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PKCε IS AN ESSENTIAL MEDIATOR OF PROSTATE CANCER BONE METASTASIS

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Abstract

The bone is a preferred site for metastatic homing of prostate cancer cells. Once prostate cancer patients develop skeletal metastases, they eventually succumb to the disease; therefore, it is imperative to identify key molecular drivers of this process. This study examines the involvement of protein kinase C epsilon (PKCε), an oncogenic protein that is abnormally overexpressed in human tumor specimens and cell lines, on prostate cancer cell bone metastasis. PC3-ML cells, a highly invasive prostate cancer PC3 derivative with bone metastatic colonization properties, failed to induce skeletal metastatic foci upon inoculation into nude mice when PKCε expression was silenced using shRNA. Interestingly, while PKCε depletion had only marginal effects on the proliferative, adhesive and migratory capacities of PC3-ML cells *in vitro* or in the growth of xenografts upon s.c. inoculation, it caused a significant reduction in cell invasiveness. Notably, PKCε was required for transendothelial cell migration (TEM) as well as for the growth of PC3-ML cells in a bone biomimetic environment. At a mechanistic level, PKCε depletion abrogates the expression of IL-1β, a cytokine implicated in skeletal metastasis. Taken together, PKCε is a key factor for driving the formation of bone metastasis by prostate cancer cells and is a potential therapeutic target for advanced stages of the disease.

Implications—This study uncovers an important new function of PKCε in the dissemination of cancer cells to the bone; thus, highlighting the promising potential of this oncogenic kinase as a therapeutic target for skeletal metastasis.

Keywords

PKCε; PC3-ML cells; prostate cancer; IL-1β; bone metastasis

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INTRODUCTION

Prostate cancer is the second leading cause of cancer-related deaths among men, with ~221,000 new cases and ~27,500 deaths estimated for 2015 in the United States, according to the American Cancer Society. The majority of prostate cancer related deaths results from metastatic spread of prostate cancer cells from the primary tumor to contiguous and distal sites. One of the preferred sites of metastatic dissemination of prostate cancer cells is the bone. Once the disease reaches this advance stage it becomes inevitably fatal, as 5-year survival for prostate cancer patients with skeletal involvement is <1%. The bone metastatic process first involves the detachment and shedding of cancer cells from the primary tumor into a blood vessel, an event called intravasation, followed by hematogenous dissemination and homing to specific niches in the bone, particularly perivascular niches of the bone marrow sinusoids where active metastatic foci form (1). The molecular mechanisms implicated in prostate cancer cell metastasis are poorly understood, which ultimately limits the development of effective therapies aimed at preventing the spread of prostate cancer cells and formation of secondary skeletal metastasis in patients.

The PKC serine-threonine kinases have been broadly implicated in the regulation of signaling pathways that control cell proliferation, differentiation, apoptosis and motility. This kinase family comprises 10 structurally related isozymes that have been classified into 3 different groups with diverse biochemical and functional properties: "classical/conventional" cPKCs (α, βI, βII, and γ), "novel" nPKCs (δ, ε, η, and Θ), and "atypical" aPKCs (ζ and λ/I). cPKCs and nPKCs are established cellular receptors for the phorbol ester tumor promoters, and at a physiological level they become activated by the lipid second messenger diacylglycerol (DAG). Although PKCs have been widely implicated in tumor promotion, it is now recognized that individual isozymes have distinctive roles in the progression of cancer, both in positive and negative manners (2-4). Among the different members of the PKC family, numerous studies in the last years unambiguously established PKCε as an oncogenic kinase and tumor biomarker (5, 6). Notably, PKCε is markedly up-regulated in epithelial cancers, including prostate, lung, breast, and head and neck cancer (2, 7-12). In lung and breast cancer cells, silencing PKCε expression using RNAi impairs their ability to grow as xenografts in nude mice (7, 12, 13). These results have been validated pharmacologically with specific PKCε inhibitors (12, 13), thus highlighting the importance of this kinase in the maintenance of tumor growth. In addition to its role in tumorigenesis, numerous reports linked PKCε to metastatic dissemination. For example, NIH 3T3 cells constitutively expressing PKCε develop invadopodial-like structures, exhibit increased pericellular metalloprotease activity, and display metastatic capacity in nude mice (14). Moreover, PKCε is required for motility and invasion in various cancer cellular models in culture as well as for metastatic dissemination *in vivo*. Most remarkably, studies using transgenic PKCε mice established this kinase as a driver of metastatic skin squamous cell carcinoma (15-19).

PKCε is barely detected in normal or benign human prostatic epithelium, whereas it is highly expressed in prostate tumors and in recurrent disease. Nearly 100% of human prostate tumors overexpress PKCε, particularly advanced metastatic tumors (10, 15, 20, 21). Accordingly, androgen-independent prostate cancer cells display significant PKCε up-

regulation relative to androgen-dependent prostate cancer cell lines or "normal" immortalized prostate epithelial cells, and ectopic expression of PKCε in androgen-dependent prostate cancer cells contributes to the acquisition of androgen independence (22, 23). In an *in vivo* context, our laboratory showed that transgenic overexpression of PKCε in the mouse prostate leads to the formation of preneoplastic lesions (23, 24). Moreover, in the transgenic mouse model of prostate adenocarcinoma (TRAMP), genetic ablation of PKCε inhibits the development of prostate cancer and spontaneous metastatic dissemination to lymph nodes, lung, and kidney (17). Regardless of the distinctive roles assigned to PKCε in different stages of disease progression, the role of PKCε in the dissemination of prostate cancer cells to the bone remains elusive.

In this study, we investigated whether PKCε could play a role in the formation of skeletal tumors in mice. We took advantage of PC3-ML cells, a subline derived from the widely used PC3 cell line that consistently produces skeletal metastasis in mice (25, 26). We present for the first time evidence that PKCε mediates the invasive capacity of these cells and is required for the formation of skeletal tumors in mice.

MATERIALS AND METHODS

Cell culture

PC3-ML cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. HUVEC cells were purchased from Lonza (Walkersville, MD) and cultured in EGM-2-BulletKit medium (Lonza), as indicated by the provider. MG-63 osteosarcoma cells were purchased from ATCC and cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The PKCε inhibitor εV1-2 (tat-fused) and its control peptide Tat were a kind gift from Dr. Daria Mochly-Rosen (Stanford University).

