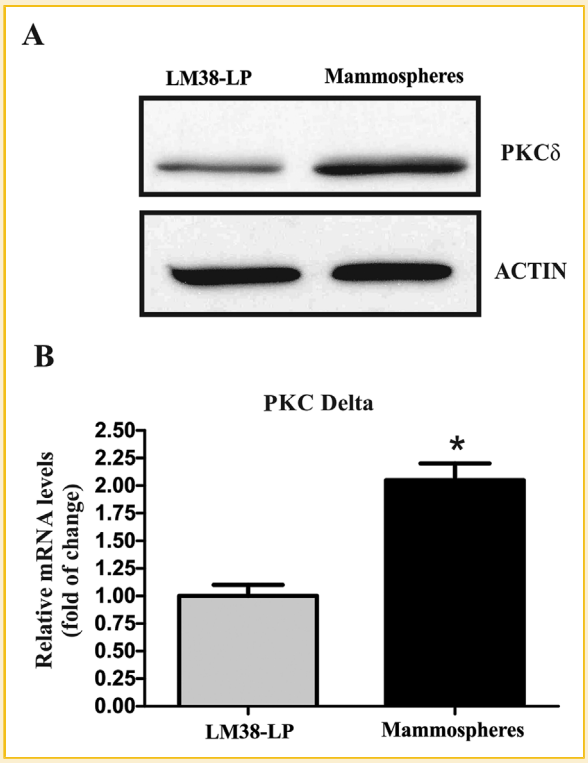

Fig. 1. A: Immunoblot analysis of PKCδ levels. LM38-LP cell and mammosphere lysates were resolved in 10% SDS-PAGE and blotted against PKCδ. Actin immunoblotting was used as a loading control. Results are representative of three independent experiments. B: RNA from LM38-LP monolayers and mammospheres was isolated and PKCδ expression level was analyzed by qPCR. Each data point represents the mean ± S.D. of triplicate determinations, $^{*}P < 0.05$ versus control (Student's *t* test). At least three independent experiments were performed with similar results.

this protein. We could determine that Rottlerin treatment induced the appearance of LC3 dots in cell cytoplasm (Fig. 2D). Autophagic flux was then evaluated using bafilomycin A1 (BAF), a specific autophagosomal lysosome activity inhibitor (Fig. 3A). While, LC3-II and its precursor LC3-I levels were equal in control cells, BAF, or Rottlerin treatments induced an important increase in LC3-II levels (Fig. 3A–B). Moreover, PKCδ pharmacological inhibition significantly increased LC3-II accumulation in BAF-treated cells, suggested that PKCδ activity is involved in the autophagy flux induction (Fig. 3A–B).

In order to confirm the specific participation of PKCδ in the autophagy process, we perform an RNAi assays (Fig. 3C). We could observe that LC3-II was markedly increased under PKCδ down-regulated conditions (Fig. 3D–E). Rottlerin did not induce autophagy in an unspecific manner since no autophagy increase was detected when PKCδ was knocked down (Fig. 3D–E).

EFFECT ON GROWTH INHIBITION UNDER IMPAIRS PKCδ ACTIVITY AND AUTOPHAGY SUPPRESSION ON LM38-LP CELL LINE

Next we evaluate whether the suppression autophagy first steps, by 3-methyladenine (3MA) treatment, could alter PKCδ inhibitory effect on LM38-LP cell proliferation. As observed in Figure 3D, the blockage of both autophagy and PKCδ activity reduces LM38-LP growth. A clear synergistic effect on cell proliferation was observed when both drugs were combined (Fig. 3F).

PHARMACOLOGICAL INHIBITION OF PKCδ INCREASES THE CSC MALIGNANT PHENOTYPE

In order to determine whether PKCδ is involved in proliferation and/or CSC self-renewal capacity, single-cell suspensions from LM38-LP cells were cultured under mammosphere conditions and treated or not with Rottlerin. As shown in Figure 4A, PKCδ pharmacological inhibition, increased CSC growth rate, determined by the increase in primary mammospheres diameter.

Furthermore, through a secondary mammosphere assay, we could determine that Rottlerin pre-treatment increased CSC self-renewal, as determined by the higher number of mammospheres formed (Fig. 4B).

Next, in order to evaluate whether Rottlerin treatment exerts a process selection on CSC from LM38-LP, we sub-cultured this cell line for a long-term period (1 week) in media supplemented with low dose of Rottlerin (0.20 μM). LM38-LP cells under these conditions were able to develop more mammospheres than control (Fig. 4C).

To validate the pharmacological approach, we performed a primary mammosphere assay using LM38-LP cells transfected with a PKCδ siRNA. As shown in Figure 4D–E, PKCδ down regulation increased the diameter and number of primary mammospheres.

