Breast Cancer Res Treat (2009) 118:469–480
DOI 10.1007/s10549-008-0299-4

PRECLINICAL STUDY

Opposite effects of protein kinase C beta1 (PKCβ1) and PKCε in the metastatic potential of a breast cancer murine model

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Received: 16 October 2008 / Accepted: 24 December 2008 / Published online: 9 January 2009

Abstract In this paper we investigated whether protein kinase C (PKC) β1 and PKCε, members of the classical and novel PKC family, respectively, induce phenotypic alterations that could be associated with tumor progression and metastatic dissemination in a murine model of breast cancer. Stable overexpression of PKCβ1 in LM3 cells altered their ability to proliferate, adhere, and survive, and impaired their tumorigenicity and metastatic capacity. Moreover, PKCβ1 induced the re-expression of fibronectin, an extracellular matrix glycoprotein which loss has been associated with the acquisition of a transformed phenotype in different cell models, and exerted an important inhibition on proteases production, effects that probably impact on LM3 invasiveness and dissemination. Conversely, PKCε overexpression enhanced LM3 survival, anchorage-independent growth, and caused a significant increase in spontaneous lung metastasis. Our results suggest PKCβ1 functions as an inhibitory protein for tumor growth and metastasis dissemination whereas PKCε drives metastatic dissemination without affecting primary tumor growth.

Keywords PKCβ1 · PKCε · Metastasis dissemination · Tumor growth · Proliferation · Survival

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Introduction

The protein kinase C (PKC) family of phospholipid-dependent serine/threonine kinases influences diverse cell functions through phosphorylation of target proteins. These cell functions involve a wide variety of fundamental physiological processes including proliferation, differentiation, and apoptosis [1–3]. Molecular cloning and biochemical studies have revealed the presence of at least 11 PKC members that can be divided into three distinct classes based on differences in their structure and biochemical properties [4, 5]: classical PKC isozymes (α, β, and γ), which can be stimulated by Ca^{2+} and diacylglycerol or phorbol esters; novel PKC isozymes (δ, ε, η, and θ), which can be activated by diacylglycerol or phorbol esters but are Ca^{2+} independent; and atypical PKC isozymes (ζ and λ/ι), which are unresponsive to Ca^{2+}, diacylglycerol and phorbol esters [1, 3, 6].

Changes in the expression of PKC isozymes have been reported in numerous human cancers including lung, colon, and breast [2, 7–10], and in many instances a correlation between elevated PKC protein levels and aggressiveness has been reported [11–13]. PKCβ1 displays a controversial role in malignant transformation. PKCβ1 expression is increased in human prostate cancer [14]. Its overexpression in rat fibroblasts resulted in transformed cells that exhibit anchorage-independent growth properties and are capable of forming tumors when inoculated into nude mice [15]. PKCβ1 is also involved in the attenuation of the pro-apoptotic responses in gastric and hepatocellular carcinomas [16, 17]. In contrast, PKCβ1 overexpression reduces the tumorigenic potential of HT29 and SW480 colon cancer cells in nude mice [15, 18], induces growth arrest and apoptosis of myeloid cells [19], and is also down-regulated during melanocyte transformation [20].

PKCε has been shown to be a transforming oncogene in fibroblasts and epithelial cells. In this regard, PKCε overexpression in NIH 3T3 fibroblasts and in rat colonic epithelial cells enhances proliferation and anchorage-independent growth, as well as it confers a highly tumorigenic in vivo phenotype [15]. Inhibition of PKCε in MDA-MB231 breast carcinoma cells drastically reduces tumor growth and metastasis development [15]. Recent evidences indicate that PKCε expression is elevated in tissue biopsies of breast, and prostate cancer patients [15, 21]. PKCε expression and/or activation provide a pro-survival signal in many different types of cancer cells, as well as it contributes to resistance of cancer cells to chemotherapy or irradiation [21, 22]. Transgenic mice overexpressing PKCε in skin develop squamous cell carcinoma and have enhanced sensitivity to UVR-induced cutaneous damage. It has been proposed that PKCε induces the activation of Stat3 which in turn induces the expression of Stat3-regulated genes (i.e., *c-myc*, *cyclin D1*, *cdc25A*, and *COX-2*) triggering an uncontrolled cell growth [23].

The extracellular matrix (ECM) constitutes an important non-cellular compartment of tumor microenvironment which not only serves as a barrier against tumor invasion but also represents a reservoir of cell binding proteins and growth factors. Loss of fibronectin (FN) matrix assembling-capacity is observed in several oncogene-transformed cells [24]. Moreover, FN loss is associated with reduced cell adhesion, alterations in cytoskeletal organization, and increased tumorigenicity and malignancy in vivo [25]. ECM degradation is dependent on proteolytic enzymes such as urokinase-type plasminogen activator (uPA) [26, 27] and matrix metalloproteinases (MMPs) [28, 29], which are capable of digesting ECM components under physiological conditions and are also involved in cancer invasion and metastasis [29]. Classical and novel PKC isoforms have been associated with an enhanced expression of uPA and MMPs [30–32], both during mammary gland differentiation and involution [33] as well as in cancer initiation and progression [34].

In this study we focused on how PKCβ1 and PKCε regulate mammary cancer progression. As mentioned before, while PKCε is considered a transforming oncogene, including in breast cancer, the precise role of PKCβ1 in the progression of mammary cancer has not been extensively studied yet. As an experimental approach we overexpressed both PKCs in LM3 cells, an established model of murine mammary adenocarcinoma-derived cells developed in our laboratory [35]. Our results revealed that while PKCε overexpression markedly increased lung metastasis, PKCβ1 caused a significant reduction in tumor growth and metastasis development. Interestingly, PKCβ1 overexpression in LM3 cells not only induced the re-expression of FN, but also exerted profound inhibitory effects on the production of proteases involved in invasiveness and metastasis.

