The role of PTEN in prostate cancer cell tropism to the bone micro-environment

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Abbreviations: MEM, minimum essential medium; HA, hemaglutinin; EGFP, enhanced green fluorescent protein.

Little is known about the role of the tumor suppressor gene phosphatase and tensin homolog deleted on chromosome 10 (PTEN) in prostate cancer bone metastasis. To explore this, we used a pTetOn PTEN cell line in which PTEN expression was reconstituted in a PTEN-null bone metastatic human prostate cancer cell line, LnCaP-C4-2. We found that C4-2 cells selectively migrated toward conditioned medium from primary mouse calvaria compared with that derived from lung fibroblasts. Further evaluation with conditioned medium from an established mouse calvaria osteoblast cell line and control non-osteoblast cell line indicates that osteoblastic characteristics convey this specific migration to C4-2 cells. We evaluated promiscuously metastatic PC-3 prostate as well as T24T and UM-3 bladder cells and found they did not have a specific migratory response to calvaria-conditioned medium as did C4-2. Induction of PTEN expression inhibited the motility of C4-2 cells toward calvaria-conditioned medium but had no effect on migration toward lung-conditioned medium and this inhibitory effect was dependent on the PTEN lipid phosphatase activity. Calvaria- but not lung-conditioned medium induced activation of the small GTPase Rac1. Constitutively active Rac1 but not focal adhesion kinase or Cdc42 could rescue cells from the inhibitory effect of PTEN on cell migration and PTEN induction was observed to inhibit Rac1 activation in response to calvaria-conditioned medium. Our results support the notion that loss of PTEN function in human prostate cancer may specifically facilitate bone rather than other organ metastasis and suggest that Rac1, as a PTEN effector, may contribute to this metastatic tropism.

Introduction

Bone is the major site of prostate cancer metastasis (1) and the frequency of bone metastasis is much higher than predicted by random tumor cell dissemination (2). Bone metastases from prostate cancer are generally osteoblastic with local stimulation of osteoblasts adjacent to the metastatic tumor cells (3). This tropism of prostate cancer to bone is important to the treatment of metastatic disease and bone-targeted therapy has shown promising results in recent years (4,5). Fortunately, the mechanisms underlying this osteotropic process are gradually being elucidated (6–8).

Cancer cell migration has been considered an important step for metastasis. Metastatic tumor cells display more active motility with increased expression of genes involved in cell motility (9). After the adhesion of tumor cells to the microvasculature of host organs, cell motility and subsequently chemotactic extravasation of adherent cells are required for cells to reach the specific organ. In prostate cancer, tumor cells could be specifically attracted by bone-specific factors and migrate preferentially to bone (8). For example, Jacob et al. (10) found that prostate cancer cells migrated preferentially to bone extracts as well as to conditioned medium of bone marrow stromal cells and they identified osteonectin/SPARC (secreted protein, acidic, and rich in cysteine) as the factor that mediated this migration.

The tumor suppressor gene phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is frequently deleted in various advanced human cancers, including prostate (11,12). While only 10–15% of all prostate tumors have PTEN mutations, PTEN inactivation has been found in up to 60% of advanced prostate cancers (12–15). In addition, the frequency of the loss of PTEN in prostate cancer is correlated with high Gleason score and advanced tumor stage (16,17), suggesting the potential role of PTEN in prostate cancer progression. Furthermore, PTEN inactivation is found to be correlated with invasiveness and metastasis in various cancers including prostate cancer (18–20). Recently, transgenic mice studies with prostate-specific deletion of PTEN further reveal a functional involvement of PTEN in prostate cancer progression and metastasis (21–23). The observation that these mice developed liver and bone metastasis may indicate that metastatic tropism is a species-dependent phenotype.

Although loss of PTEN is common in advanced prostate cancer, little is known about its functional role specifically in prostate cancer bone metastasis. To explore the role of PTEN in this process, we used a pTetOn PTEN cell line in which PTEN expression was reconstituted in a PTEN-null bone metastatic human prostate cancer cell line, LnCaP-C4-2 (24). Using a Boyden chamber motility assay and conditioned medium from primary mouse calvaria or lung fibroblast cells, we sought to address the following questions: (i) do prostate cancer cells preferentially migrate toward bone-conditioned medium compared with that from other organs and is this related to their bone metastatic tropism? (ii) does PTEN have a regulatory role in this migratory process and is this effect specific to the bone micro-environment? (iii) is the lipid phosphatase activity of PTEN necessary in prostate cancer cell migration toward bone-conditioned medium and finally (iv) what are the signaling molecules involved in the PTEN-mediated effects on bone-conditioned medium-specific migration.

