PKCε Is an Essential Mediator of Prostate Cancer Bone Metastasis

Alvaro Gutierrez-Uzquiza, Cynthia Lopez-Haber, Danielle L. Jernigan, Alessandro Fatatis, and Marcelo G. Kazanietz

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 IL1β, 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. Mol Cancer Res; 13(9):1336–46. ©2015 AACR.

Introduction

Prostate cancer is the second leading cause of cancer-related deaths among men, with approximately 221,000 new cases and approximately 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 advanced 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 protein kinase C (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 three different groups with diverse biochemical and functional properties: “classical/conventional” cPKCs (α, β, βII, and γ), “novel” nPKCs (δ, ε, η, and θ), and “atypical” aPKCs (ζ and λ). cPKCs and nPKCs are established cellular receptors for the phorbol ester tumor promoters, and at a physiologic 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 upregulated 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
PKCe and Prostate Cancer Bone Metastasis

as well as for metastatic dissemination. For example, NIH 3T3 cells constitutively expressing PKCe develop invadopodial-like structures, exhibit increased pericellular metalloprotease activity, and display metastatic capacity in nude mice (14). Moreover, PKCe 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 PKCe mice established this kinase as a driver of metastatic skin squamous cell carcinoma (15–19).

PKCe 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 PKCe, particularly advanced metastatic tumors (10, 15, 20, 21). Accordingly, androgen-independent prostate cancer cells display significant PKCe upregulation relative to androgen-dependent prostate cancer cell lines or “normal” immortalized prostate epithelial cells, and ectopic expression of PKCe 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 PKCe 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 PKCe inhibits the development of prostate cancer and spontaneous metastatic dissemination to lymph nodes, lung, and kidney (17). Regardless of the distinctive roles assigned to PKCe in different stages of disease progression, the role of PKCe in the dissemination of prostate cancer cells to the bone remains elusive. In this study, we investigated whether PKCe could play a role in the formation of skeletal metastases in mice. We present for the first time evidence that PKCe 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 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 PKCe inhibitor sV1-2 (tat-fused) and its control peptide Tat were a kind gift from Dr. Daria Mochly-Rosen (Stanford University, Stanford, CA).

Lentiviral infections

Stable depletion of PKCe in PC3-ML cells was achieved by infection with shRNA lentiviruses (MISSION shRNA Lentiviral Transduction particles; Sigma). As a control, we used the MISSION nontarget shRNA lentivirus. Selection of stable cell lines was carried out with puromycin (0.3 µg/mL, 3 weeks).

Western blot assays

Western blot analysis and densitometric analyses were carried out as previously described (27). The following antibodies (1:1,000 dilution) were used: anti-PKCe (sc-214; Santa Cruz Biotechnology), anti-Rac1 (05-389; Upstate Biotechnology), anti-interleukin-1β (sc-1251; Santa Cruz Biotechnology), 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), 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). 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 hours at 37°C, membranes were recovered and cells on the upper side (nonmigratory) 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). Migratory cells in each well were counted by contrast microscopy in five 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 interleukin-1β (II1β, 10 nmol/L/R&D Systems), or incubate them with anti-II1β blocking antibody (1 µmol/L/R&D Systems) or control IgG (Santa Cruz Biotechnology).

Rac-GTP pull-down assays

Determination of Rac-GTP levels was carried out essentially as previously described (28). Briefly, cells growing at 80% confluence were lysed in pull-down buffer (20 mmol/L Tris–HCl, pH 7.4, 150 mmol/L NaCl, 5 mmol/L MgCl2, 0.5% NP40, 5 mmol/L β-glycerophosphate, 1 mmol/L DTT, and protease inhibitors) containing 15 µg/mL of PBD-GST. Lysates were cleared by centrifugation (10 minutes at 4°C, 13,000 × g) and incubated with glutathione-Sepharose 4B beads (GE Healthcare) for 45 minutes 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 analysis 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 five random fields cells using the ImageJ software.