Finally, to determine whether the pharmacological inhibition of PKCδ is able to alter differentiation properties of mammospheres derived cells, we performed 3D cultures in Matrigel. By phase-contrast microscopy we determined that 3D cultures derived from Rottlerin treated mammospheres developed a "grape-like" morphology, usually associated with a malignant phenotype, in contrast to 3D cultures without treatment, which present a "mass" morphology (Fig. 4F).

In order to confirm this possible increase in malignancy induced by PKCδ inhibition, we performed an experimental metastasis assay. As shown in Fig. 5A, a significant increase in the number of lung metastatic foci was observed in mice inoculated with mammosphere derived cells pre-treated with Rottlerin as compared with control animals, injected with vehicle pre-treated cells. Surprisingly, a significantly loss weight corresponding to 10% aprox appeared in the mice group that received Rottlerin pretreated mammospheres, showing cachexia symptoms (Fig. 5B).

PHARMACOLOGICAL INHIBITION OF PKCδ INDUCES NANOG EXPRESSION IN LM38-LP CSC

Next we determined whether changes induced by PKCδ inhibition in mammospheres number and/or size respond to pluripotent genes expression modulation. In a previous report we have demonstrated that LM38-LP mammospheres expressed high levels of several pluripotent genes including Nanog and Sox2, confirming an enrichment in cancer stem/progenitor cells [Berardi et al., 2015]. The pharmacological inhibition of PKCδ in mammospheres clearly increased Nanog expression levels but no changes were observed in Sox2 expression (Fig. 6).