Materials and methods

Cell culture

LnCaP, PC-3 and immortalized mouse pre-osteoblast cell lines (MC3T3 clones 1 and 24) were obtained from the American Type Culture Collection (Rockville, MD). C4-2b cells were from UroCore (Oklahoma City, OK). The inducible pTetOn PTEN, wild-type (WT) PTEN, and pTetOn PTEN (G129E), mutant PTEN C4-2 cells were described previously (24). Both LnCaP and C4-2b cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). PC-3 cells were cultured in Dulbecco’s modified Eagle’s medium as did C4-2. Induction of PTEN expression inhibited the motility of C4-2 cells toward calvaria-conditioned medium but had no effect on migration toward lung-conditioned medium and this inhibitory effect was dependent on the PTEN lipid phosphatase activity. Calvaria- but not lung-conditioned medium induced activation of the small GTPase Rac1. Constitutively active Rac1 but not focal adhesion kinase or Cdc42 could rescue cells from the inhibitory effect of PTEN on cell migration and PTEN induction was observed to inhibit Rac1 activation in response to calvaria-conditioned medium. Our results support the notion that loss of PTEN function in human prostate cancer may specifically facilitate bone rather than other organ metastasis and suggest that Rac1, as a PTEN effector, may contribute to this metastatic tropism.

Abbreviations: FAK, focal adhesion kinase; FBS, fetal bovine serum; PTEN, phosphatase and tensin homolog deleted on chromosome 10; WT, wild type; MEM, minimum essential medium; HA, hemaglutinin; EGFP, enhanced green fluorescent protein; PKA-1, p21 activated kinase 1; P3K, phosphoinositide 3-kinase; EGF, epidermal growth factor; PDGF, platelet-derived growth factor.
Cultures of primary mouse calvaria cells were isolated from the calvaria by the use of a procedure published previously (30), with modifications as follows. After dissection, calvaria cells were minced and digested sequentially for 8 and 10 min on a rotator at 37°C in α-MEM containing 1 mg/ml collagenase P (Roche Applied Science, Indianapolis, IN) and 0.25% trypsin (Life Technologies). The digestion medium and any released cells were then discarded. The digestion was repeated for 30 min, and the cell suspension was transferred to new tubes and washed with α-MEM plus 10% FBS. This 30 min digestion was repeated one more time and cell suspension was collected and pooled with previous digested cells. Cells were counted and plated in 10 cm plates with a density of 1.2 x 10⁴ cells/cm² in α-MEM plus 10% FBS. After 48 h, cells were trypsinized and replated for expansion and the expanded cells were plated in culture dishes to perform the experiment. To obtain lung fibroblasts, we isolated lungs from same mice that we got calvaria cells. Lungs were

Fig. 1. C4-2 cell migration toward conditioned medium from either calvaria or lung fibroblast cells. (A) Boyden chambers were plated with 40 000 pTetOn PTEN C4-2 cells in α-MEM–0.5% FBS. Cells were allowed to migrate toward serum-free α-MEM (SF), primary mouse calvaria cell-conditioned medium (calvaria CM), primary mouse lung fibroblast-conditioned medium (lung CM) or α-MEM–10% FBS (10% FBS) for 24 h. Cells on the upper surface of the filter were removed and cells on the lower surface were fixed, stained and counted. (B) Protein concentrations of the different medium used in experiment (A) were determined with NanoOrange assay as described in Materials and methods. Inset: migration data in (A) normalized by protein concentration of conditioned media or added serum from (B). (C) pTetOn PTEN C4-2 cells (40 000) were plated into Boyden chambers in α-MEM–0.5% FBS. Cells were allowed to migrate toward conditioned medium (CM) from primary mouse lung fibroblast, primary mouse calvaria or established mouse calvaria cell lines MC3T3-4 and MC3T3-24. Cells on the lower surface of the filter were fixed, stained and counted after the removal of the cells on the upper surface. Same number of cells was plated in 96-well plate in the context of different conditioned medium for plating control and cell numbers were quantitated by CyQuant assay. (D) Growth study of pTetOn PTEN C4-2 cells in different conditioned medium. Cells were plated in α-MEM, lung fibroblast-conditioned medium or calvaria-conditioned medium with 0.5% FBS in all of them. Cell numbers were quantified by MTT assay at the indicated time points. Error bars indicate standard deviation for a representative experiment performed in duplicate (A, B and C) or triplicate (D). P value was derived from t-test. All experiments were repeated at least three times.
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Lakes, NJ) in a 24-well tissue culture plate. The lower chambers contained 0.65 ml serum-free, α-MEM with 10% FBS or conditioned medium. For plating control, same number of cells was added in duplicate in wells of 96-well plate (roughly the same growth area as compared with the upper chamber of transwell) containing 100 μl conditioned medium. After 24 h of plating, cells remaining on the upper surface of the filters were removed with cotton swabs and cells on the lower surface were fixed with 100% methanol, stained with crystal violet and counted with the aid of a grid coverslip (Belloco Biotechnol.