Materials and methods

Reagents and antibodies

Media for cell culture, agarose, G418, and Lipofectamine Plus were from Gibco BRL Laboratories (Rockville, MD). Oligofectamine was obtained from Invitrogen (Carlsbad, CA). Fetal calf serum was from GBO (Buenos Aires, Argentina). Plasminogen was purchased to Chromogenix (Molndal, Sweden). Human urokinase was a gift from Serono (Buenos Aires, Argentina). Triton X-100 was obtained from J. T. Baker (Phillipsburg, NJ). Monoclonal anti-PKCα, β, δ, and ζ, and anti-retinoblastoma protein (RB) and phospho-RB antibodies (pRB, Ser 795) were purchased from BD Biosciences (San Diego, CA). Monoclonal antibodies for ERK, phospho-ERK (pERK), and β1-integrin, as well as polyclonal antibodies for actin and cyclin D1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies for Akt, phospho-Akt (pAkt, Ser 473), focal adhesion kinase (FAK) and phospho-FAK (pFAK, Tyr 576/577) were purchased from Cell Signaling Technology (Beverly, MA). Anti-fibronectin antibodies, acrylamide, horseradish peroxidase conjugated anti-mouse antibodies and PMA were obtained from Sigma (St Louis, MO). Hybond-P membranes and chemiluminescence reagents (ECL) were from GE Healthcare Bio-Sciences (Little Chalfont, UK). Other reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad (Richmond, CA).

Cell line and culture conditions

The LM3 cell line was established from primary cultures of the spontaneous mammary adenocarcinoma M3 [35]. While M3 tumor and primary cultures were able to express and deploy FN in the ECM, once this cell line was established, no FN could be detected either in the ECM or in the culture media [35]. LM3 cells were cultured at 37°C in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and 80 μg/ml gentamycin in a humidified atmosphere with 5% CO_2.

Expression vectors, transfection and selection

LM3 cells were stably transfected with 5 μg of expression vectors encoding for PKCβ1 and PKCε in pMTH vector (LM3-PKCβ1 and LM3-PKCε, respectively) using Lipofectamine Plus. LM3 cells transfected with the empty vector were used as control (LM3-vector). Forty-eight hours after transfection, cells were selected using 500 μg/ml

G418. After selection, resistant clones were pooled to avoid clonal variations [36].

Western blot

Semiconfluent monolayers were washed twice with ice-cold PBS and then lysed with 1% Triton X-100 in PBS by scrapping with a teflon scrapper. Samples were denatured by boiling in sample buffer with 5% β-mercaptoethanol and run in 10% SDS–PAGE (6% for RB, FAK and β1-integrin detection). A volume of 50 μg of protein were loaded in each lane. Gels were blotted to Hybond-P membranes. Membranes were incubated for 1 h with PBS containing 5% skim-milk plus 0.1% Tween-20. Membranes were incubated with the first antibody overnight at 4°C, and then with a secondary antibody coupled to horseradish peroxidase. Detection was performed by ECL. Bands were quantified by scanning on a digital GS-700 densitometer and Molecular Analyst software (Bio-Rad).

Immunofluorescence

Semiconfluent monolayers grown on glass coverslides were fixed with 10% formaldehyde in PBS for 15 min, blocked with 5% BSA in PBS for 30 min and then incubated at room temperature for 1 h with an anti-FN antibody in a moist environment. Cells were incubated for 1 h with a secondary FITC-conjugated antibody, and coverslips were then mounted onto microscope slides.

Animals

For the in vivo assays, randomized inbred female BALB/c mice, 2–4 months old, obtained from the Animal Care Area of the Institute of Oncology "A. H. Roffo" were employed. Food and water were administered ad libitum. All animal studies were conducted in accordance with the NIH "Guide for the Care and the Use of Laboratory Animals".

Tumorigenicity and metastasis studies

Analysis of tumor cell growth and metastasis was performed essentially as described [35]. Briefly, trypsinized cells were resuspended in MEM at a final concentration of 1×10^6 cells/ml. A volume of 0.2 ml of each transfectant-cell suspension was injected s.c. in the left flank of female BALB/c mice. The size of the tumors was measured with a sliding caliper twice a week by measuring length (L) and width (W) and then calculating the volume using the formula $V = \frac{3}{4}(\pi \times L \times 2W)$, where L is the longest and W is the shortest diameter. Latency was defined as the time between the injection of tumor cells and the palpation of external tumors in 50% of the mice. Mice were sacrificed 35 days after tumor inoculation and lungs removed and fixed in Bouin's solution. The number and size of surface lung metastatic nodules was determined under a dissecting microscope. Tumors were fixed in 10% formalin and embedded in paraffin. A total of 5 μm sections were stained with hematoxilyn and eosin for histopathological studies. Liver, kidney, and spleen were also examined for the presence of metastatic nodules.

To study the experimental lung metastasizing ability, 2×10^5 cells were injected into the tail vein of syngeneic mice using a 27-gauge needle. Mice were monitored daily and sacrificed 21 days later. Lungs were removed and the number and size of superficial lung colonies were recorded as mentioned above. 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.

Cell proliferation

Population doubling-time was determined by assessing cell number during the exponential growth phase of unsynchronized monolayer cultures. Briefly, 1×10^5 cells were seeded onto 35 mm multiplates in MEM supplemented with 10% FCS, 80 μg/ml gentamicin, and 250 μg/ml G418. At different times after seeding, cells from triplicate wells were washed twice with PBS, trypsinized, and counted using a hemocytometer and trypan blue exclusion.

Clonogenic assay

Clonal cell growth was studied as previously described [36]. Briefly, single-cell suspensions containing 500–1,000 cells were seeded onto 60 mm plastic dishes in MEM with 10% FCS. Medium was changed every 72 h. After 10 days of culture, cells were washed, fixed with methanol, and stained with hematoxylin. The number of colonies was determined using an inverted microscope. Plating efficiency was defined as the percentage of cells able to grow as colonies having more than ten cells.

Effect of serum starvation on cell survival

Subconfluent monolayers in 96-well plates were subjected to serum starvation (48 h in serum-free MEM). Cell viability was evaluated with the MTS assay (Celltiter 96^{TM} Non Radioactive Proliferation Assay, Promega), as described by the manufacturer.

Anchorage-independent growth

For soft agar assays, 60 mm Petri dishes were prepared with 2 ml base feeder layer of 0.6% agar in complete

medium and a semisolid top layer (0.3% agar in complete medium) containing 10^5 monodispersed cells detached from log phase cultures. Fifteen days after seeding, cultures were fixed with 10% formaldehyde in PBS, and the number of colonies with more than 20 cells was determined using an inverted microscope. Colony efficiency was determined as the percentage of cells able to form colonies in soft agar.

Adhesion assay

Suspensions of 4×10^5 cells were seeded onto 35 mm Petri dishes in FCS-free culture media and incubated at 37°C. After 60 min the medium was removed and adherent cells were washed twice with PBS, trypsinized, and counted using a hemocytometer. Determinations were carried out by triplicate. Adhesion efficiency was expressed as the percentage of the total number of cells seeded.