Lentiviral infections

Stable depletion of PKCε in PC3-ML cells was achieved by infection with shRNA lentiviruses (MISSION® shRNA Lentiviral Transduction particles, Sigma, St Louis, MO). As a control we used the MISSION® non-target shRNA lentivirus. Selection of stable cell lines was carried out with puromycin (0.3 μg/ml, 3 weeks).

Western blot assays

Western blots and densitometric analyses were carried out as previously described (27). The following antibodies (1:1000 dilution) were used: anti-PKCε (sc-214; Santa Cruz Biotechnology, Dallas, TX), anti-Rac1 (05-389; Upstate Biotechnology, Billerica, MA), anti-interleukin-1β (sc-1251; Santa Cruz Biotechnology, Dallas, TX), and anti-vinculin (V9131 Sigma). Bands were visualized by the enhanced chemiluminescence (ECL) Western blotting detection system. Images were captured using a FUJIFILM LAS-3000 system and the LAS-2000 software.

Cell growth assays

Cells (1×10^5) were seeded onto 96-well plates. At different times, cell viability in triplicate samples was determined with the CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI), as previously described (28).

Migration and invasion assays

Cells were trypsinized, suspended in 0.1% BSA/RPMI, and seeded (1.5×10^4 cells/well) in the upper compartment of a Boyden chamber (NeuroProbe, Gaithersburg, MD). Polycarbonate membranes of 12 μM pore diameter were used to separate the upper and lower compartments. In the lower chamber, RPMI medium containing 10% FBS was used. After an incubation period of 18 h at 37°C, membranes were recovered and cells on the upper side (non-migratory) were wiped off the surface. Cells on the lower side of the membrane were fixed and stained with the DIFF Quik Stain Set (Dade Behring, Deerfield, IL). Migratory cells in each well were counted by contrast microscopy in 5 random fields. Invasion assays were carried out in Boyden chambers using Matrigel-coated polycarbonate membranes, as previously described (18). For some assays, we preincubate cells with IL-1β (10 nM, R&D Systems, Minneapolis, MN), or incubate them with anti-IL-1β blocking antibody (1 μM, R&D Systems) or control IgG (Santa Cruz Biotechnology, Dallas, TX).

Rac-GTP pull-down assays

Determination of Rac-GTP levels was carried out essentially as previously described (28). Briefly, cells growing at 80% confluency were lysed in pull-down buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% NP40, 5 mM β-glycerophosphate, 1 mM DTT, and protease inhibitors) containing 15 μg/ml of PBD-GST. Lysates were cleared by centrifugation (10 min at 4°C, 13,000 × g) and incubated with glutathione-Sepharose 4B beads (GE Healthcare, Buckinghamshire, UK) for 45 min at 4°C. After centrifugation, the beads were washed twice with pull-down buffer and run on SDS-PAGE. Rac1 in the beads (Rac1-GTP) was detected by Western blot using an anti-Rac1 antibody.

Adhesion assays

Plates were coated with 500 μg/ml collagen, poly-L-lysine, or Matrigel, and blocked with 0.5% BSA. Cells (3×10^4 cells/well in 0.1% BSA/RPMI) were seeded in triplicate. FBS (10%) was added to stimulate adhesion. After incubation at 37°C for different times, unattached cells were washed with PBS. Attached cells were fixed and stained with DAPI. Plates were visualized by fluorescence microscopy, and the number of nuclei from attached cells was quantified in 5 random fields cells using the ImageJ software.

Quantitative real-time PCR (qPCR)

For the determination of IL-1β and PKCε mRNA levels, total RNA was extracted from subconfluent cell cultures using the RNeasy kit (Qiagen, Valencia, CA). One μg of RNA from each sample was reverse transcribed using the TaqMan reverse transcription reagent kit (Applied Biosystems, Branchburg, NJ) with random hexamers used as primers. PCR primers and 5′-end 6-carboxyfluorescein-labeled probes for PKCε and IL-1β were purchased from Applied Biosystems. PCR was performed using an ABI PRISM 7700 detection system

in a total volume of 25 μl containing TaqMan universal PCR MasterMix (Applied Biosystems), commercial target primers (300 nM), the fluorescent probe (200 nM), and 1 μl cDNA. PCR product formation was continuously monitored using the sequence detection system software version 1.7 (Applied Biosystems) (29). The 6-carboxyfluorescein signal was normalized to endogenous tRNA 18S. Ct was obtained by subtracting the cycle threshold (CT) of 18S or ubiquitin C from that of PKCε. (Ct) was determined by subtracting the control Ct from the sample Ct. Fold-changes were calculated using Data Assist software from Life Technologies, and expressed as $2^{-(\ Ct)}$.

mRNA levels for 84 invasive and metastatic genes, including extracellular matrix (ECM) proteases and protease inhibitors, were determined by qPCR using the Human Tumor Metastasis RT2 Profiler PCR Array and a RT2 SYBR Green/5-carboxy-X-rhodamine (ROX) qPCR master mix (Qiagen). Data were normalized using ACTB, GAPDH, B2M, HPRT1, and RPLP0 housekeeping genes provided in the PCR array. The relative mRNA levels were calculated using the CT method, as indicated in the RT2 Profiler PCR Array Data Analysis Webportal.

Tumor growth and bone metastasis in nude mice

Studies were carried out in strict accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals from NIH. Protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania. For tumorigenesis experiments, male athymic mice (6-8 weeks, 10 mice/group, Harlan Laboratories) were injected *s.c.* with PC3-ML cells (1.2×10^6 or 4×10^6) resuspended in 0.1 ml PBS. For determination of tumor growth, the width and length of tumors were measured with a caliper at different times, and tumor volume calculated as previously described (13).

Bone metastasis experiments were carried out essentially as previously described (26, 30). Briefly, PC3-ML cell lines were infected with ZsGreen Lentivirus, which had been produced using Lenti-X high-titer lentiviral packaging systems (Lenti-X 293T cell line, Lenti-X™ HTX Packaging System, pLVX-IRES-ZsGreen1 vector from Clontech, Mountain View, CA). 5×10^4 cells from the brightest sorted population were inoculated in the left cardiac ventricle of athymic nude mice. Delivery of cells into the systemic blood circulation was corroborated by co-injection of blue fluorescent 10 μm polystyrene beads (Molecular Probes, Eugene, OR). Animals were randomly assigned to different experimental groups and sacrificed either at 72 h or 3 weeks following inoculation. The homogeneous and numerically consistent distribution of the beads in adrenal glands and lungs collected at necropsy and inspected by fluorescence microscopy were used as discrimination criteria for the inclusion of animals in the studies.