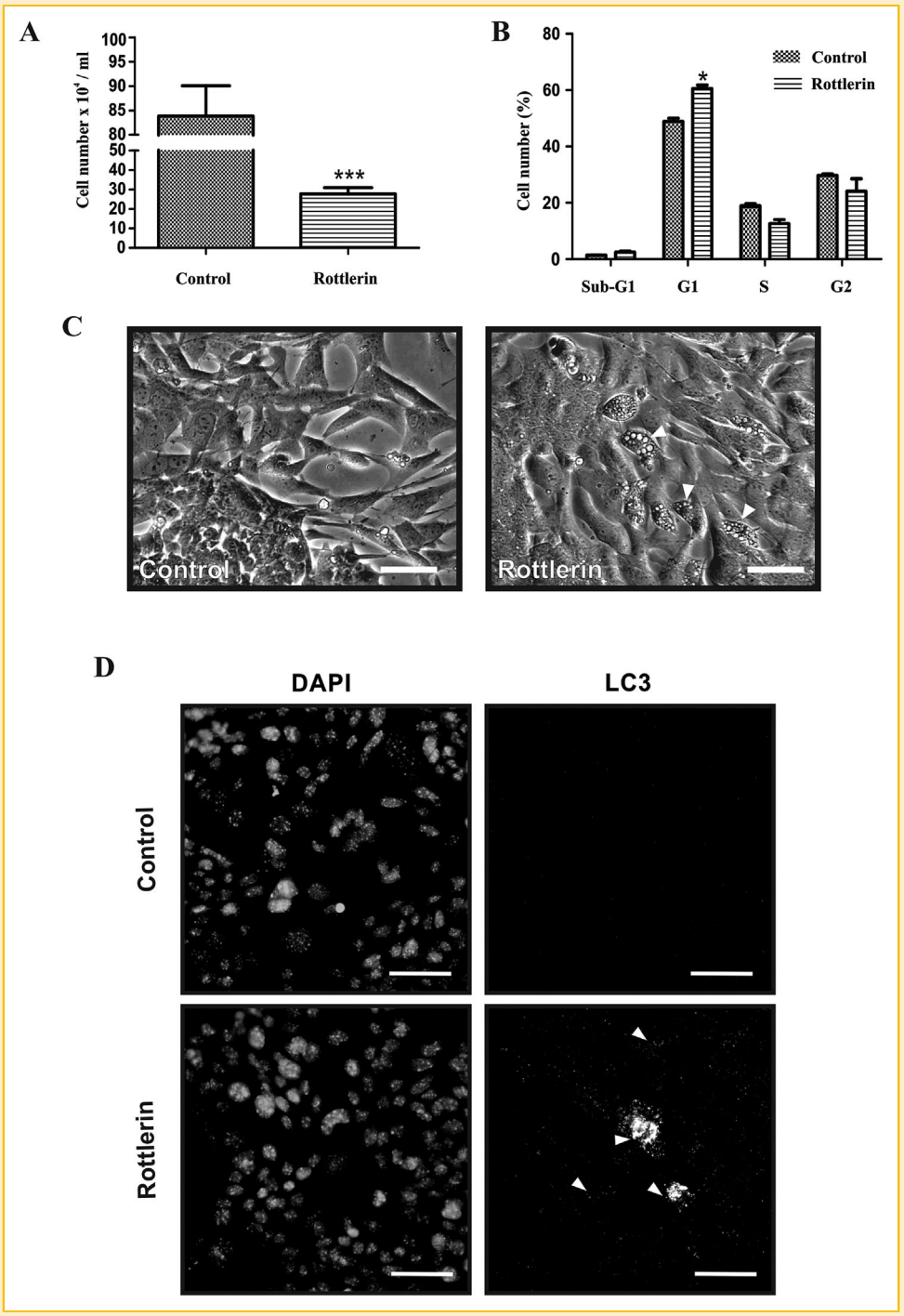

Fig. 2. Effect of Rottlerin on in vitro cell proliferation. A: LM38-LP cell number was assessed 96 h after the treatment with Rottlerin. Each data point represents the mean ± S.D. of triplicate determinations, $^{*}P < 0.05$ versus control (Student's *t* test). At least three independent experiments were performed with similar results. B: LM38-LP cell cycle analysis by flow cytometry after Rottlerin treatment. Each data point represents the mean ± S.D. of triplicate determinations, $^{*}P < 0.05$ versus control (Student's *t* test). At least three independent experiments were performed with similar results. C: Cells were treated with Rottlerin and morphologic changes were observed by phase contrast microscopy (×200 magnification, scale bar: 50 μm). Results are representative of three independent experiments. D: Immunofluorescence analysis of LC3. Cells were treated with Rottlerin during 48 h and LC3 expression/accumulation was observed as cytoplasmic dots by epiflorescence. Arrows indicate the presence of the mentioned points (×400 magnification, scale bar: 50 μm). Results are representative of three independent experiments.