β1-integrin RNAi

A β1-integrin RNAi duplex (Invitrogen; Carlsbad, CA) was transfected into LM3-PKCβ1 cells following the manufacturer's instructions. The following sequence was used: AAUAGGGUAAUCUUCAGCCCUCUUG. An irrelevant RNAi duplex was used as control: AAGGUAUUGACAG GGAUCUGA. Twenty-four hours after transfection, cells were detached and used to evaluate proliferation, adhesion, clonal, and anchorage-independent growth following the same protocols described above.

Quantification of uPA and MMPs activities of conditioned media (CM)

Semiconfluent cell monolayers growing in 35 mm plastic Petri dishes were extensively washed with PBS. Serum-free medium (1 ml) was added and incubation was continued for 24 h. CM were harvested, the remaining monolayers were lysed with 1% Triton-X100 in PBS, and cell protein content was determined using the Bio-Rad Protein Assay. CM samples were stored at −40°C and used only once after thawing. To determine uPA activity, a radial caseinolysis assay was used, as previously described [37]. Briefly, 4 mm wells were punched in the plasminogen-rich casein-agarose gels and 10 µl of CM were loaded. Gels were incubated for 24 h at 37°C in a humidified atmosphere. The diameter of lytic zones was measured, and the areas of degradation were referenced to a standard curve of purified urokinase (0.1–50 IU/ml) and normalized to the original cell culture protein content. Specificity of uPA activity was determined by blocking its activity with B428 (18 µM), a uPA specific catalytic inhibitor [38].

MMPs activity was determined on substrate-impregnated gels as previously described by Aguirre-Ghiso et al. [39]. Briefly, CM samples were run on 9% SDS polyacrylamide slab gels containing 1 mg/ml of gelatin, under non-reducing conditions. After electrophoresis, gels were washed for 30 min in 2.5% Triton x-100 and subsequently incubated for 48 h at 37°C in a buffer containing 0.25 M Tris–HCl pH 7.4, 1 M NaCl, and 25 mM $CaCl_2$. Non-specific activity was detected using gels incubated in the same buffer solution but supplemented with 40 mM EDTA. After incubation, gels were fixed and stained with Coomasie Brilliant Blue G-250. Gelatinolytic bands were measured using a digital densitometer (Bio-Rad GS-700) and data were expressed as arbitrary units and normalized to the original cell culture protein content.

Results

Overexpression of PKCβ1 and PKCε in LM3 cells

In order to investigate whether the overexpression of the classical (β1) and novel (ε) PKC isozymes can modulate malignant phenotype and metastatic potential of LM3 cells, a murine mammary tumor-derived cell line, we used a stable transfection approach. Overexpression of both PKC isoforms was achieved by transfecting LM3 cells with the expression vectors pMTH-PKCβ1 and pMTH-PKCε followed by selection with G418. To avoid clonal variations, G418-resistant clones were pooled 2 weeks after transfection. Western blot analysis revealed a 10.4- and 4.3-fold increase in PKCβ1 and PKCε expression levels, respectively, relative to vector transfected cells (Fig. 1). Overexpression of PKCβ1 and PKCε did not alter the expression of other PKC isozymes expressed by LM3 cells (Fig. 1).

Contrasting effects of PKCβ1 and PKCε in tumor and metastasis development

To determine whether overexpression of the different PKC isoforms alters in vivo cell behavior, LM3 transfectants were inoculated s.c. in syngeneic BALB/c mice. LM3-PKCβ1 cells induced the formation of tumors that, compared with vector transfected cells, displayed longer latency, a decreased tumor take, and a significant reduction in the growth rate (Fig. 2a; Table 1). These alterations in tumor behavior were also associated with an important reduction both in the incidence and number of spontaneous and experimental lung metastasis (Fig. 2b, c; Table 1). On the other side, inoculation of PKCε overexpressors did not cause significant changes in tumor growth but significantly increased the incidence and number of spontaneous and experimental lung metastases relative to control cells (Fig. 2a–c; Table 1). Histopathological analysis revealed

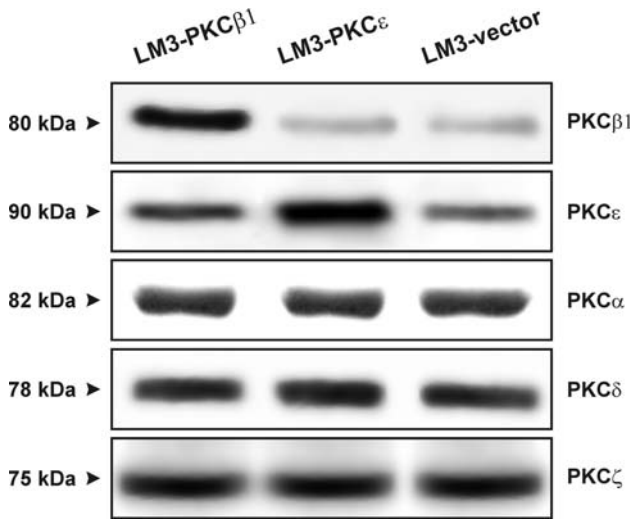

Fig. 1 PKCβ1 and PKCε overexpression in LM3 cells. Whole cell lysates prepared from LM3 cells transfected with pMTH-PKCβ1, pMTH-PKCε or pMTH were resolved on 10% SDS–PAGE and blotted with different antibodies against individual PKC isozymes (50 μg/lane). A representative experiment is shown. Similar results were observed in three independent experiments

that in all cases mice developed very poorly differentiated adenocarcinomas constituted by solid cords of heterogeneous epithelioid cells almost devoid of stroma around them. Moderate local invasiveness, with the tumoral cells invading dermis and muscular tissues, was observed in all cases. No differences between control and PKC-transfected cells were found in the amount and type of infiltrating host cells in and around the primary tumors or in the metastatic foci in lungs (data not shown).

Effect of PKCβ1 and PKCε overexpression on LM3 cell proliferation in vitro

Next, we analyzed the proliferative capacity of LM3 transfectants in unsynchronized monolayer cultures. Unexpectedly, LM3-PKCβ1 cells proliferated at a higher rate, as revealed by an important reduction in doubling time (DT) relative to LM3-vector cells (LM3-PKCβ1: 18.24 ± 1.4 h; LM3-vector: 22.4 ± 0.9 h, $P < 0.05$). On the other hand, PKCε overexpression did not alter DT (23.1 ± 1.2 h, $P = 0.65$) (Fig. 3a). We also explored whether PKC isozyme overexpression conferred an advantage under more stringent growth conditions such as clonal cell growth. As shown in Fig. 3b, PKCβ1 transfectants showed higher clonogenic ability than control cells and PKCε overexpressors. Analysis of mitogenic signaling pathways in vitro by Western blot revealed that, compared to vector transfected cells, PKCβ1 overexpressors have elevated cyclin D1 levels, as well as enhanced pRb and pERK. These effects were not observed in PKCε overexpressors (Fig. 3c).