Bones were collected and fixed in 4% paraformaldehyde solution for 24 h and transferred into fresh formaldehyde for an additional period of 24 h. Bones were decalcified in 0.5M EDTA for 7 days followed by incubation in 30% sucrose. Bones were maintained at 4°C for all aforementioned steps and frozen in optimum cutting temperature medium (OCT) by placement over dry ice chilled 2-methylbutane. Serial sections of 80 μm thickness were obtained using a Microm HM550 cryostat. Femur and tibia in each knee joint were cut entirely through, resulting in approximately 30 sections per specimen made available for

analysis. Fluorescent images of skeletal metastases were acquired using a Zeiss AX70 microscope (Carl Zeiss) connected to a Nuance Multispectral Imaging System from 30 bone sections (80 μm) per animal. Digital images were analyzed, processed with the Nuance Software (v. 2.4), and the number of micrometastasis was determined. Microscope and software calibration for size measurement was conducted using a TS-M2 stage micrometer (Oplenic Optronics, Hangzhou, China).

Bone marrow cultures

Bone marrow from mouse tibia and femur were flushed after 3 weeks after intracardiac inoculation. Cells were dissociated using a syringe (19G needle) and incubated with RPMI media supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.3 μg/ml puromycin. After 7 days in culture, the presence of ZsGreen fluorescent colonies was determined using an inverted microscope.

Transendothelial assays

We used the *in vitro* assay described by Okada *et al.* (31). Briefly, HUVEC endothelial cells were seeded on the top chamber of a 12 mm insert Millicell Standing Insert (Catalogue # PIXP01250; Millipore, Billerica, MA) with a 12 μm polycarbonate porous membrane coated with 200 μl of Matrigel (0.5 mg/ml), and cultured until they reached 100% confluence. Then, 300 μl of a suspension containing ZsGreen labeled PC3-ML cells (5×10^5 cells/ml) were added on top of the endothelial monolayer, and 10% FBS was added to the lower chamber. After 18 h incubation at 37°C, unattached cells were removed by washing with PBS, and attached cells were fixed and stained with DAPI. The invasion of tumor cells across the endothelium was determined by counting the number of ZsGreen-labeled cells that migrated to the lower chamber, as determined by fluorescence microscopy. In addition, confocal microscopy was used for immunofluorescent visualization of migrating cells. Transendothelial migrations in 3D were visualized under a Zeiss LSM 710 AxioObserver inverted confocal microscope (Carl Zeiss, Oberkochen, Germany). Images were obtained at 20x magnification using two lasers (405 nm and 488 nm, in sequential mode). ZEN 2011 software was used during all image acquisition procedures. The whole Z dimension was scanned, and the 3D images from each assay were then built by stacking approximately 100 cross sections.

To determine adhesion to endothelial cells, PC3-ML cells were labeled with [^{3}H]thymidine (1 mCi) for 18 h. Labeled PC3-ML cells were centrifuged and resuspended in 2.5 ml of RPMI, and 100 μl of this suspension were added to 24-well plates that had been previously seeded with HUVEC cells (100% confluency). After 30 or 60 min, non-adherent PC3-ML cells were removed by washing three times with 250 μl PBS. The remainder was lysed with 250 μl 0.1 M NaOH, and radioactivity counted in a scintillation counter.

Co-culture experiments

Osteoblast co-culture experiments were carried out essentially as described (32). Briefly, PC3-ML cells (5×10^3) expressing ZsGreen were seeded over a confluent monolayer of MG-63 osteosarcoma cells. After different times, non-attached cells were rinsed with PBS. Three weeks later the number of ZsGreen positive foci was quantified by fluorescence

microscopy, and the number of green foci in 10 random fields was quantified with the ImageJ software.

To determine adhesion to MG-63 osteosarcoma cells, PC3-ML cells were labeled with [^{3}H]thymidine (1 mCi) for 18 h. Labeled PC3-ML cells were centrifuged and resuspended in 2.5 ml of RPMI, and 100 μl of this suspension were added to 24-well plates that had been previously seeded with MG-63 cells (100% confluency). After 30 or 60 min, non-adherent PC3-ML cells were removed by washing three times with 250 μl PBS. The remainder was lysed with 250 μl 0.1 M NaOH, and radioactivity counted in a scintillation counter.

RESULTS

PKCε mediates metastasic dissemination of PC3-ML prostate cancer cells to the bone

Emerging data from several laboratories established essential roles for PKCε in the progression of cancer, including prostate cancer (2, 5, 6, 10, 17). PKCε has been implicated in cell invasiveness, yet it is not clear whether this kinase plays a role in metastasis to the bone, a main site of prostate cancer cell dissemination. To this end, we took advantage of a well-established model of experimental metastasis that uses PC3-ML human prostate cancer cells, a cell line with high bone metastatic potential derived from the PC3 parental cell line (25, 26). As previously reported for parental androgen-independent PC3 and DU145 cells (11, 23), the PC3-ML subline displays elevated levels of PKCε relative to "normal" immortalized prostate epithelial cells (Fig. 1a). To ascertain the role of PKCε in prostate cancer bone metastasis, we used shRNA lentiviruses to stably knockdown PKCε from PC3-ML cells, followed by selection with puromycin. Two different shRNA lentiviruses were used (ε1 and ε2) in order to generate PC3-ML-KDε1 and PC3-ML-KDε2 cell lines. As a control, we generated a PC3-ML cell line infected with a non-target control shRNA lentivirus (PC3-ML-NTC). As shown in Fig. 1b, ~ 85% and 70% depletion in PKCε levels was achieved in PC3-ML-KDε1 and PC3-ML-KDε2 cell lines, respectively (n=3).

To address the relevance of PKCε in a metastasis model *in vivo*, cell lines were engineered to stably express the GFP variant ZsGreen using a lentiviral approach. ZsGreen expression did not significantly affect PKCε levels in PC3-ML cells (Fig. 1a). Studies showed that upon direct inoculation in the blood circulation of immunodeficient mice, these cells generate metastatic foci primarily in femur and tibia, jaws and ribs (25). Parental PC3-ML, PC3-ML-NTC, PC3-ML-KDε1 and PC3-ML-KDε2 cells were inoculated in the left cardiac ventricle of athymic nude mice, as previously described (33). Mice were sacrificed after 3 or 21 days, and their femur and tibiae harvested, fixed, and analyzed for the presence of micrometastatic foci. As previously reported (33), we found microscopic tumors in the tibia and/or femur upon intracardiac inoculation of PC3-ML cells. Similar results were observed with PC3-ML-NTC cells. A representative picture is shown in Fig. 1c. Quantification analysis revealed that PKCε-depletion from PC3-ML cells severely impaired the formation of micrometastasis, as essentially no bone metastatic foci could be detected in the femur/tibia upon injection of PC3-ML-KDε1 and PC3-ML-KDε2 cells (Fig. 1d).