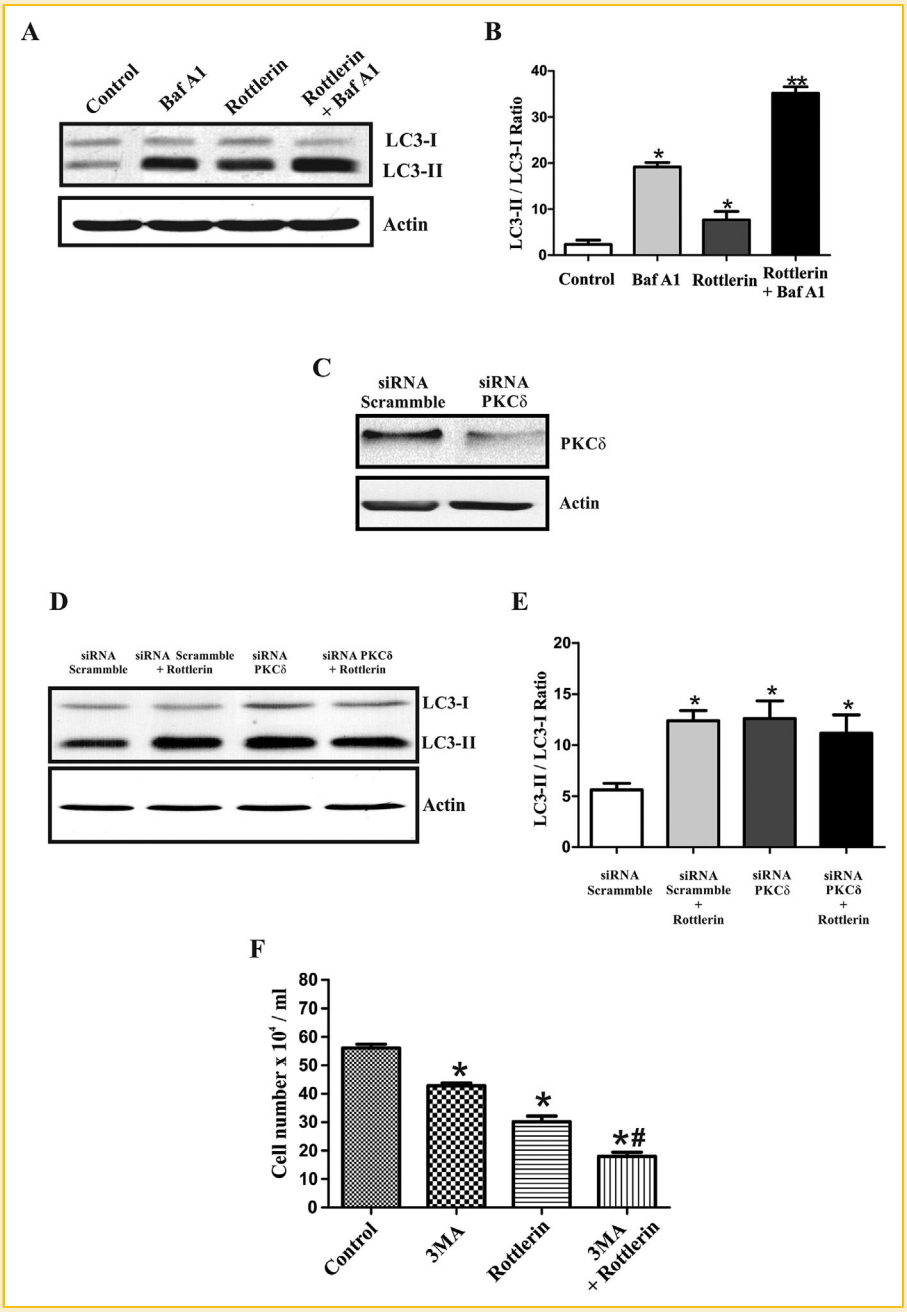

Fig. 3. Effect of PKCδ inhibition on autophagy. A: Immunoblot analysis of LC3-I and LC3-II levels. LM38-LP cells were incubated in complete medium with Rottlerin and in the presence or absence of BafA1. Whole cell lysates prepared from LM38-LP cells and were resolved in 10% SDS-PAGE and blotted with LC3 antibody. Actin immunoblotting was used as a loading control. B: Densitometric analysis of A. Each data point represents the mean ± S.D. of triplicate determinations, $^{*}P < 0.05$ versus control (ANOVA TEST). Results are representative of three independent experiments. The increase of LC3 was determined as the ratio between the LC3-II and LC3-I. C: Immunoblot analysis of PKCδ levels. LM38-LP cells were transfected with PKCδ- or scramble-siRNA. Whole cell lysates prepared from transfected LM38-LP cells was resolved in 10% SDS-PAGE and blotted against PKCδ. Actin immunoblotting was used as a loading control. Results are representative of three independent experiments. D: Immunoblot analysis of LC3 I and LC3 II levels. LM38-LP cells were transfected with PKCδ- or scramble-siRNA and incubated in the presence or absence of Rottlerin. Whole cell lysates prepared from transfected LM38-LP cells was resolved in 10% SDS-PAGE and blotted against LC3. Actin immunoblotting was used as a loading control. E: Densitometric analysis of D. Each data point represents the mean ± S.D. of triplicate determinations, $^{*}P < 0.05$ versus control (ANOVA TEST). Results are representative of three independent experiments. The increase of LC3 was determined as the ratio between the LC3-II and LC3-I. F: LM38-LP cell number was assessed 96 h after treatment with Rottlerin in the presence or absence of 3-MA. Each data point represents the mean ± S.D. of triplicate determinations, $^{*}P < 0.05$ versus control and $\#P < 0.05$ versus Rottlerin (ANOVA test). At least three independent experiments were performed with similar results. The data are presented as mean ± S.D. of three independent experiments.