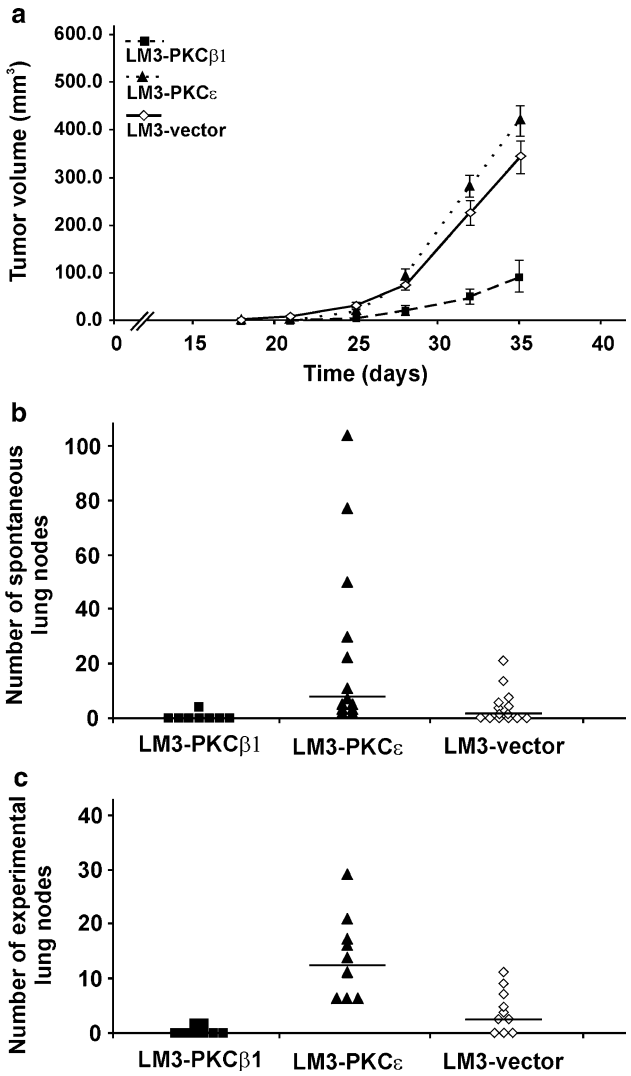

Fig. 2 **a** In vivo tumor growth of LM3 PKC-transfected cells. LM3 control and PKC transfectants were harvested from subconfluent cultures and inoculated s.c. in the left flank of BALB/c mice. The size of the two perpendicular diameters was recorded and used to calculate tumor volume. Tumor growth was followed up for 35 days. **b** Spontaneous lung metastatic capacity. To investigate the presence of spontaneous metastases, tumor-bearing mice were sacrificed and lungs were removed and fixed in Bouin's solution. The number and size of surface lung nodules was determined under a dissecting microscope. The *figure* shows the results of one experiment representative of three independent assays. *$P < 0.05$ versus control cells. Kruskal–Wallis test. **c** Experimental lung metastasizing capacity. LM3 transfectants were i.v. inoculated into BALB/c mice and after 21 days mice were sacrificed and the number and size of lung foci was recorded. The *figure* shows the results of one experiment representative of three independent assays. *$P < 0.05$ versus control cells. Kruskal–Wallis test

Role of PKCβ1 and PKCε overexpression in anchorage-independent growth and survival of LM3 cells

The acquisition of resistance towards cell death constitutes an important feature of cancer cells. This prompted us to

Table 1 Tumor growth and dissemination parameters

	LM3-PKCβ1	LM3-PKCε	LM3-vector
Latency (days) Median (range)	18 (11–21)*	5.5 (4–11)*	12.5 (4–14)
Tumor take (%) (mean ± S.E.)	35 ± 10*	90 ± 14	85 ± 21
Volume doubling time (h)	55.2 ± 5#	44.2 ± 7	46.7 ± 5
Spontaneous lung metastasis[a]			
Incidence (%) (mean ± S.E.)	14 ± 2*	90 ± 0*	57 ± 6
Number Median (range)	0 (0–4)#	5 (1 to >100)#	1 (0–34)
Experimental lung metastasis			
Incidence (%) (mean ± S.E.)	40 ± 3*	100 ± 0*	70 ± 5
Number Median (range)	0 (0–2)#	12 (6–28)#	2 (0–11)

Each group consisted of at least ten female mice. Values are the average of two independent experiments. * $P < 0.05$ versus LM3-vector, Chi Square Test, # $P < 0.05$ versus LM3-vector, Kruskal–Wallis test

[a] Only those animals that developed s.c. tumors were considered for the evaluation of spontaneous lung metastases incidence and number

explore whether PKCs could alter survival of LM3 cells in response to serum-starvation. Figure 4a shows that overexpression of PKCβ1 leads to a marked reduction in LM3 cell resistance to serum starvation. On the other hand, PKCε overexpression induced a slight, not statistically significant, increase in cell death resistance compared to control LM3-vector cells.

As the PI3K effector Akt is a crucial mediator of cell survival, we assessed the levels of pAkt by Western blot. Basal pAkt levels were significantly higher in LM3-PKCε cells relative to LM3-vector cells; however, no appreciable changes in basal pAkt levels were observed in LM3-PKCβ1 cells (Fig. 4b). No significant changes were observed in total Akt levels in any case.

The ability to grow independently of substrate attachment represents a hallmark of cell transformation. Thus, we next assayed whether PKCβ1 or PKCε overexpression could alter LM3 anchorage-independent growth in a soft agar assay. Even though control LM3 cells, either untransfected (data not shown) or vector-transfected, are able to grow and form colonies in soft agar, overexpression of PKCβ1 severely impaired anchorage-independent growth, while on the other hand PKCε overexpression caused a remarkable elevation in the number and size of colonies in soft agar (Fig. 4c).