Quantitative real-time PCR

For the determination of IL1β and PKCe mRNA levels, total RNA was extracted from subconfluent cell cultures using the RNeasy Kit (Qiagen). One microgram of RNA from each sample
was reverse transcribed using the TaqMan reverse transcription reagent kit (Applied Biosystems) with random hexamers used as primers. PCR primers and 5′-end 6-carboxyfluorescein-labeled probes for PKCζ and II1β 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 nmol/L), the fluorescent probe (200 nmol/L), and 1 μL cDNA. PCR product formation was continuously monitored using the sequence detection system software version 1.7 (Applied Biosystems; ref. 29). The 6-carboxyfluorescein signal was normalized to endogenous trRNA 18S. ΔCt was obtained by subtracting the cycle threshold (Ct) of 18S or ubiquitin C from that of PKCζ. ΔΔCt was determined by subtracting the control ΔCt, from the sample ΔCt. Fold-changes were calculated using Data Assist software from Life Technologies, and expressed as 2−ΔΔCt.

mRNA levels for 84 invasive and metastatic genes, including extracellular matrix (ECM) proteases and protease inhibitors, were determined by quantitative real-time PCR (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 ΔΔCt 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 (Philadelphia, PA). For tumorigenesis experiments, male athymic mice (6–8 weeks, 10 mice/group; Harlan Laboratories) were injected s.c. with PC3-ML cells (1.2 × 106 or 4 × 105) 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 Lent-X high-titer lentiviral packaging systems (Lenti-X 293T cell line, Lenti-X HTX Packaging System, pLVX-IRES-ZsGreen1 vector from Clontech). A total of 5 × 105 cells from the brightest sorted population were inoculated in the left cardiac ventricle of athymic male athymic mice (6–8 weeks; 10 mice/group). HUVEC endothelial cells were seeded on the top chamber of a 12-mm insert Millicell Standing Insert (cat no. P8501250; Millipore) 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 × 105 cells/mL) were added on top of the endothelial monolayer, and 10% FBS was added to the lower chamber. After 18 hours 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 AxiObserver inverted confocal microscope (Carl Zeiss). Images were obtained at ×20 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 [3H]thymidine (1 μCi) for 18 hours. 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% confluence). After 30 or 60 minutes, nonadherent PC3-ML cells were removed by washing three times with 250 μL PBS. The remainder was lysed with 250 μL 0.1 mol/L NaOH, and radioactivity counted in a scintillation counter.

Coculture experiments

Osteoblast coculture experiments were carried out essentially as described previously (32). Briefly, PC3-ML cells (5 × 103) expressing ZsGreen were seeded over a confluent monolayer of MG-63 osteosarcoma cells. After different times, nonattached 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 [3H]thymidine (1 mCi) for 18 hours. 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 minutes, nonadherent PC3-ML cells were removed by washing three times with 250-μL PBS. The remainder was lysed with 250 μL 0.1 mol/L NaOH, and radioactivity counted in a scintillation counter.

Results
PKCε mediates metastatic 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 (KDε1 and KDε2) 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 nontarget control shRNA lentivirus (PC3-ML-NTC). As shown in Fig. 1B, approximately 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 PKCe 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-KDr1, and PC3-ML-KD2 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 PKCe 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-KDr1 and PC3-ML-KD2 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 tibia and femurs were flushed and cultured in vitro. Although 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-KDr1 or PC3-ML-KD2 cells (Fig. 2). Thus, PKCe is required for bone metastatic dissemination of PC3-ML cells.

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

PKCe 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 PKCe on the growth properties of PC3-ML cells in culture, we noticed only a marginal reduction in cell number in PC3-ML-KDr1 and PC3-ML-KD2 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; data not shown), the formation of tumors by PKCe-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 PKCe 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 PKCe-depleted and control PC3-ML cells (Fig. 3C).

In a recent study using lung cancer models, we showed a critical requirement of PKCe for motile activity. Moreover, we established that PKCe 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 PKCe 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 PKCe depletion. PC3-ML cells display constitutively elevated levels of active Rac-GTP levels compared with nontransformed prostate epithelial cells (data not shown). However, and consistent with the lack of involvement of PKCe in PC3-ML cell migration, silencing PKCe fails to reduce Rac-GTP levels in this cell line (Fig. 3E).