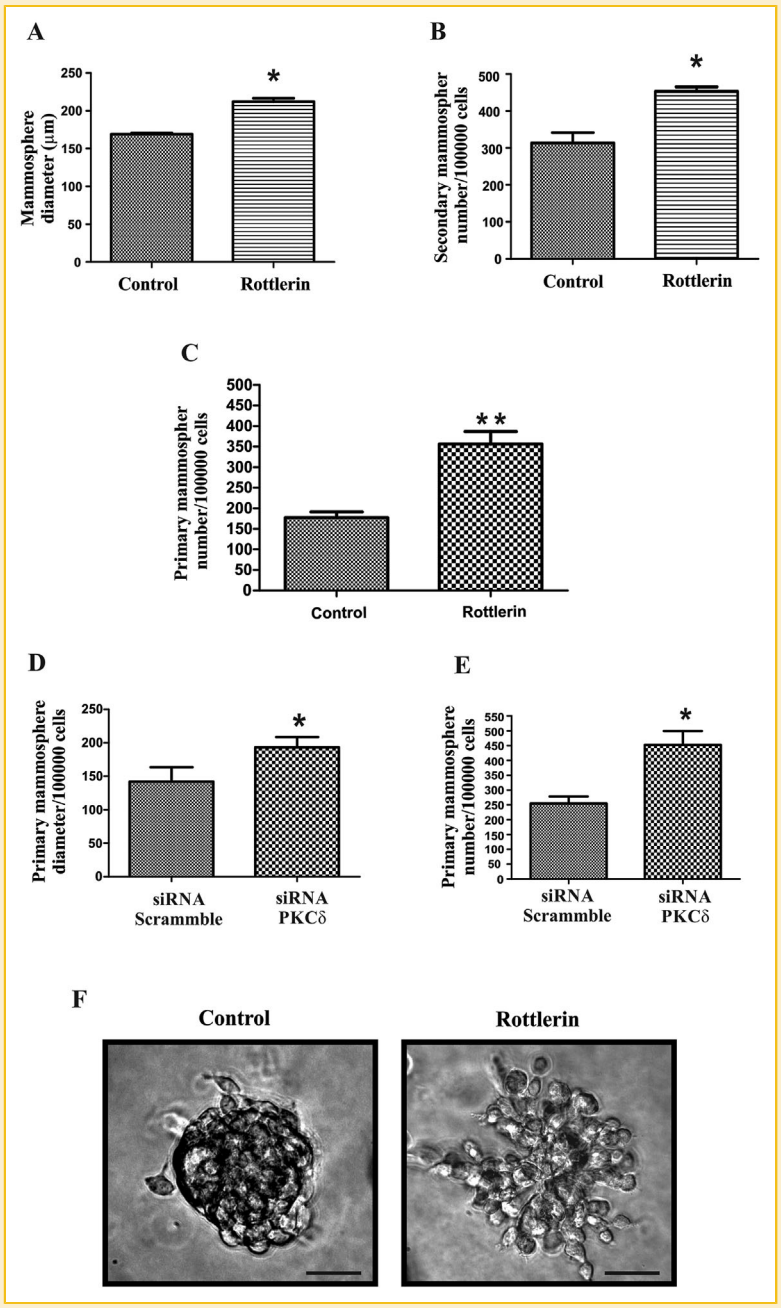

Fig. 4. Effect of Rottlerin in the proliferation and self-renewal of LM38-LP CSC. A: Primary mammospheres treated or not with Rottlerin were enzymatically dissociated in order to obtain a single-cell suspension. Cells were then seeded and secondary mammospheres were formed. The size of the secondary mammospheres was quantified. Each data point represents the mean ± S.D. of triplicate determinations, $^{*}P < 0.05$ versus control (Student's *t* test). At least three independent experiments were performed with similar results. B: Primary mammospheres treated or not with Rottlerin were enzymatically dissociated in order to obtain a single-cell suspension. Cells were then seeded and secondary mammospheres were formed. The number of the secondary mammospheres was quantified. Each data point represents the mean ± S.D. of triplicate determinations, $^{*}P < 0.05$ versus control (Student's *t* test). At least three independent experiments were performed with similar results. C: LM38-LP cells were treated or not with Rottlerin for 1 week and were enzymatically dissociated in order to obtain a single-cell suspension. Cells were then seeded and primary mammospheres were formed. The number of the primary mammospheres was quantified. Each data point represents the mean ± S.D. of triplicate determinations, $^{*}P < 0.05$ versus control (Student's *t* test). At least three independent experiments were performed with similar results. D: LM38-LP cells were transfected with PKCδ- or scramble-siRNA. Single-cell suspensions were cultured under mammosphere conditions and the diameter of primary mammospheres was analyzed. Each data point represents the mean ± S.D. of triplicate determinations, $^{*}P < 0.05$ versus control (Student's *t* test). At least three independent experiments were performed with similar results. E: LM38-LP cells were transfected with PKCδ- or scramble-siRNA. Single-cell suspensions were cultured under mammosphere conditions and the number of primary mammospheres was analyzed. Each data point represents the mean ± S.D. of triplicate determinations, $^{*}P < 0.05$ versus control (Student's *t* test). At least three independent experiments were performed with similar results. F: Primary LM38-LP mammospheres treated or not with Rottlerin were enzymatically dissociated in order to obtain a single-cell suspension. Cells were then seeded into Matrigel in order to obtain 3D cultures. Representative phase-contrast photographs of LM38-LP 3D cultures treated or not with Rottlerin are shown (×400 magnification, scale bar: 100 μm).