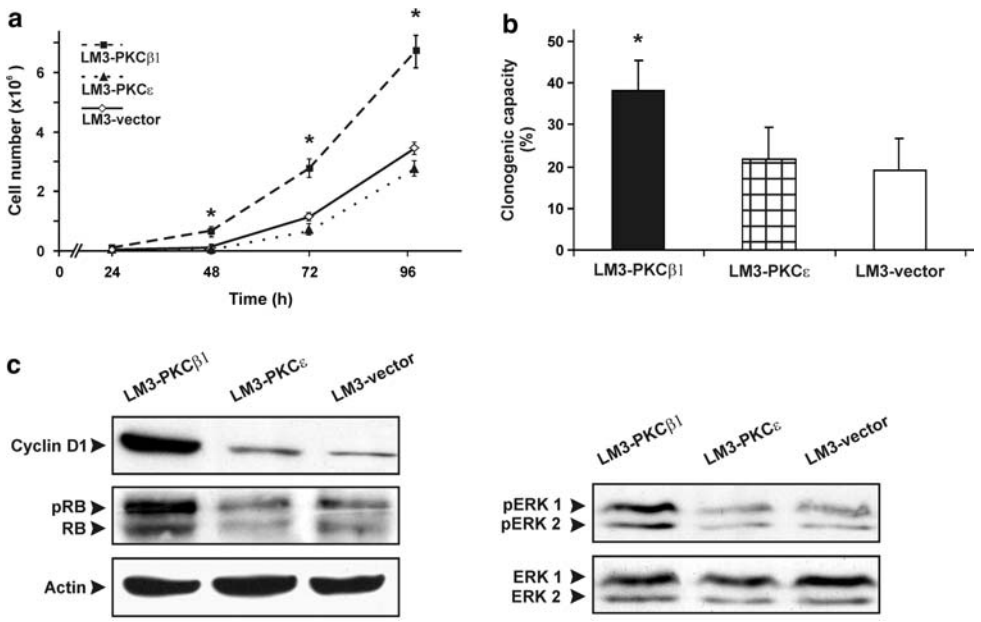

Fig. 3 **a** Effect of PKCβ1 and PKCε on cell population doubling time. Cell number was assessed at different times during the exponential growth phase of unsynchronized LM3 transfected cultures. Each data point represents the mean ± SD of triplicate determinations. *$P < 0.05$ versus LM3-vector cells (Student's *t* test). The *figure* shows the results of one experiment representative of three independent assays. **b** Clonogenic capacity. Clonal cell growth was studied as previously described in Sect. "Materials and methods" in PKCβ1 and PKCε overexpressors. Colony number was assessed using an inverted microscope 10 days after seeding. Data are expressed as the mean ± SD of triplicate determinations. Three independent experiments gave similar results. *$P < 0.05$ versus LM3-vector cells (Student's *t* test). **c** Expression of mitogenic pathway-related molecules. Cell lysates prepared from LM3-PKCβ1, LM3-PKCε, and vector-transfected cells, were subjected to Western blot analysis using antibodies against cyclin D1, pRB, and pERK (50 μg protein/lane). A representative experiment is shown. Similar results were observed in three independent experiments

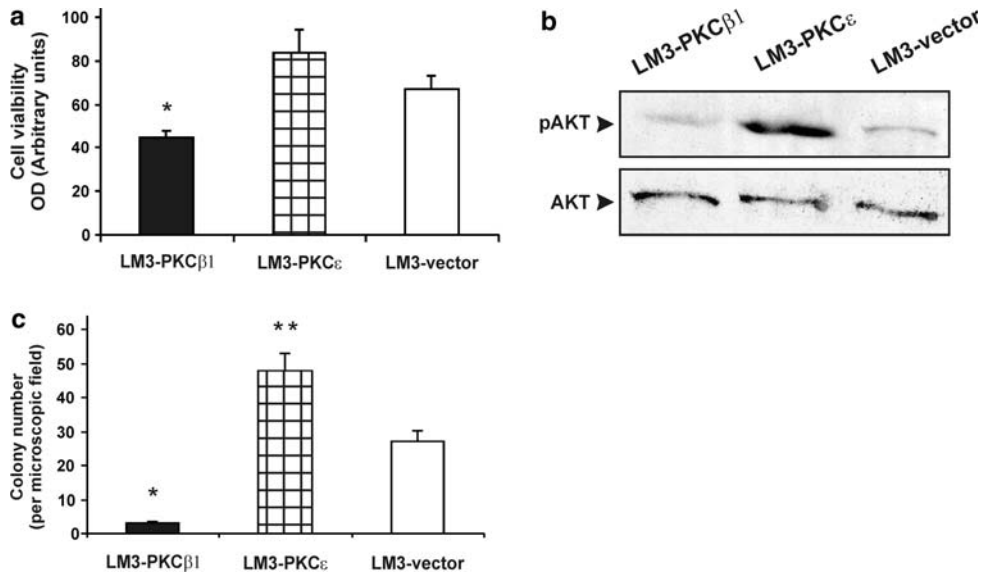

Fig. 4 **a** Effect of PKCβ1 and PKCε on cell death susceptibility. Cells were deprived of serum for 48 h and viability was evaluated using the MTS assay. Data are expressed as the mean ± SD of triplicate determinations. The *figure* shows the results of one experiment representative of three independent assays. *$P < 0.05$ versus LM3-vector cells (Student's *t* test). **b** Evaluation of pAkt levels in PKC overexpressors. Cell lysates prepared from control and PKC overexpressing cells were subjected to Western blot analysis using anti-pAkt and anti-Akt antibodies (50 µg protein/lane). Results are representative of three independent experiments. **c** Effect of PKC overexpression on LM3 anchorage-independent growth. LM3 transfectants were seeded in agar and colony number was assessed using an inverted microscope after 15-day culture. Data are expressed as the mean ± SD of triplicate determinations. The *figure* shows the results of one experiment representative of three independent assays. *$P < 0.05$ versus LM3-vector cells, **$P < 0.05$ versus LM3-PKCβ1 and LM3-vector cells (Student's *t* test)

PKCβ1 overexpression increases adhesiveness and induces the re-expression of FN in LM3 cells

The observed higher ability of LM3-PKCβ1 cells to proliferate in monolayer could be the consequence of an increase in their adhesiveness favoring an earlier entrance in cell cycle. This possibility prompted us to examine the adhesive behavior of the different cell lines. Our analysis revealed that 60 min after plating a significantly higher number of PKCβ1-overexpressors adhered to the plastic surface relative to control cells, while no differences in adhesiveness could be detected for LM3-PKCε cells (Fig. 5a). Time-dependence analysis of adhesion revealed that 100% of LM3-PKCβ1 cells were adhered at 5 h while it took more than 10 h for PKCε- and vector-transfected cells to achieve full adhesion (data not shown). Analysis in the expression of adhesiveness-related molecules by Western blot revealed an important increase in β1-integrin and active FAK levels in PKCβ1 overexpressing LM3 cells (Fig. 5b). In addition, immunofluorescence assays revealed that PKCβ1 expression was associated with the presence of FN fibrils organized in an incipient ECM, which were absent in LM3-vector and LM3-PKCε cells (Fig. 5c).