PKCe is required for PC3-ML cell invasiveness: a role for IL1b

In the next set of experiments, we sought to examine the potential involvement of PKCe 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-KDr1 and PC3-ML-KD2 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 PKCe in PC3-ML invasiveness we used the PKCe inhibitor eV1-2, a Tat-fused permeable peptide that specifically prevents PKCe translocation to the membrane and therefore inhibits its activation (18, 34). Consistent with results using PKCe 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 ECM. To evaluate a role of PKCe in the expression of matrix metalloproteinase (MMP) 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 PKCe knockdown in PC3-ML cells, which are depicted in Fig. 4C. The complete list of 84 genes is shown in Supplementary Fig. S1. Among the most notable changes, we found a significant down-regulation in the expression of MMPs (MMP7, MMP11, and MMP13). Another notable change in PKCe-depleted PC3-ML cells is the downregulation of IL1b. PC3-ML cells indeed express high levels of IL1b, and this cytokine was shown to have a fundamental role in the bone metastatic properties of these cells (26). The involvement of PKCe in the control of IL1b expression in PC3-ML cells was validated using a silencing approach, both by qPCR (Fig. 4D) and Western blot analysis (Fig. 4E). It has been recently established that IL1b is upregulated in prostate cancer.
cells; moreover, silencing IL1β expression interferes with the bone metastatic potential of PC3-ML cells (26). As our results suggest IL1β as a PKCe target, we reasoned that inhibition of IL1β signaling should also affect PC3-ML invasiveness. Indeed, incubation of PC3-ML with an anti-IL1β blocking antibody significantly reduced invasion of PC3-ML cells through Matrigel (Fig. 4F). Addition of IL1β rescued the effect of PKCe knockdown on invasion in these cells (Fig. 4G). This suggests that PKCe-mediated IL1β production is important for PC3-ML cell invasiveness.

PKCe 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 PKCe depletion on migration through endothelial cells, we used an in vitro transendothelial assay (31). Remarkably, PC3-ML-KD1 and PC3-ML-KD2 cells have a major deficiency in their ability to transmigrate through a monolayer of endothelial cells (HREVEC) relative to parental PC3-ML or PC3-ML-NTC cells (Fig. 5A). The reduced invasive capacity of PKCe-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-ML cells and PC3-ML-NTC cells but not for PKCe-depleted PC3-ML cells (Fig. 5B). The impaired transmigratory properties of PKCe silenced PC3-ML cells is not due to weakened adhesion, as determined by the...
similar capacity of control and PKCe-silenced PC3-ML cells to attach to confluent monolayers of nonstimulated (Fig. 5C) or stimulated (LPS-activated) HUVEC cells (data not shown).

To analyze whether PKCe confers a survival advantage in a bone biomimetic microenvironment, we examined the ability of PKCe-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, nonattached cells were rinsed with PBS, and 3 weeks later, the number of ZsGreen fluorescent PC3-ML foci was quantified. Whereas there were no

Figure 4. PKCe mediates PC3-ML cell invasiveness: a role for IL1-beta. A, invasion of PC3-ML cell lines in response to 10% FBS (16 hours) using a Boyden chamber with Matrigel-coated membrane. Left, representative micrographs. Right, quantitative analysis. Data, mean ± SEM of three individual experiments. **, P < 0.01. B, effect of the PKCe inhibitor eV1-2 on invasion of PC3-ML cells. Experiments were carried out in the presence of eV1-2 or its control Tat peptide (1 μmol/L). Left, representative micrographs. Right, quantitative analysis. Data, mean ± SEM of three individual experiments. **, P < 0.01. C, analysis of metastasis genes by qPCR in control (NTC) and PKCe-depleted cells, using the Human Tumor Metastasis RT2 Profiler PCR array (Qiagen). The figure shows genes up- or downregulated >1.3 times in PKCe-depleted cells (relative to NTC). Genes that achieve statistical significance according to the RT2 Profiler PCR Array Data Analysis Webportal are shown. D, IL1-beta and PKCe mRNA levels in PC3-ML cells subject to either control (NTC) or PKCe (KD1 and KD2) shRNA, as determined by qPCR. Results are expressed as a percentage relative to NTC. Data, mean ± SEM of three individual experiments. **, P < 0.01. E, Western blot analysis depicting the expression levels of IL1-beta in PC3-ML cell lines. Top band, pro-IL1-beta; bottom band, IL1-beta. A representative experiment is shown. Similar results were observed in two experiments. F, effect of an IL1-beta blocking antibody (1 μmol/L) on invasion of PC3-ML cells. IgG was used as a control. Left, representative micrographs. Right, quantitative analysis. Data, mean ± SEM of three individual experiments. **, P < 0.01. G, IL1-beta (10 nmol/L) rescues the effect of PKCe depletion in PC3-ML cells. Left, representative micrographs. Right, quantitative analysis. **, P < 0.01.
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 PKCe as a novel player in skeletal metastasis of prostate cancer cells. Our results clearly show that silencing the expression of PKCe 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 PKCe in key steps leading to prostate cancer cell colonization in the bone.