As a complementary approach, we analyzed bone marrows for the presence of fluorescent ZsGreen labeled cells. Three weeks after intracardiac inoculation of PC3-ML cells into

athymic mice, bone marrows from tibiae and femurs were flushed and cultured *in vitro*. While a significant number of ZsGreen labeled cells could be detected in bone marrows from mice injected with either parental PC3-ML or PC3-ML-NTC cells, fluorescent cells could not be isolated from bone marrows from mice inoculated with either PC3-ML-KDε1 or PC3-MLKDε2 cells (Fig. 2). Thus, PKCε is required for bone metastatic dissemination of PC3-ML cells.

PKCε depletion has limited effects on PC3-ML cell growth, adhesiveness, and motility

PKCε is known to mediate proliferation in a number of cancer cells, such as lung and breast cancer cellular models (12, 13, 16). When we examined the effect of silencing PKCε on the growth properties of PC3-ML cells in culture, we noticed only a marginal reduction in cell number in PC3-ML-KDε1 and PC3-ML-KDε2 cells relative to control cell lines (Fig. 3a). Similar results were observed when we analyzed the tumorigenic properties of these cells *in vivo*. Indeed, upon *s.c.* inoculation into nude mice (1.2×10^6 cells), the formation of tumors by PKCε-depleted cell lines was somehow slower, although there were no statistically significant differences between the different cell lines (Fig. 3b). Similar results were observed in experiments using a higher number of cells (4×10^6 cells) (data not shown).

The capacity of cancer cells to successfully metastasize is frequently associated with changes in their adhesive properties and their ability to migrate. We therefore examined the effect of PKCε depletion on the adhesive capacity of PC3-ML cells to different substrates. These experiments revealed essentially no significant differences in adhesion to Matrigel, collagen or poly-L-lysine coated plates between PKCε-depleted and control PC3-ML cells (Fig. 3c).

In a recent study using lung cancer models, we showed a critical requirement of PKCε for motile activity. Moreover, we established that PKCε controls the activation of Rac1, a small GTPase widely implicated in cytoskeletal rearrangements and cell migration (18). Thus, we asked if silencing the expression of PKCε could impair the motility of PC3-ML cells. As illustrated in Fig. 3d, the ability of PC3-ML cells to migrate, as determined by means of a Boyden chamber assay, remained essentially unchanged as a consequence of PKCε depletion. PC3-ML cells display constitutively elevated levels of active Rac-GTP levels compared to non-transformed prostate epithelial cells (data not shown). However, and consistent with the lack of involvement of PKCε in PC3-ML cell migration, silencing PKCε fails to reduce Rac-GTP levels in this cell line (Fig. 3e).

PKCε is required for PC3-ML cell invasiveness: a role for IL-1β

In the next set of experiments, we sought to examine the potential involvement of PKCε in invasiveness by assessing the ability of cells to migrate through Matrigel in a Boyden chamber (18). It has been previously reported that PC3 and PC3-ML cells are highly invasive (25). Notably, PC3-ML-KDε1 and PC3-ML-KDε2 cells showed a major impairment in invasiveness relative to parental PC3-ML cells and PC3-ML-NTC cells (Fig. 4a). Similar results were observed in DU-145 cells (data not shown). To further establish a role of PKCε in PC3-ML invasiveness we used the PKCε inhibitor εV1-2, a Tat-fused permeable peptide that specifically prevents PKCε translocation to the membrane and

therefore inhibits its activation (18, 34). Consistent with results using PKCε RNAi, this inhibitor markedly reduced PC3-ML cell invasion through Matrigel relative to the control Tat peptide (Fig. 4b).

Cancer cell invasion is associated with the production and release of proteases required for remodeling the extracellular matrix (ECM). To evaluate a role of PKCε in the expression of metalloproteases (MMPs) and other key proteins implicated in cell invasiveness, we used the Human Tumor Metastasis RT2 Profiler PCR array (Qiagen), which allows for the simultaneous determination of the expression of 84 genes implicated in invasion and metastasis. This analysis revealed multiple changes as a consequence of PKCε knockdown in PC3-ML cells, which are depicted in Fig. 4c. The complete list of 84 genes is shown in Suppl. Fig. 1. Among the most notable changes, we found a significant down-regulation in the expression of MMPs (MMP7, MMP11, MMP13). Another notable change in PKCε-depleted PC3-ML cells is the down-regulation of interleukin-1β (IL-1β). PC3-ML cells indeed express high levels of IL-1β, and this cytokine was shown to have a fundamental role in the bone metastatic properties of these cells (26). The involvement of PKCε in the control of IL-1β expression in PC3-ML cells was validated using a silencing approach, both by qPCR (Fig. 4d) and Western blot (Fig. 4e). It has been recently established that IL-1β is up-regulated in prostate cancer cells; moreover, silencing IL-1β expression interferes with the bone metastatic potential of PC3-ML cells (26). As our results suggest IL-1β as a PKCε target, we reasoned that inhibition of IL-1β signaling should also affect PC3-ML invasiveness. Indeed, incubation of PC3-ML with an anti-IL-1β blocking antibody significantly reduced invasion of PC3-ML cells through Matrigel (Fig. 4f). Addition of IL-1β rescued the effect of PKCε knockdown on invasion in these cells (Fig. 4g). This suggests that PKCε-mediated IL-1β production is important for PC3-ML cell invasiveness.