Role of β1-integrin knock-down on in vitro growth properties of LM3-PKCβ1 cells

In order to evaluate whether the increased ability of PKCβ1 transfectants to adhere and proliferate could be consequence of increased adhesiveness, we performed β1-integrin RNAi depletion in LM3-PKCβ1 cells. Using this methodology we achieved a 70% reduction in β1-integrin levels as compared with cells transfected with a control RNAi (Fig. 6a). The reduction in β1-integrin levels was clearly associated with a decrease in the ability of PKCβ1-overexpressors to adhere to the plastic surface. Under these conditions, LM3-PKCβ1 adhesive capacity was reduced by 51% (Fig. 6b) and it is in the same range as those from control and LM3-PKCε cells (compare Figs. 6b, 5a). The proliferative and clonogenic capacities of LM3-PKCβ1 cell monolayers were affected by β1-integrin down-modulation, thus indicating that this ECM receptor affects adhesive behavior which in turn modulates proliferative and clonogenic capacities (Fig. 6c, d). As expected, the low anchorage-independent growth capacity displayed by LM3-PKCβ1 cells was not modulated by the inhibition of β1-integrin expression (data not shown).

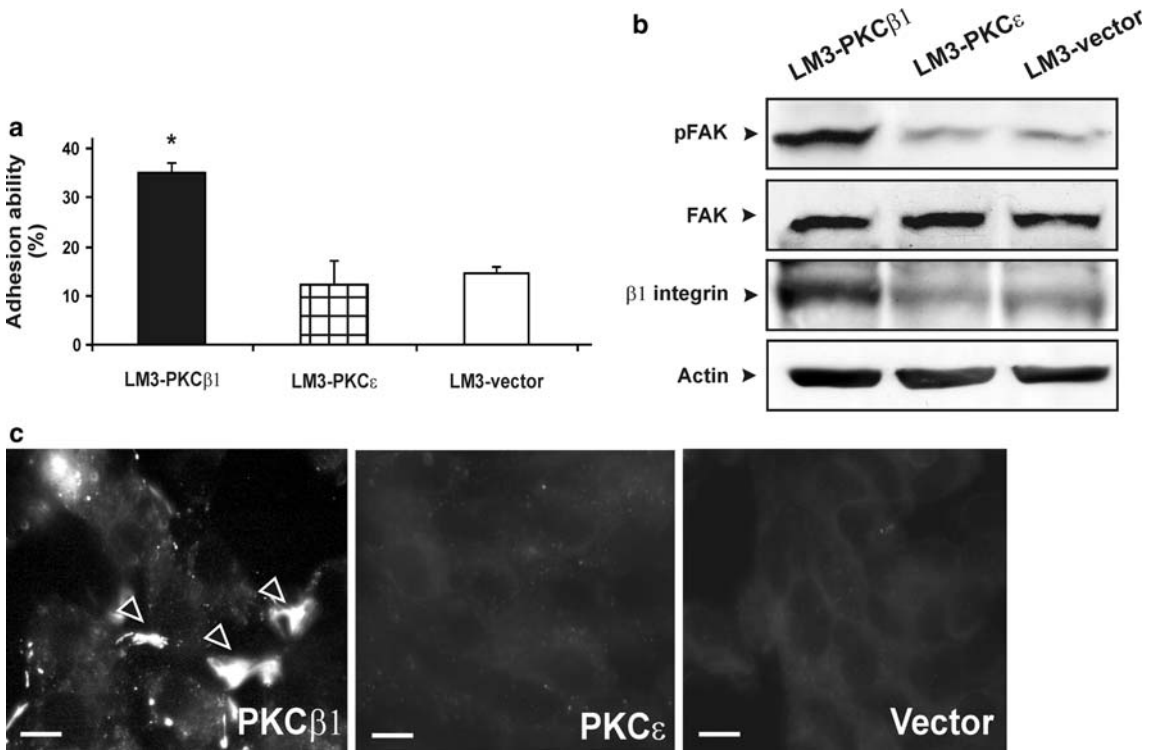

Fig. 5 **a** Effect of PKC overexpression on LM3 adhesive capacity. Control and PKC transfectants were assayed for their adhesive capacity as described in Sect. "Materials and methods". The rate of adhesion was expressed as the percentage of the total number of cells seeded. Data are expressed as the mean ± SD of triplicate determinations. Three independent experiments gave similar results. $^{*}P < 0.05$ versus LM3-vector cells (Student's t test). **b** Regulation of adhesiveness-related molecules. Cell lysates prepared from LM3-PKCβ1, LM3-PKCε, and vector-transfected cells, were subjected to Western blot analysis using antibodies against pFAK and β1-integrin (50 μg protein/lane). A representative experiment is shown. Three independent experiments gave similar results. **c** Detection of FN by immunofluorescence. Vector and PKC transfectant cells were plated on glass coverslips. Forty-eight hours after seeding, monolayers were fixed and immunostained using anti-FN antibody as described in Sect. "Materials and methods". *Photographs* shown are representative of three independent experiments. *Scale bar* 20 μm

PKCβ1 overexpression down-modulates the production of proteases in LM3 cells

Finally, we assessed the effect of overexpressing PKCs on the production of proteases. Interestingly, we observed a marked decrease both in uPA and MMP-2 secreted activity in LM3-PKCβ1 cells (Fig. 7a, b, d). On the other hand, PKCε overexpression did not cause any noticeable effect on the production of uPA and MMPs. The effect seems to be specific for these proteases, as MMP-9 activity was not changed either in PKCβ1 or PKCε transfectants (Fig. 7a, c).

Discussion

Our studies report for the first time that overexpression of PKCβ1 in mammary cells greatly impacts their ability to form tumors and metastasize. Using the metastatic murine mammary tumor model LM3 [35] we found that overexpression of this PKC causes marked phenotypic changes in LM3 cells. The effects observed are in sharp contrast to those found upon overexpression of the novel PKCε, a PKC isozyme that has been widely implicated in tumorigenesis, including prostate, skin, and breast tumors, and that has been found to be up-regulated in various types of cancers as well as linked to poor prognosis in some cases, such as in breast cancer [15].