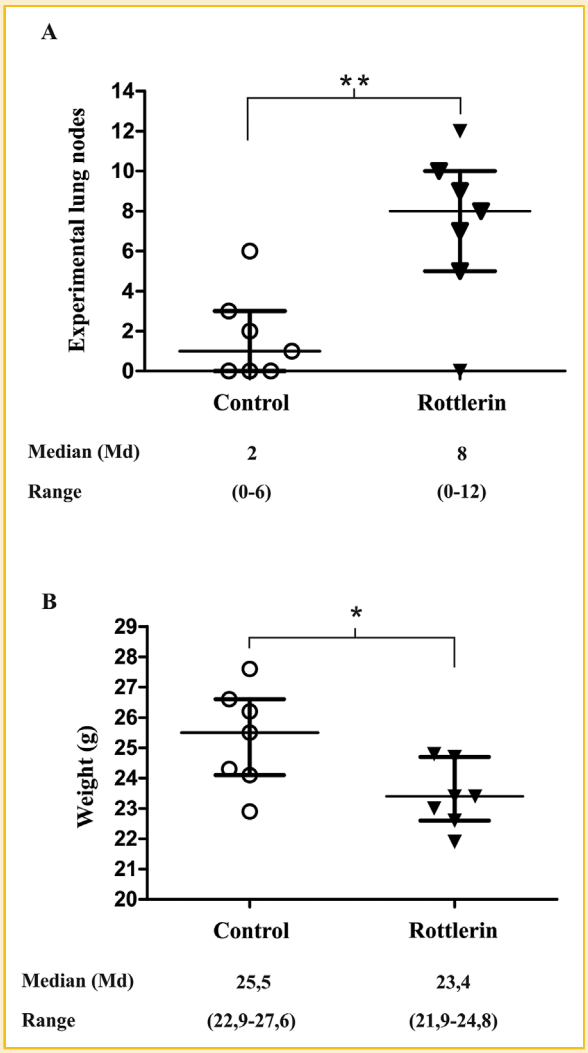

Fig. 5. Experimental lung metastasis assays and weight measurement. A: mammospheres derived cells were pretreated in vitro during 96 h with Rottlerin or DMSO as vehicle and then cells were i.v. inoculated into BALB/c mice. Twenty-one days post-inoculation, mice were sacrificed and the number of lung foci was recorded. The figure shows the results of one experiment representative of three independent assays $^{*}P < 0.05$ versus control, (Kruskal–Wallis test). B: The weight of mice that received mammosphere derived cells pretreated or not with Rottlerin was recorded at day 21 before sacrifice. The figure shows the results of one experiment representative of three independent assays $^{*}P < 0.05$ versus control, (Kruskal–Wallis test).

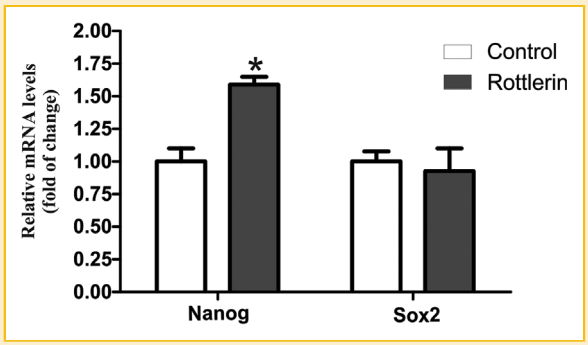

Fig. 6. Modulation of pluripotent genes expression. RNA from LM38-LP mammospheres treated or not with Rottlerin was isolated and expression of different pluripotent genes was analyzed by qPCR. Each data point represents the mean ± S.D. of triplicate determinations, $^{*}P < 0.05$ versus control (Student's t test). At least three independent experiments were performed with similar results.

AUTOPHAGY PROMOTED BY PKCδ ACTIVITY INHIBITION IS INVOLVED IN CSC SUBPOPULATION INCREMENT

Finally, with the aim to evaluate whether autophagy induced by PKCδ inhibition, was involved in the selection of CSC subpopulation, we perform a WB assay in order to analyze LC3-I–LC3-II levels. Interestingly, although mammosphere presented a baseline autophagy flux, evidenced by LC3II levels, Rottlerin treatment induced an increment of this molecule (Fig. 7A–B). In order to validate if autophagy is involved in CSC enrichment when PKCδ activity was inhibited, LM38-LP cells were pre-treated with Rottlerin and/or 3MA during 96 h in order to perform a mammosphere assay. We could observe that an increment in the diameter and number of mammospheres was reverted under 3MA treatment. This would suggest that autophagy, promoted by PKCδ impairment, would be necessary for CSC self-renewal induction (Fig. 7C–D).

DISCUSSION

Autophagy is a catabolic process, where cytoplasmic components are sequestered into autophagosomes and fused with lysosomes in other to degrade their content [Nezis et al., 2014].

Excessive autophagy and/or the downregulation of this process have been involved in the pathogenesis of several diseases [Mizushima et al., 2008]. Nevertheless, autophagy might have a dual role, since it supports both suppression and promotion of cancer growth [Gonzalez et al., 2014]. In similar manner, a dual role has been described for PKCδ, since this PKC isoform may function both as an inductor or as an inhibitor of cell growth in different cancer cell models [Grossoni et al., 2007] depending on cell type and stimulus [Chen et al., 2009].

Besides, PKCδ-dependent signal pathway not only represents a mechanism to induce autophagy to protect cells from stress conditions and a mechanism or to promote cell death in order to eliminate damaged cells, but it also provides a mechanism to switch or regulate the balance between cells survival and death.

In this paper, we have evaluated another dual role of PKCδ on the proliferation and maintenance of CSC through autophagy mechanism in a hormone-independent mammary cancer model LM38-LP.

The importance of this hormone-independent cell line lies in its unique characteristic that is the presence of luminal and myoepithelial cells, as well as a bi-potential progenitor CSC [Bumaschny et al., 2004; Berardi et al., 2015].