PKCε is required for transendothelial migration and growth of PC3-ML cells in a bone biomimetic microenvironment

The ability of cancer cells to migrate through the endothelial cell layer represents a key event in tumor metastasis. Prostate cancer cell extravasation to bone requires tethering to the bone marrow endothelium and transmigration (35). To address the effect of PKCε depletion on migration through endothelial cells, we used an *in vitro* transendothelial assay (31). Remarkably, PC3-ML-KDε1 and PC3-ML-KDε2 cells have a major deficiency in their ability to transmigrate through a monolayer of endothelial cells (HUVEC) relative to parental PC3-ML or PC3-ML-NTC cells (Fig. 5a). The reduced invasive capacity of PKCε-depleted cells through endothelial cells could be readily observed in 3D pictures taken by confocal microscopy, which show clear migration underneath the endothelial cells only for parental PC3 cells and PC3-MLNTC cells but not for PKCε-depleted PC3-ML cells (Fig. 5b). The impaired transmigratory properties of PKCε silenced PC3-ML cells is not due to weakened adhesion, as determined by the similar capacity of control and PKCε-silenced PC3-ML cells to attach to confluent monolayers of non-stimulated (Fig. 5c) or stimulated (LPS-activated) HUVEC cells (data not shown).

To analyze whether PKCε confers a survival advantage in a bone biomimetic microenvironment, we examined the ability of PKCε depleted cells or their corresponding

control cell lines to adhere and proliferate when seeded on top of a confluent layer of human MG-63 osteoblast cells. At different times, non-attached cells were rinsed with PBS, and three weeks later the number of ZsGreen fluorescent PC3-ML foci was quantified. Whereas there were no significant differences in the ability of all PC3-ML cell lines to adhere to MG-63 osteoblast cells (Fig. 6a), a reduced ability to grow under these conditions was observed for PC3-ML-KDε1 and PC3-ML-KDε2 cells (Fig. 6b).

DISCUSSION

The mechanisms implicated in prostate cancer initiation and progression have been extensively studied, and significant advances in our understanding of the genes and signaling pathways that contribute to the various steps of disease progression have been elucidated, particularly those concerning the growth of the primary tumor. Unfortunately, the mechanisms leading to metastatic dissemination of prostate cancer cells, particularly to the bone, remain poorly understood. In this study, we identified PKCε as a novel player in skeletal metastasis of prostate cancer cells. Our results clearly show that silencing the expression of PKCε from PC3-ML cells, a subline that metastasizes with high propensity to the bone, prevents the formation of skeletal metastatic foci upon inoculation into nude mice, thus reflecting the requirement of PKCε in key steps leading to prostate cancer cell colonization in the bone.

PKCε has been widely associated with the development of epithelial cancers, and has been originally described as an oncogenic kinase that is able to transform fibroblasts through the activation of the Ras-Raf1 signaling pathway and autocrine secretion of TGF-β (19, 36, 37). Numerous laboratories underscored important roles for PKCε in cell cycle progression and the control of cell survival mechanisms (2, 5, 6). For example, forced expression of PKCε in LNCaP prostate cancer cells accelerates proliferation due to constitutive activation of the Erk cascade and protects cells against apoptotic stimuli (21, 22). Our laboratory found that PKCε modulates Bad phosphorylation to protect LNCaP cells against phorbol ester- and TNFα-induced apoptosis (38). In other cell models, such as lung cancer cells, PKCε regulates the expression of genes implicated in cell death and survival, and plays essential roles in anchorage-dependent and anchorage-independent growth (13). PKCε overexpression is a signature of many cancer types, including breast, lung, and head and neck cancer (2, 7-10, 12). Several lines of evidence indicate that PKCε is frequently up-regulated in prostate cancer cell lines and tumor specimens, and is a predictive biomarker of prostate cancer; moreover, its expression correlates with aggressiveness and recurrence (10, 20, 21). Notably, overexpression of PKCε in androgen-dependent LNCaP cells initiates tumor growth in intact and castrated nude mice, arguing for a potential role of this kinase in the progression to androgen independence (22). Transgenic mice generated in our laboratory, in which PKCε was overexpressed in the prostate under the control of a probasin promoter, develop preneoplastic lesions (prostatic intraepithelial neoplasia or PIN) and have reduced apoptotic death in response to androgen ablation, suggesting also a role for PKCε in tumor initiation. Lesion formation is accompanied by hyperactivation of a number of pro-survival signaling pathways, namely Akt, NF-κB, and Stat3 (23, 24). PKCε up-regulation is also observed in prostates from TRAMP mice, a model that spontaneously develops invasive prostate cancer, and genetic ablation of PKCε in these mice inhibits prostate cancer

development and metastasis, as well as the expression of a number of proliferative and survival markers such as cyclin D1, Bcl-xL, and Stat3 (17).

There is a growing body of evidence for the involvement of PKCε in cancer cell migration and invasion, as well as in the regulation of Rho GTPases that govern these processes, as we recently reported in lung cancer cells (18). PKCε depletion or inhibition significantly impairs motility in a number of cancer cell models, namely lung, breast, and colon cancer (7, 18, 19, 39). Strikingly, PKCε overexpression has been associated with elevated incidence of spontaneous and experimental metastases in mouse models. For example, overexpression of PKCε enhances spontaneous lung metastasis of breast cancer cells (16), and targeted transgenic overexpression of PKCε in skin promotes the development of squamous cell carcinomas which rapidly metastasize to regional lymph nodes (15). Our results in PC3-ML cells, revealed significant roles for PKCε in invasion regardless of a lack of involvement in cell motility and Rac activity, possibly highlighting distinctive roles for PKCε in different cellular models. The effect of PKCε silencing on invasiveness could be recapitulated by pharmacological treatment with a specific PKCε inhibitor. PKCε RNAi depletion from PC3-ML cells significantly reduced the expression of MMPs required for ECM modeling, which may conceivably contribute to the impaired invasive capacity of PC3-ML-KDε1 and PC3-ML-KDε2 cells. There is significant evidence that MMPs contribute to the formation of osteolytic metastatic lesions through multiple mechanisms, including the recruitment of osteoclasts at metastatic sites, cleavage of receptor activator of NF-κB ligand (RANKL) required for the activation of newly recruited osteoclasts, and chemoattraction of tumor cells to perpetuate a tumor-stromal vicious cycle of bone matrix degradation (40). Quite remarkably, we found PKCε to be required for transendothelial migration. Prostate cancer cell bone extravasation is a multistep process that involves tethering and rolling on bone marrow endothelial cells, firm adhesion, and transmigration (35). Whereas our studies did not reveal major defects in the capacity of PKCε-deficient PC3-ML cells to adhere to endothelial cells, a process that depends on multiple players such as the ligand E-selectin and integrins β1 and αvβ3 (35), we observed a major impairment in their ability to transmigrate through the endothelial layer. Although a detailed mechanistic analysis would be required to decipher how PKCε drives this transmigratory process, we speculate that MMPs regulated by PKCε may be involved in the activation/shedding of chemokines implicated in prostate cancer cell extravasation of prostate cancer cells, such as CX3CL1/fractalkine (41).