PKCβ has been implicated in several types of cancer including prostate, colon, and hematological cancers. On the other hand, the precise role for this PKC in the modulation of mammary tumor growth and dissemination is not well understood. PKCβ has been proposed as a target for cancer therapy, and there are several clinical trials for the PKC beta inhibitor Enzastaurin; however, none of them involves mammary cancer patients.

Overexpression of PKCβ1 in LM3 cells leads to an important reduction in their local tumor growth and metastatic capacities, while elevated PKCε levels exert only an important increase in LM3 cell metastasis without affecting primary tumor growth. This last in vivo effect paralleled phenotypic changes observed in vitro, including alterations in cell survival and anchorage-independent growth. These

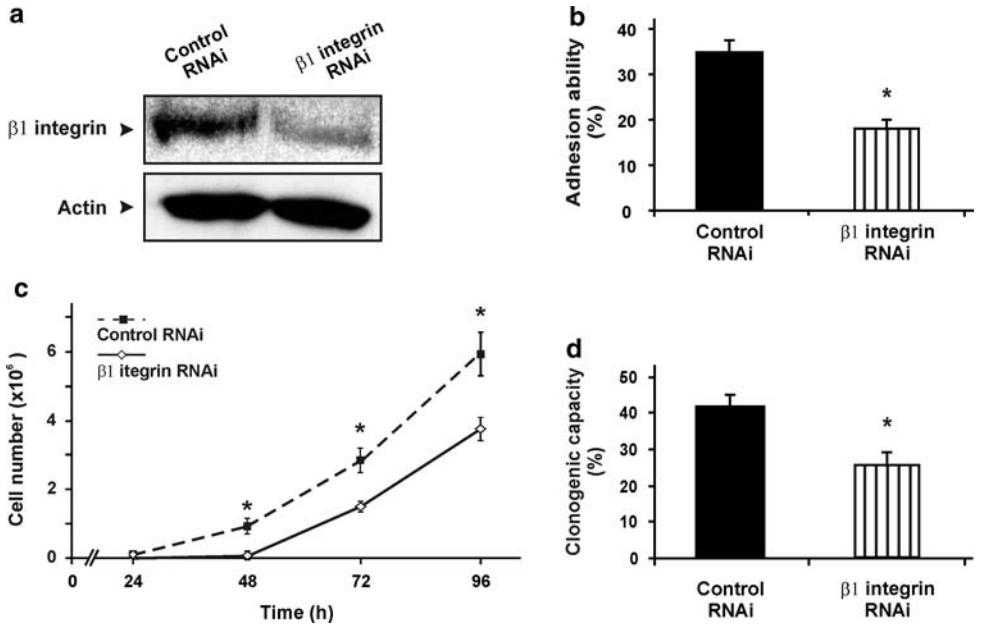

Fig. 6 **a** β1-integrin knock-down in LM3-PKCβ1 cells. LM3-PKCβ1 cells were transfected with either β1-integrin or a control RNAi. Forty-eight hours after transfection cells were harvested and subjected to Western blot analysis using antibodies against β1-integrin (50 μg protein/lane). A representative experiment is shown. Three independent experiments gave similar results. **b** Effect of β1-integrin RNAi on LM3-PKCβ1 adhesive capacity. Control and β1-integrin RNAi transfectants were assayed for their adhesive capacity as described in Sect. "Materials and methods". The rate of adhesion was expressed as the percentage of the total number of cells seeded. Data are expressed as the mean ± SD of triplicate determinations. Two independent experiments gave similar results. $^*P < 0.05$ versus LM3-PKCβ1 cells transfected with a control RNAi (Student's t test). **c** Effect of β1-integrin RNAi on the proliferation of LM3-PKCβ1 cells. Each data point represents the mean ± SD of triplicate determinations. $^*P < 0.05$ versus LM3-PKCβ1 cells transfected with a control RNAi (Student's t test). The *figure* shows the results of one experiment representative of three independent assays with similar results. **d** Effect of β1-integrin RNAi on LM3-PKCβ1 clonogenic capacity. Clonal cell growth was studied as previously described in Sect. "Materials and methods" in PKCβ1 overexpressors subjected to β1-integrin RNAi depletion. Colony number was assessed using an inverted microscope 10 days after seeding. Data are expressed as the mean ± SD of triplicate determinations. Two independent experiments gave similar results. $^*P < 0.05$ versus LM3-PKCβ1 cells transfected with a control RNAi (Student's t test)

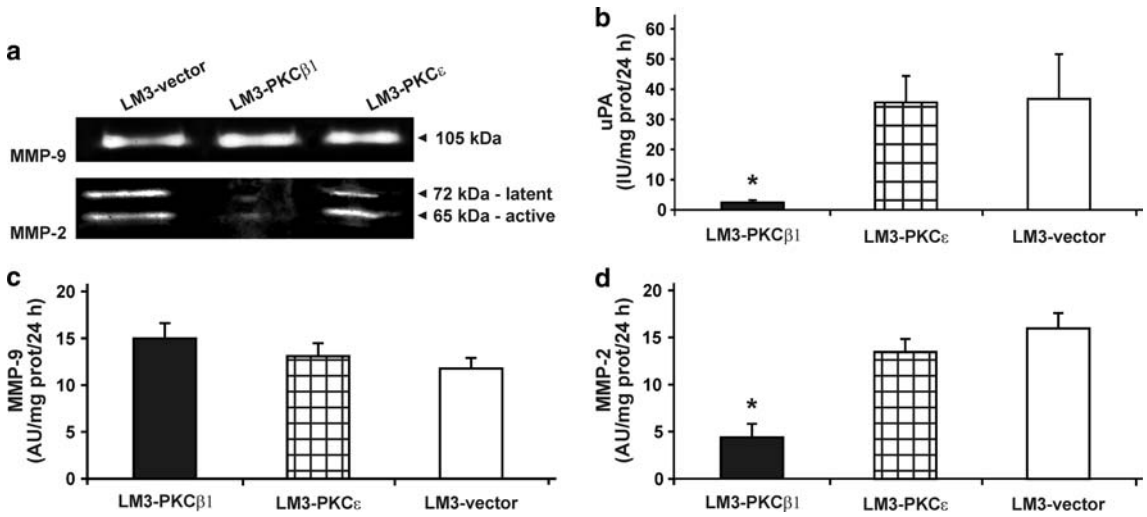

Fig. 7 **a** Quantification of MMPs activity by zymography. MMP-2 and MMP-9 activities were analyzed by zymography in CM from PKC- and vector-transfected cultures. Zymograms are representative of three independent experiments. **b** Quantification of uPA activity by radial caseinolysis. uPA secreted activity was quantified by radial caseinolysis in CM from PKC transfectants and control cell cultures. Data are expressed as the mean ± SD of triplicate determinations. Three independent experiments gave similar results. $^*P < 0.01$ versus LM3-vector cells (Student's t test). **c** and **d** Densitometric analysis of proteolytic bands in panel **a**. Lytic bands were measured with a digital densitometer and data are expressed as the mean ± SD in arbitrary units. $^*P < 0.05$ versus LM3-vector cells (Student's t test). Results are representative of three independent experiments

