PKCε Is an Essential Mediator of Prostate Cancer Bone Metastasis

Alvaro Gutierrez-Uzquiza1, Cynthia Lopez-Haber1, Danielle L. Jernigan2, Alessandro Fatatis2,3, and Marcelo G. Kazanietz1

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.

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PKCε and Prostate Cancer Bone Metastasis

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 upregulation 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 neoplastic 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 immortalized prostate epithelial cells, and ectopic expression of PKCε in 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 neoplastic 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.

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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 IL18 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 mRNA 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 × 10² or 4 × 10²) 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-IRE-ZsGreen1 vector from Clontech). A total of 5 × 10⁴ 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 coinjection of blue fluorescent 10-μm polystyrene beads (Molecular Probes). Animals were randomly assigned to different experimental groups and Sacrificed either at 72 hours 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 hours and transferred into fresh formaldehyde for an additional period of 24 hours. Bones were decalcified in 0.5 mol/L 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 micrometastases was determined. Microscope and software calibration for size measurement was conducted using a TS-M2 stage micrometer (Oplenic Optronics).

Bone marrow cultures

Bone marrow from mouse tibia and femur were flushed after 3 weeks after intracardiac inoculation. Cells were dissociated using a syringe (19-Gy needle) and incubated with RPMI media supplemented with 10% FBS, 2 mmol/L 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 and colleagues (31). Briefly, HUVEC endothelial cells were seeded on the top chamber of a 12-mm insert Millicell Standing Insert (cat no. PIKP01250; 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 × 10⁴ 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 AxioObserver 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 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 the lower chamber. 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 × 10⁴) 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% 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.

Results

PKCe mediates metastatic dissemination of PC3-ML prostate cancer cells to the bone

Emerging data from several laboratories established essential roles for PKCe in the progression of cancer, including prostate cancer (2, 5, 6, 10, 17). PKCe 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 PKCe relative to “normal” immortalized prostate epithelial cells (Fig. 1A). To ascertain the role of PKCe in prostate cancer bone metastasis, we used shRNA lentiviruses to stably knockdown PKCe from PC3-ML cells, followed by selection with puromycin. Two different shRNA lentiviruses were used (e1 and e2) in order to generate PC3-ML-KD1 and PC3-ML-KD2 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 PKCe levels was achieved in PC3-ML-KD1 and PC3-ML-KD2 cell lines, respectively (n = 3). To address the relevance of PKCe 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). Parenteral PC3-ML, PC3-ML-NTC, PC3-ML-KDe1, and PC3-ML-KDe2 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-KDe1 and PC3-ML-KDe2 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-KDe1 or PC3-ML-KDe2 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-KDe1 and PC3-ML-KDe2 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 somewhat slower, although there were no statistically significant differences between the different cell lines (Fig. 3B).

Figure 2.
PKCe-depleted PC3-ML cells do not accumulate in the bone marrow. Male athymic nude mice were inoculated by intracardiac injection with parental PC3-ML (P), or PC3-ML cells subject to infection with either nontarget control (NTC) or PKCe (KDe1 and KDe2) 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.

PKCe is required for PC3-ML cell invasiveness: a role for IL1β

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 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 IL1β

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 assay (18). It has been previously reported that PC3 and PC3-ML cells are highly invasive (25). Notably, PC3-ML-KDe1 and PC3-ML-KDe2 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 εV1-2, 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 downregulation in the expression of MMPs (MMP7, MMP11, and MMP13). Another notable change in PKCe-depleted PC3-ML cells is the downregulation of IL1β. PC3-ML cells indeed express high levels of IL1β, 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 IL1β 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 IL1β is upregulated in prostate cancer.

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PKCe depletions 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 nontarget control (NTC) or PKCe (KD1 and KD2) shRNA lentiviruses was determined at the indicated times. Data, mean ± SD (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, mean ± SD (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 minutes. Data, mean ± SEM (n = 3). D, migration of PC3-ML cell lines in response to 10% FBS (16 hours) using a Boyden chamber. Left, representative micrographs. Right, quantitation of three independent experiments. Data are expressed mean ± S.E.M. (n = 3). E, Rac-GTP levels in the different PC3-ML cell lines, as determined using a pull-down assay. Two additional experiments gave similar results.

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 cell lines 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 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 PKCε-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 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, 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.** PKCε mediates PC3-ML cell invasiveness: a role for IL1β. 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. **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 μmol/L). Left, representative micrographs. Right, quantitative analysis. Data, mean ± SEM of three individual experiments. **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 downregulated >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, IL1β and PKCε mRNA levels in PC3-ML cells subject to either control (NTC) or PKCε (KD1 and KD2) shRNA, as determined by qPCR. Results are expressed as a percentage relative to NTC. Data, mean ± SEM of three individual experiments. **E**, Western blot analysis depicting the expression levels of IL1β in PC3-ML cell lines. Top band, pro-IL1β; bottom band, IL1β. A representative experiment is shown. Similar results were observed in two experiments. F, effect of a IL1β 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. **G**, IL1β (10 nmol/L) rescues the effect of PKCε 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-KD
e1 and PC3-ML-KD
e2 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 PKC
e as a novel player in skeletal metastasis of prostate cancer cells. Our results clearly show that silencing the expression of PKC
e 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 PKC
e in key steps leading to prostate cancer cell colonization in the bone.

PKC
e 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n (19, 36, 37). Numerous laboratories underscored important roles for PKC
e in cell-cycle progression and the control of cell survival mechanisms (2, 5, 6). For example, forced expression of PKC
e 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 PKC
e modulates Bad phosphorylation to protect LNCaP cells against phorbol ester- and TNF
t-induced apoptosis.
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; 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 prosurvival signaling pathways, namely Akt, NF-κB, and Stat3 (23, 24). PKCε upregulation 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 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-KD1 and PC3-ML-KD2 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 chemotraction 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/frac-talkine (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 PKCe 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 PKCe-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 PKCe, 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 PKCe effector gene in prostate cancer cells (24), we hypothesize that PKCe 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 PKCe may play key roles. The enhanced survival of PKCe expressing PC3-ML cells in a bone mimetic microenvironment is consistent with this premise. Because IL1β has been shown to induce PKCe expression (45), and PKCs (including PKCe) are also downstream effectors of PGE2 and IL1β (46, 47), it is reasonable to speculate that sustained activation of PKCe in prostate cancer cells (and possibly bone stromal cells) may contribute to a vicious cycle that facilitates skeletal metastasis.

In conclusion, our studies identified PKCe 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 PKCe inhibitors with anticaner 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-κB activation (50). Thus, an attractive possibility is that PKCe 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.

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References
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