A notable alteration that we identified in PKCε-depleted PC3-ML cells is a marked reduction in IL-1β expression. Although the mechanisms by which PKCε controls IL-1β expression are not known, it has been reported that in prostate cancer this kinase activates pathways such as NF-κB and Erk (23)(24). Accordingly, NF-κB and the Ras-Raf cascade have been implicated in IL-1β transcription and secretion (42). IL-1β is solely active in its secreted form and is highly abundant at tumor sites, thus affecting tumor growth, invasiveness and the pattern of tumor-microenvironment interactions. It has been reported that expression of IL-1β contributes to the tumorigenic potential of malignant cells and potentiates carcinogenesis by promoting local inflammatory responses. Moreover, IL-1β plays essential roles in cancer cell invasiveness, and neutralization of secretable IL-1β is

sufficient to limit tumor invasiveness (42). More recently, Liu *et al.* (26) established a functional association between IL-1β expression and the acquisition of bone metastatic capabilities of prostate cancer cells. Indeed, IL-1β is up-regulated in highly bone metastatic PC3-ML cells relative to low non-metastatic PC3-N cells. Moreover, shRNA mediated silencing of IL-1β from PC3-ML cells leads to a marked inhibition of skeletal metastasis, whereas PC3-N cells engineered to ectopically overexpress IL-1β have enhanced bone tropism and acquire bone metastatic capacity. Hence, taken together, these data strongly suggest that skeletal metastasis driven by PKCε may be mediated by IL-1β. Although this has yet to be formally demonstrated, our results clearly reveal that a blocking IL-1β antibody significantly reduces migration of PC3-ML cells through Matrigel, thus supporting the involvement of PKCε-mediated IL-1β production in PC3-ML cell invasiveness. Since IL-1β is known to stimulate the bone-resorption activity of osteoclasts (43), it may be possible that PKCε, by controlling the synthesis of IL-1β, promotes bone matrix turnover. IL-1β derived from malignant cells stimulates the production of a pro-inflammatory environment and increases COX-2 expression and PGE_2 production in bone marrow-derived mesenchymal stem cells (44). As COX2 is a PKCε effector gene in prostate cancer cells (24), we hypothesize that PKCε overexpression is a dominant event in the control of autocrine and paracrine effects in the bone microenvironment that contribute to creating a niche for the survival and growth of prostate cancer cells, and in this context, IL-1β and other local mediators controlled by PKCε may play key roles. The enhanced survival of PKCε expressing PC3-ML cells in a bone mimetic microenvironment is consistent with this premise. Since IL-1β has been shown to induce PKCε expression (45), and PKCs (including PKCε) are also downstream effectors of PGE_2 and IL-1β (46, 47),it is reasonable to speculate that sustained activation of PKCε in prostate cancer cells (and possibly bone stromal cells) may contribute to a vicious cycle that facilitates skeletal metastasis.