two biological assays measure the ability of LM3 cells to grow and survive under stress conditions, similar to those that cells must undergo during the first stages after in vivo inoculation. Signaling through PI3K and its downstream kinase Akt has been widely implicated in cell survival [40], and a correlation between active Akt levels and survival was found in our experimental models. The relationship between PKC expression and Akt function has been the subject of intense investigation by several laboratories. It became clear that a strict isozyme- and cell type-dependency exists since PKCs can either activate Akt or promote Akt dephosphorylation (i.e., inactivation) depending on the cell model [41, 42]. A similar paradigm has been recently described in keratinocytes, in this case involving PKCδ and PKCε [43]. The fact that PKCε increases the ability of LM3 cells to grow and form colonies in soft agar suggests that this novel PKC may have an important role in mammary carcinogenesis and tumor progression. In fact, overexpression of PKCε was clearly associated with an increase both in the incidence as well as in the number of spontaneous and experimental lung metastasis upon inoculation in syngeneic BALB/c mice.

Our studies also show that LM3-PKCβ1 cells have increased proliferative properties both in monolayer and in low density cultures, and accordingly, a marked elevation in cyclin D1 levels, Rb phosphorylation, and ERK activation was found in these cells. This contradiction between in vitro and in vivo growth capacity of LM3-PKCβ1 cells is currently a subject of intense research in our laboratory, but we speculate that it is probably related to the complexity in the molecular and cellular interactions occurring in an in vivo environment, probably involving signals from stroma or another yet unidentified mechanisms. Notably, Bumaschny et al. [44] have reported similar discrepancies between in vivo and in vitro growth properties using a different murine mammary adenocarcinoma cell line.

To further analyze some of the mechanisms implicated in the changes induced by PKCβ1 overexpression, we pursued studies on adhesiveness and the expression of adhesion-related molecules and ECM components. Our results revealed that β1-integrin and FN expression together with FAK activation are modulated by PKCβ1 overexpression. All these molecules have been previously reported as important regulatory components of focal contacts [24]. It has been proposed that integrins are involved in the modulation of FN matrix assembly [45], but the underlying mechanisms are not completely understood. In agreement with our findings, ectopic expression of $\alpha 5\beta 1$ integrin in CHO cells results in increased FN fibril formation [24]. Our results clearly show that β1-integrin function not only as an adhesion molecule but also shows profound effects on LM3 cell growth behavior. In fact, here we demonstrate that its enhanced expression induced by PKCβ1 as well as its inhibition, by RNAi, induced important changes in LM3 proliferative and clonogenic capacities. In addition, the increased β1-integrin levels in PKCβ1 overexpressors may play a role in extracellular fibril organization of FN observed in LM3-PKCβ1 cells, since this integrin is the main FN receptor. Early studies showed that FN is deposited into the ECM of normal cells but that their malignantly transformed counterparts often failed to express or deposit a FN matrix [46]. In this regard, we have previously demonstrated that FN re-expression is associated with a reduction in metastatic potential of mammary tumor cells [25]. FN fragments may stimulate the activation of different PKCs by mechanisms that involve integrin signaling [47]. Additionally, other report indicates that PKC expression and/or activation leads to a progressive accumulation of FN in the ECM, whereas PKC inhibitors markedly inhibited FN fibrilogenesis [48]. Since LM3 cells are unable to express and deploy FN in the ECM [49], our results are in line with this last observation. Thus, our results argue for a potential role of FN re-expression in the reduction of tumorigenic and metastatic properties of mammary tumor cells as a consequence of PKCβ1 overexpression. To our knowledge this is the first report showing that PKCβ1 may function as an ECM modulator both by inducing FN expression and inhibiting its degradation by altering proteases secretion.

It is known that expression of proteases involved in ECM remodeling plays fundamental roles in normal physiology as well as in the malignant transformation and tumor progression of the mammary gland [34, 50]. Furthermore, the expression of proteases has been associated with poor prognosis of breast cancer patients [51, 52]. It has been reported in various cellular models that overexpression of different PKCs augments the secretion of both uPA and MMPs [36, 53, 54]. However, our studies show that overexpression of the classical PKCβ1 reduces the secretion of uPA and MMP-2 in LM3 mammary tumor cells. Conversely, we found that PKCβ1 overexpression in normal murine mammary NMuMG cells causes the opposite effect, as it favors the secretion of both uPA and MMP-2 (unpublished observations). Taken together, these data are consistent with the idea that individual PKC isozymes have distinct and even opposing functions within different cell contexts [55, 56]. It is important to note that there are only few reports describing PKCs as negative modulators of proteases production. In this sense, our group was the first to describe this effect induced by PKCδ overexpression in NMuMG mammary cells [36]. We have shown several years ago that FN re-expression is able to reduce the ability of uPA to bind to its receptor (uPAR), thus leading to an important inhibition of its proteolytic capacity [37]. It is conceivable that this could generate a positive ECM-deposition feedback which would be ultimately

responsible for the reduction in metastatic potential observed in LM3-PKCβ1 cells. PKCε overexpressors showed an increase in lung metastasis incidence and number that is not associated with changes in proteases activity suggesting alternative mechanisms driven by PKCε overexpression. We cannot rule out that PKCε modulate the activity of other proteases not studied here such as heparanase, cathepsins and other MMPs.

Altogether, our results suggest that PKCβ1 overexpression may function as a growth-inhibitory protein in vivo, preventing mammary tumor and metastasis development, whereas PKCε drives metastatic dissemination of already established mammary cancers without affecting primary tumor evolution.

Acknowledgments The authors thank Dr. Marina Simian for kindly providing β1-integrin RNAi and Martin Krasnapolski for his constant support and useful discussions. This work was supported by grants from ANPCyT-FONCYT, Argentina (PICT 14088 and PICT 417, Préstamo BID 1728/OC-AR), and UBACyT, Argentina (M068 and M003) to Elisa Bal de Kier Joffé. Alejandro J. Urtreger, Laura B. Todaro, and Elisa Bal de Kier Joffé are members of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

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