In order to evaluate PKCδ participation on cell growth, the proliferation rate was analyzed under Rottlerin treatment. An important decrease in LM38-LP cell number could be detected under these conditions. This inhibitory effect was associated with an increase of cells arrested in G1 phase of the cell cycle. These

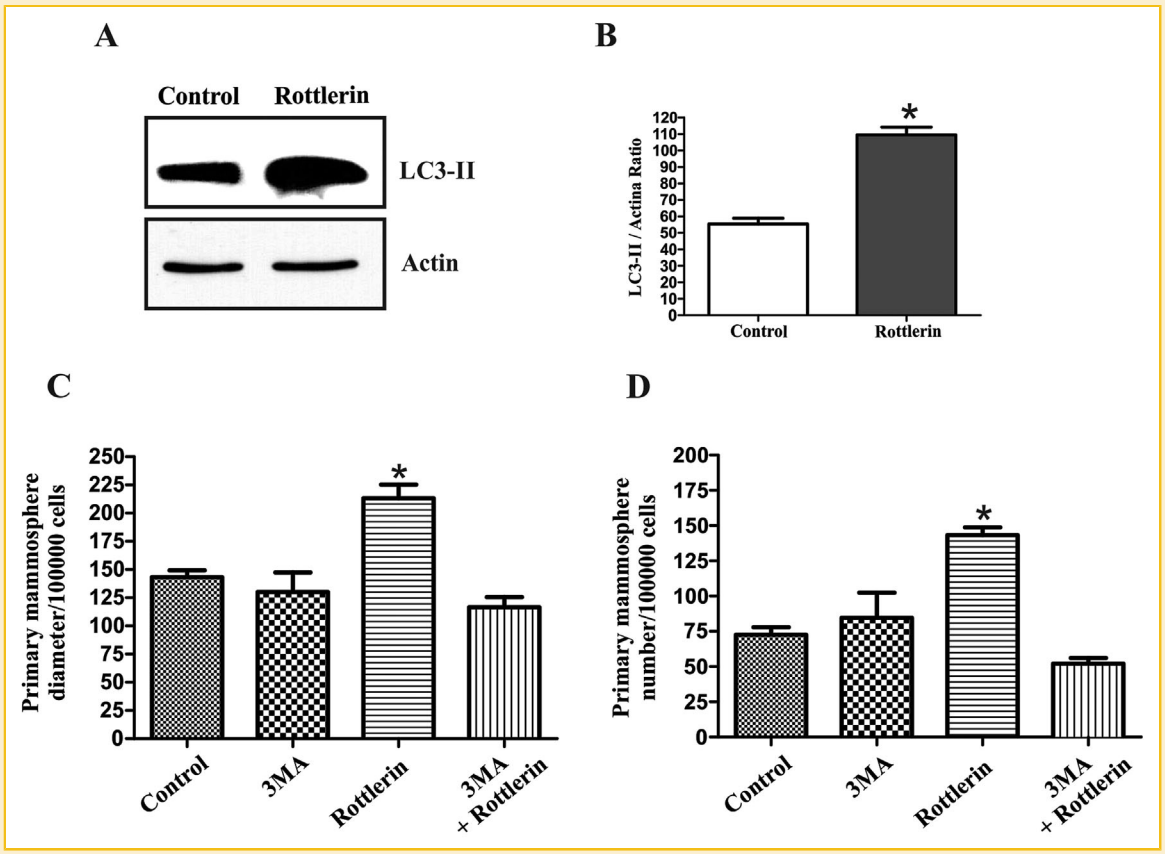

Fig. 7. Involvement of PKCδ in CSC induction thought autophagy. A: Immunoblot analysis of LC3 II levels. Mammospheres were incubated or not with Rottlerin. Whole cell lysates prepared from mammoespheres and were resolved in 10% SDS-PAGE and blotted with LC3 antibody. Actin immunoblotting was used as a loading control. B: densitometric analysis of A. Each data point represents the mean ± S.D. of triplicate determinations, $^{*}P < 0.05$ versus control (Student's *t* test). Results are representative of three independent experiments. The increase of LC3 was determined as the ratio between the LC3-II and Actin. C: LM38-LP cells were treated with Rottlerin and/or 3MA for 96 h. Single-cell suspensions from each treatment were cultured under mammosphere conditions and the number of primary mammospheres was analyzed. Each data point represents the mean ± S.D. of triplicate determinations, $^{*}P < 0.05$ versus control (ANOVA test). At least three independent experiments were performed with similar results. D: LM38-LP cells were treated with Rottlerin and/or 3MA for 96 h. Single-cell suspensions from each treatment were cultured under mammosphere conditions and the diameter of primary mammospheres was analyzed. Each data point represents the mean ± S.D. of triplicate determinations, $^{*}P < 0.05$ versus control (ANOVA test). At least three independent experiments were performed with similar results.

results are consistent with previous reports where PKCδ inhibition induced G1 phase cell cycle arrest in NCI-H28 cells [Okuwa et al., 2012].