In conclusion, our studies identified PKCε as an important mediator of prostate cancer skeletal metastasis, possibly acting at different levels, including the migration of prostate cancer cells to the bone and their survival in the bone microenvironment. These findings may have significant therapeutic implications, as PKCε inhibitors with as anti-cancer activity have been generated in the last years, and some are well tolerated in humans (12, 48, 49). It is also worth noting that PKCs have been implicated in osteoclast formation, and recent studies highlighted a potential therapeutic use of pharmacological blockade of PKC-dependent pathways in osteolytic diseases. For example, RANKL, a key signal regulator that is currently targeted in the clinical management of bone metastatic disease, exerts its effect via PKC, and PKC inhibition attenuates osteoclastogenesis, bone resorption and RANKL-induced NF-κB activation (50). Thus, an attractive possibility is that PKCε inhibitors may have therapeutic benefit for the treatment of bone metastatic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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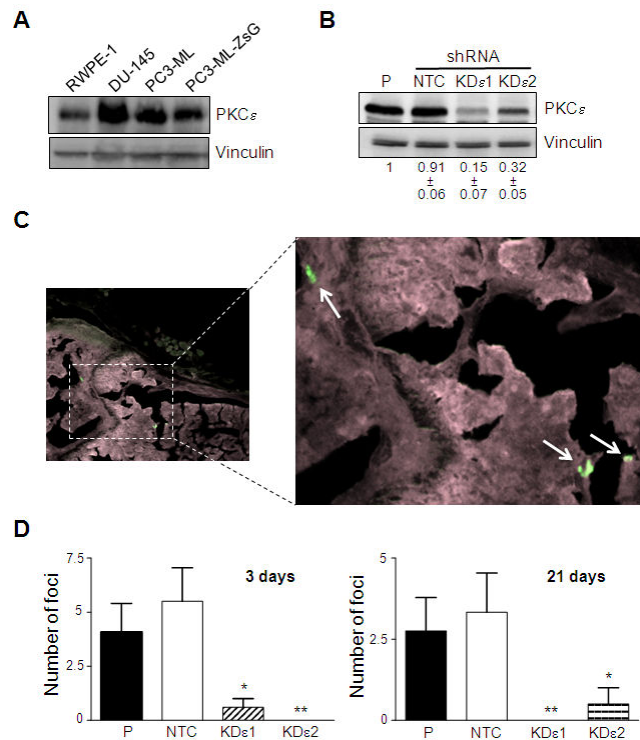
Figure 1. PKCε silencing from PC3-ML cells impairs metastasic dissemination to the bone
(*a*) PKCε expression in "normal" immortalized RWPE-1 prostate epithelial cells and
prostate cancer cells (DU145, PC3-ML, and PC3-ML expressing ZsGreen), as determined
by Western blot. (*b*) PC3-ML cell lines were generated upon infection with either PKCε
RNAi (*KDε1* and *KDε2*) or non-target control (*NTC*) shRNA lentiviruses. Densitometric
analysis of PKCε expression, normalized to vinculin (loading control) is indicated. *P*,
parental PC3-ML cells. Data are expressed as mean ± S.D. (n= 3). (*c*) and (*d*) Male athymic
nude mice were inoculated by intracardiac injection with the different PC3-ML cell lines.
After 3 or 21 days, skeletal micrometastasis were identified by fluorescence
stereomicroscopy. (*c*) A representative picture showing ZsGreen PC3-ML-NTC cell
micrometastases (*arrows*) at 3 days is shown. (*d*) Number of skeletal micrometastasis 3 and
21 days days after injection. Data are expressed mean ± S.D. (n = 5). *, p<0.05; **, p<0.01.
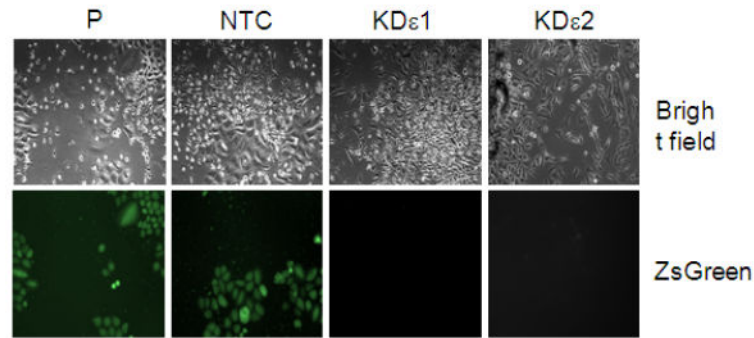
Figure 2. PKCε depleted PC3-ML cells do not accumulate in the bone marrow
Male athymic nude mice were inoculated by intracardic injection with parental PC3-ML (*P*), or PC3-ML cells subject to infection with either non-target control (*NTC*) or PKCε (*KDε1* and *KDε2*) shRNA lentiviruses. In all cases, cells express an enhanced variant of ZsGreen. Twenty-one days after intracardiac injection, cells from bone marrow were flushed and cultured for 7 days. Representative micrographs are shown.
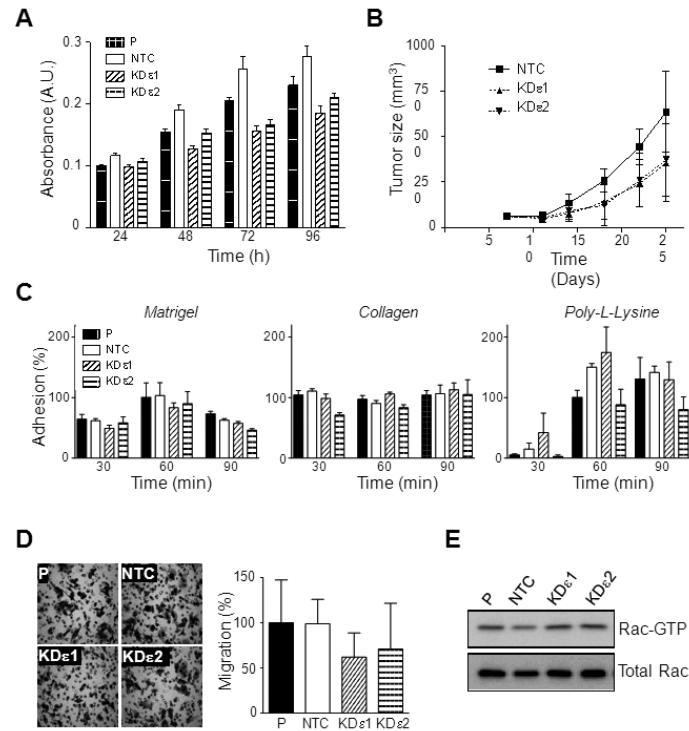
Figure 3. PKCε depletion does not affect growth, adhesiveness and motility of PC3-ML cells
(*a*) Proliferation of parental PC3-ML cells (*P*), or PC3-ML cells subject to infection with either non-target control (*NTC*) or PKCε (*KDε1* and *KDε2*) shRNA lentiviruses was determined at the indicated times. Data are expressed as mean ± S.D. (n=3). A second experiment gave similar results. (*b*) PC3-ML cell lines were injected *s.c.* into male athymic mice, and tumor growth was determined. Data are expressed as mean ± S.D. (n=10). (*c*) Different PC3-ML cell lines were seeded over culture plates coated with Matrigel, collagen or Poly-L-lysine. At the indicated times, cells were rinsed, fixed, and stained with DAPI. The number of cells adhered to the plates was quantified. Results are normalized to adhesion in parental cells at 60 min. Data are expressed as mean ± S.E.M. (n=3). (*d*) Migration of PC3-ML cell lines in response to 10% FBS (16 h) using a Boyden chamber. *Left panel*, representative micrographs. *Right panel*, quantitation of 3 independent experiments. Data are expressed mean ± S.E.M. (n=8). (*e*) Rac-GTP levels in the different PC3-ML cell lines, as determined using a pull-down assay. Two additional experiments gave similar results.
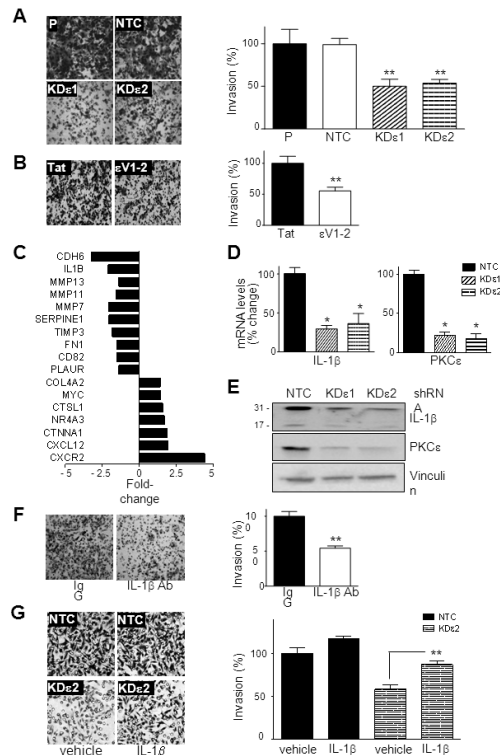
Figure 4. PKCε mediates PC3-ML cell invasiveness: a role for IL-1β