Vineland, NJ). Cell numbers in plating control wells of 96-well plates were determined with CyQuant (Molecular Probes) according to the manufacturer’s instructions.

Western blot analysis and Rac activation assay

Protein extracts were prepared from pTetOn PTEN C4-2 cells after the PTEN induction with 48 h of doxycycline (MP Biomedicals, Irvine, CA) treatment or 48 h after transfection with Rac1-V12, Cdc42-V12 and PAK. Western blot was carried out as described (26) using commercial chemiluminescence reagents (SuperSignal West Femto, Pierce Biotechnology, Rockford, IL) and photographic film. The anti-HA (hemaglutinin) monoclonal antibody was purchased from Covance (Berkeley, CA) and used at 1:3000 dilution. The polyclonal anti-GFP (green fluorescent protein) (BD Biosciences, Palo Alto, CA) antibody was used at 1:4000 dilution. The monoclonal anti-α-tubulin antibody was purchased from Oncogene (San Diego, CA) and was diluted at 1:2000. The horse-

radish peroxidase-conjugated anti-mouse (Pierce) or anti-rabbit (New England Biolabs, Beverly, MA) secondary antibody was used at 1:2000 dilution. The Rac 1 activation assay was described previously (31) and the active GTP-bound Rac was pulled down with PAK1 (p21 activated kinase 1) agarose beads according to the manufacturer’s protocol (Upstate, Charlotteville, VA).

Statistical analysis

Values shown are representative of three or more experiments carried out independently. Treatment group comparisons were evaluated using Student’s t-test. Error bars are standard deviation of the means. Values of P < 0.05 were taken as a significant difference between means.

Results

C4-2 prostate cancer cells preferentially migrated toward conditioned medium from osteoblast cells

Previously, we established a conditional PTEN expression cell line, pTetOn PTEN C4-2 (24), in which PTEN expression was under the control of TetOn system in a Pten-null bone metastatic human prostate cancer cell line, LnCaP-C4-2 (14). To explore the possible mechanism involved in prostate cancer bone metastasis, we assessed the ability of pTetOn PTEN C4-2 cells in migrating toward conditioned medium from primary mouse calvaria or lung fibroblast cells in the Boyden chamber. Minimal motility activity was observed in the presence of lung fibroblast-conditioned medium in the bottom well that was similar to the cell motility in the presence of serum-free medium. Although the motility activity of C4-2 cells was still lower in calvaria-conditioned medium compared with that in 10% FBS medium, it was stimulated >10-fold by the calvaria-conditioned medium compared with lung fibroblast-conditioned medium (Figure 1A). No significant difference in cell attachment and spreading was observed in the presence of different medium in the bottom well under microscopy observation (data not shown). Although the conditioned medium was collected from same number of calvaria or lung fibroblast cells as

Fig. 2. In vitro migration patterns toward calvaria-conditioned medium versus lung fibroblast-conditioned medium in cell lines with different in vivo metastatic characterization. (A) Prostate cancer cell lines, LnCaP, C4-2 and PC3, as well as bladder cancer cell lines, T24T, and UMUC-3, were plated into Boyden chamber in α-MEM–0.5% FBS. Cells were allowed to migrate toward either calvaria- or lung fibroblast-conditioned medium (CM) for 24 h. (B) The ratio of calvaria CM/LnCaP vs. lung fibroblast CM/LnCaP.

Minced and digested for 30 min twice on a rotator at 37°C in 5 ml α-MEM containing 1 mg/ml collagenase P and 0.25% trypsin. Cell suspension from these two digestions was pooled together and plated as we did for calvaria cells.

Conditioned medium preparation from calvaria, lung fibroblasts and pre-osteoblast MC3T3 cells

For conditioned medium collection from undifferentiated subconfluent cultures, 5 × 10^6 primary mouse calvaria, lung fibroblast or immortalized mouse pre-osteoblast MC3T3 clones 4 and 24 cells were plated into 10 cm tissue culture plate in α-MEM plus 10% FBS. At the same time, cells with similar density were plated into 12-well plate for cell number determination. Twenty-four hours later, cells were washed in phosphate-buffered saline two times and were switched to 7 ml (10 cm plate) or 0.5 ml (12-well plate) serum-free α-MEM. After 24 h incubation, conditioned medium in 10 cm plate with cell density ~80% was collected and stored at ~80°C in 1 ml aliquot. CyQuant assay (Molecular Probes, Eugene, OR) was performed according to the manufacturer’s instructions to determine cell numbers in 12-well plate between calvaria, lung fibroblast and MC3T3 cells and result showed similar cell numbers between different cell types at the time of conditioned medium collection. The protein concentration of different conditioned medium was determined with NanoOrange assay (Molecular Probes) according to the manufacturer’s instructions.