PKCe has been widely associated with the development of epithelial cancers, and has been originally described as an oncoprotein that is able to transform fibroblasts through the activation of the Ras–Raf1 signaling pathway and autocrine secretion of TGFβ (19, 36, 37). Numerous laboratories underscored important roles for PKCe in cell-cycle progression and the control of cell survival mechanisms (2, 5, 6). For example, forced expression of PKCe 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 PKCe modulates Bad phosphorylation to protect LNCaP cells against phorbol ester- and TNFα-induced apoptosis.
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 that 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 pharmacologic 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 IL1β expression. Although the mechanisms by which PKCε controls IL1β 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 IL1β transcription and secretion (42). IL1β 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 IL1β contributes to the tumorigenic potential of malignant cells and potentiates carcinogenesis by promoting local inflammatory responses. Moreover, IL1β plays essential roles in cancer cell invasiveness, and neutralization of secretable IL1β is sufficient to limit tumor invasiveness (42). More recently, Liu and colleagues (26) established a functional association between IL1β expression and the acquisition of bone metastatic capabilities of prostate cancer cells. Indeed, IL1β is upregulated in highly bone

(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 upregulated in prostate cancer cell lines and tumor specimens, and is a predictive biomarker of prostate cancer (23, 24). PKCε overexpression is also observed in prostate from prostates from transgenic overexpression of PKCε in skin promotes the development of squamous cell carcinomas that 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 pharmacologic 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 IL1β expression. Although the mechanisms by which PKCε controls IL1β 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 IL1β transcription and secretion (42). IL1β 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 IL1β contributes to the tumorigenic potential of malignant cells and potentiates carcinogenesis by promoting local inflammatory responses. Moreover, IL1β plays essential roles in cancer cell invasiveness, and neutralization of secretable IL1β is sufficient to limit tumor invasiveness (42). More recently, Liu and colleagues (26) established a functional association between IL1β expression and the acquisition of bone metastatic capabilities of prostate cancer cells. Indeed, IL1β is upregulated in highly bone
metastatic PC3-ML cells relative to low metastatic PC3-N cells. Moreover, shRNA-mediated silencing of IL1β from PC3-ML cells leads to a marked inhibition of skeletal metastasis, whereas PC3-N cells engineered to ectopically overexpress IL1β 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 IL1β. Although this has yet to be formally demonstrated, our results clearly reveal that a blocking IL1β antibody significantly reduces migration of PC3-ML cells through Matrigel, thus supporting the involvement of PKCε-mediated IL1β production in PC3-ML cell invasiveness. Because IL1β is known to stimulate the bone-resorption activity of osteoclasts (43), it may be possible that PKCε, by controlling the synthesis of IL1β, promotes bone matrix turnover. IL1β derived from malignant cells stimulates the production of a proinflammatory environment and increases COX-2 expression and PGE2 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, IL1β 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. Because IL1β has been shown to induce PKCε expression (45), and PKCs (including PKCγ) are also downstream effectors of PGE2 and IL1β (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 anticancer 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 pharmacologic 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-kB activation (50). Thus, an attractive possibility is that PKCε inhibitors may have therapeutic benefit for the treatment of bone metastatic disease.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: A. Gutierrez-Uzquiza, A. Fatatis, M.G. Kazanietz
Development of methodology: A. Gutierrez-Uzquiza, C. Lopez-Haber, A. Fatatis, M.G. Kazanietz
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Gutierrez-Uzquiza, C. Lopez-Haber, D.L. Jernigan, A. Fatatis, M.G. Kazanietz
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Gutierrez-Uzquiza, C. Lopez-Haber
Writing, review, and/or revision of the manuscript: A. Gutierrez-Uzquiza, C. Lopez-Haber, A. Fatatis, M.G. Kazanietz
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Gutierrez-Uzquiza, C. Lopez-Haber
Study supervision: A. Fatatis, M.G. Kazanietz
Other (technical training): D.L. Jernigan

Acknowledgments
The authors thank Dr. Lorena Benedetti for helping with confocal microscopy.

Grant Support
This work is supported by grants R01-CA89202 from the NIH (M.G. Kazanietz) and PC080987 from the U.S. Department of Defense Congressionally Directed Medical Research Program (DOD CDMRP; to A. Fatatis). A. Gutierrez-Uzquiza was supported by a postdoctoral fellowship (PC102041) from the U.S. DOD CDMRP.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 4, 2015; revised May 1, 2015; accepted May 20, 2015; published OnlineFirst May 28, 2015.

References


PKCε Is an Essential Mediator of Prostate Cancer Bone Metastasis

Alvaro Gutierrez-Uzquiza, Cynthia Lopez-Haber, Danielle L. Jernigan, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-15-0111

Cited articles
This article cites 50 articles, 18 of which you can access for free at:
http://mcr.aacrjournals.org/content/13/9/1336.full.html#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.