(*a*) Invasion of PC3-ML cell lines in response to 10% FBS (16 h) using a Boyden chamber with Matrigel-coated membrane. *Left panel*, representative micrographs. *Right panel*, quantitative analysis. Data are expressed as mean ± S.E.M. of 3 individual experiments. **, p<0.01. (*b*) Effect of the PKCε inhibitor εV1-2 on invasion of PC3-ML cells. Experiments were carried out in the presence εV1-2 or its control Tat peptide (1 μM). *Left panel*, representative micrographs. *Right panel*, quantitative analysis. Data are expressed as mean ± S.E.M of 3 individual experiments. **, p<0.01. (*c*) Analysis of metastasis genes by qPCR in control (NTC) and PKCε-depleted cells, using the Human Tumor Metastasis RT2 Profiler PCR array (Qiagen). The figure shows genes up- or down- regulated >1.3 times in PKCε-depleted cells (relative to NTC). Genes that achieve statistical significance according to the RT2 Profiler PCR Array Data Analysis Webportal are shown. (*d*) IL-1β and PKCε mRNA levels in PC3-ML cells subject to either control (*NTC*) or PKCε (*KDε1* and *KDε2*) shRNA, as determined by qPCR. Results are expressed as % relative to NTC. Data are expressed as mean ± S.E.M of 3 individual experiments. *, p<0.05. (*e*) Western blot depicting the expression levels of IL-1β in PC3-ML cell lines. *Upper band*, pro-IL-1β; *lower band*, IL-1β. A representative experiment is shown. Similar results were observed in 2 experiments. (*f*) Effect of a IL-1β blocking antibody (1 μM) on invasion of PC3-ML cells. IgG was used as a control. *Left panel*, representative micrographs. *Right panel*, quantitative analysis. Data are expressed as mean ± S.E.M. of 3 individual experiments. **, p<0.01. (*g*) IL-1β (10 nM) rescues the effect of PKCε depletion in PC3-ML cells. *Left panel*, representative micrographs. *Right panel*, quantitative analysis. **, p<0.01.
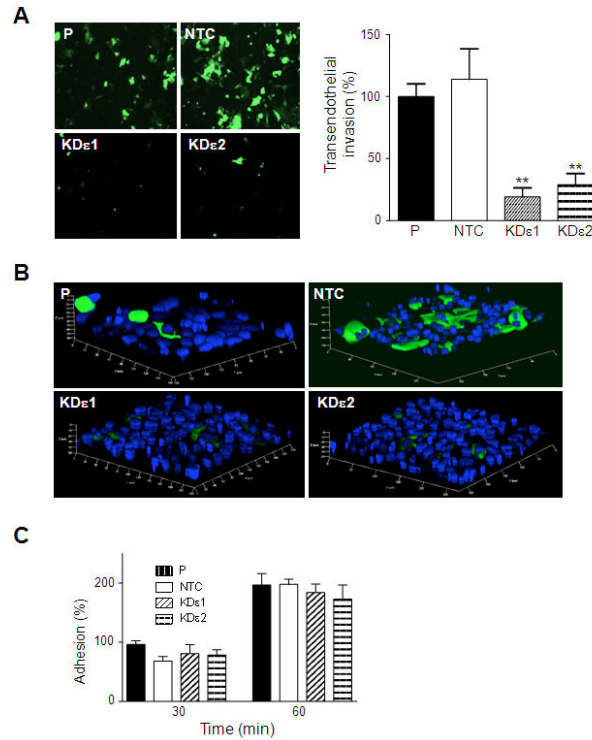
Figure 5. PKCε mediates transendothelial migration of PC3-ML cells
(*a*) Cells expressing ZsGreen were infected with either PKCε (*KDε1* and *KDε2*) or non-target control (*NTC*) shRNA lentiviruses. *P*, parental cells. Migration in response to 10% FBS (16 h) was determined using a 12 mm insert (Millipore) with a 12 µm polycarbonate porous membrane coated with a 100% confluent monolayer of HUVEC endothelial cells. *Left panel*, representative micrographs. *Right panel*, quantative analysis. Data are expressed as mean ± S.E.M. of 3 independent experiments. **, p<0.01. (*b*) Visualization of migratory cells by confocal microscopy (inverted view). *Blue*, nuclear staining using DAPI; *green*, ZsGreen expressed by PC3-ML cells. (*c*) Adhesion of PC3-ML cells to a 100% confluent monolayer of HUVEC endothelial cells. Data are expressed as mean ± S.E.M of 3 independent experiments.
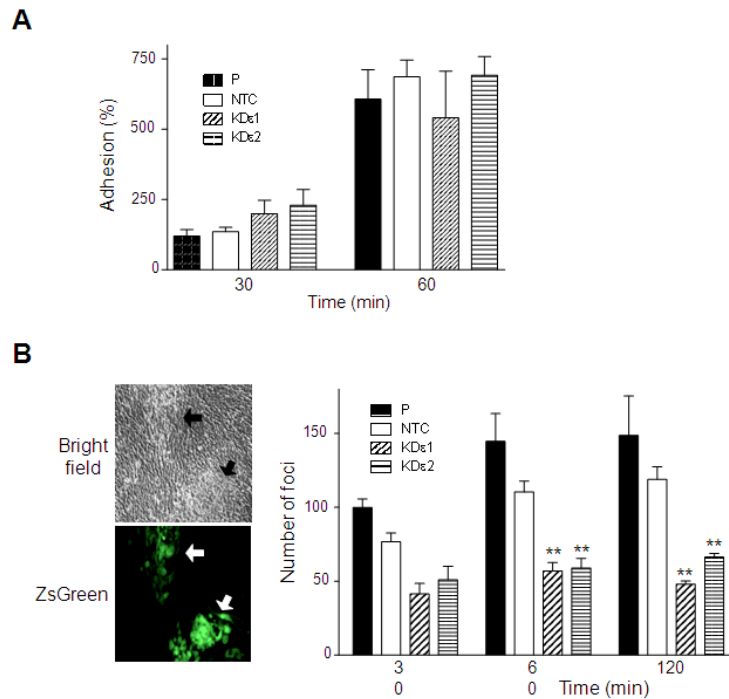
Figure 6. PKCε depletion impairs viability of PC3-ML cells in a bone biomimetic microenvironment

(*a*) Different PC3-ML cell lines expressing ZsGreen fluorescent protein were seeded over a 100% confluent monolayer of osteosarcoma human cells (MG-63). At the indicated times, non-attached cells were rinsed with PBS. Adhesion was determined as indicated in "Materials and Methods". Results are normalized to adhesion in parental cells at 30 min. Data are expressed as mean ± S.E.M. of 3 independent experiments. (*b*) After seeding PC3-ML cell lines for different times, non-attached cells were rinsed with PBS. The number of green foci was counted 3 weeks later by fluorescence microscopy. *Right panel*, representative micrographs. *Left panel*, quantitative analysis. Results were normalized to parental cells at 30 min. Data are expressed as mean ± S.E.M. of 3 independent experiments.