Moreover, we could observe that PKCδ inhibition induced autophagy. A clear PKCδ dependence could be inferred since either pharmacological inhibition or PKCδ knockdown using siRNA promoted autophagy with similar intensity.

Interestingly, a recent report showed that Cyclin D1, a cell cycle regulator that functionally integrates multiple mitogenic signals and whose activity is required for G1/S transition, acts as an autophagy suppressor in mammary epithelium [Brown et al., 2012]. So, it would be possible that autophagy was promoted by the G1 phase cell cycle arrest, where Cyclin D1 levels are decreased.

The role of several members of the PKC family in autophagy regulation has been previously pointed out [Huang et al., 2010]. Here it was clearly demonstrated the participation of PKCδ in this process.

Next, we wondered whether the autophagy increment by PKCδ inhibition had a survival effect or might promote a type of programmed cell death different from apoptosis, termed type II programmed cell death [Berardi et al., 2011; Yang et al., 2013; Nagelkerke et al., 2014]. It has been reported that, while an excessive autophagy may promote cell death, mild autophagy induced by starvation or hypoxia, seems to have a cell protective effect [Amaravadi et al., 2011; Gonzalez et al., 2014].

Our results revealed that autophagy blockage synergize with the impairment of cell proliferation induced by PKCδ inhibition. Thus in our model, autophagy mechanism might have a survival effect, since the autophagy inhibition potentiates Rottlerin treatment effect.

Accumulating evidence support an essential role of autophagy in the maintenance, pluripotency, and the self-renewal of CSCs in several histological types of cancer including breast, glioblastoma, and pancreatic malignancies. [Ahmed Hamaï, 2014].

An accurate CSC model is critical for studying the importance of autophagy in CSC biology. Using LM38-LP cell line, we determined that mammospheres had higher PKCδ levels than monolayers and interestingly the pharmacological inhibition of this isoform significantly increased their diameter, as a measure of CSC growth potential.

Moreover, Rottlerin treatment increased the number of secondary mammospheres and Nanog expression, indicating both a rise in their self-renewal potential and the enrichment in CSC. Similar results in the number of mammospheres were obtained after PKCδ knockdown by siRNA. This result agrees with a recent study that showed that Nanog expression is downregulated by PKC [Chu et al., 2013].

Altogether these data suggested that PKC was also involved in maintaining a low rate of proliferation in the stem/progenitor component, emphasizing the dual role of this isoform.

On the other hand, major differences were found when LM38-LP CSC, treated or not with Rottlerin, were grown on Matrigel. As described by Kenny et al. [2007], the 3D structures formed under this culture conditions are representative of cell differentiation or their malignancy features. Among the morphological classes, LM38-LP cells form a "Mass" structure but, upon Rottlerin treatment 3D colonies switches to "Grape-Like" structures, usually associated with a malignant phenotype [Gong et al., 2013]. The increase in malignant potential, suspected by the above mentioned morphologic changes, was confirmed thought an experimental metastasis assay. Mice inoculated with CSC pretreated with Rottlerin showed significant increase metastasic nodes, and presented cachexia. It well-know that advanced stage of cancer (metastasic dissemination) induced paraneoplasic syndromes, like cachexia [Tarin, 2013].

Therefore we can hypothesize that PKCδ blockage reduces cell-cell adhesion, as observed in grape-like colonies. This may reflect the ability to break away from their neighbors in the primary tumor acquiring a metastatic phenotype.

Next we evaluated in CSC whether autophagy was induced by PKCδ inhibition, such as occurs in LM38-LP monolayers. We could observed as described by Gong et al. [2013], mammospheres had an elevated basal autophagy flux, but under PKCδ inhibition, an increase in autophagy could be detected. Finally, it was evaluated whether autophagy was involved in CSC maintenance by PKCδ activity. In fact, blocked autophagy was able to reverse the self-renewal capacity induced by Rottlerin treatment. This result is in line with an study where it describes that the autophagy regulator ATG4A was essential for the maintenance of a sub-population with cancer stem cell properties [Wolf et al., 2013].

In sum PKCδ exerts a dual role through the autophagy mechanism, decreasing proliferative capacity of mammary tumor cells but regulating tumor stem cell self-renewal.

An important lesson derived from these studies is that PKCδ exerts paradoxical responses that may greatly impact in the rationale design of isozyme specific PKC modulators as therapeutic agents. Although, PKCδ inhibitors could be useful in the management of breast cancer patients since they limit tumor growth under some circumstances, the surviving cells could present cancer stem features with higher malignant potential. Therefore, it is imperative to elucidate the molecular events controlling such disparate effects to avoid non-desired effects in a clinical setting.

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