Cell chemotaxis and plating efficiency assays

Cells were harvested, counted in a hemacytometer and re-suspended in α-MEM containing 0.5% FBS. Cells (40 000) were added in duplicate to the upper chamber of transwell filters (8.0 μm pores, Becton Dickinson, Franklin Lakes, NJ) in a 24-well tissue culture plate. The lower chambers contained 0.65 ml serum-free, α-MEM with 10% FBS or conditioned medium. For plating control, same number of cells was added in duplicate in wells of 96-well plate (roughly the same growth area as compared with the upper chamber of transwell) containing 100 μl conditioned medium. After 24 h of plating, cells remaining on the upper surface of the filters were removed with cotton swabs and cells on the lower surface were fixed, stained and counted. The migration ratio toward calvaria-conditioned medium versus lung fibroblast-conditioned medium of different cell lines as shown in (A). Error bars indicate standard deviation for a representative experiment performed in duplicate. All experiments were repeated at least three times.

Four hours later, cells were washed in phosphate-buffered saline two times and were switched to 7 ml (10 cm plate) or 0.5 ml (12-well plate) serum-free α-MEM containing 0.5% FBS. Cells were added in duplicate to the 96-well plate (roughly the same growth area as compared with the upper chamber of transwell) containing 100 μl conditioned medium. After 24 h of plating, cells remaining on the upper surface of the filters were removed with cotton swabs and cells on the lower surface were fixed with 100% methanol, stained with crystal violet and counted with the aid of a grid coverslip (Belloco Biotechnol.

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from three separate experiments with AlphaInnotech software. Levels of active Rac1 were normalized by levels of total Rac1.

Z.Wu et al. were repeated at least three times. CyQuant assay. Error bars indicate standard deviation for a representative experiment performed in duplicate.

Plated in 96-well plate in the context of lung- or calvaria-conditioned medium with or without doxycycline treatment for 24 h. Cell numbers were quantitated with

were removed and cells on the lower surface were fixed, stained and counted. (a) Wild type

were fixed, stained and counted. (B) C4-2 pTetOn PTEN (C) Cells were either untreated (no DOX) or treated with 1 $\mu$g/ml doxycycline for 16 h to induce the expression of WT PTEN and G129E mutant PTEN, respectively. The same number of cells (40 000) was plated into Boyden chamber in α-MEM–0.5% FBS with or without the addition of 1 $\mu$g/ml doxycycline. Cells were allowed to migrate toward either calvaria- or lung fibroblast-conditioned medium (CM) for 24 h. Cells on the upper surface of the filter were removed and cells on the lower surface were fixed, stained and counted. (D) Plating control for migration experiment of (B). Same numbers of cells were plated in 96-well plate in the context of lung- or calvaria-conditioned medium with or without doxycycline treatment for 24 h. Cell numbers were quantitated with CyQuant assay. Error bars indicate standard deviation for a representative experiment performed in duplicate. $P$ value was derived from t-test. All experiments were repeated at least three times.

determined by CyQuant assay (31 337 ± 2336 versus 31 116 ± 1070 fluorescence units for lung and calvaria cells, respectively). The protein concentration of the calvaria-conditioned medium was 50% more than that of lung fibroblast-conditioned medium as determined by NanoOrange assay (Figure 1B). Normalization of migration effect as a function of protein concentration did not alter the overall cell migration pattern toward lung and calvaria medium and further confirmed the preferential effect of bone-derived conditioned medium compared with lung-derived conditioned medium (Figure 1B).

To determine whether the selective migration of C4-2 cells toward calvaria-conditioned medium was related to osteoblastic features of calvaria cells, similar migration experiment was carried out with conditioned medium from two immortalized mouse pre-osteoblast cell lines, MC3T3 clones 4 (MC3T3-4) and 24 (MC3T3-24). MC3T3-4

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cells were able to fully differentiate into osteoblast in vitro whereas MC3T3-24 could not (32). Similar to calvaria-conditioned medium, subconfluent MC3T3-4-conditioned medium significantly increased motility of C4-2 cells whereas MC3T3-24-conditioned medium had less effect (Figure 1C) suggesting that C4-2 cells preferentially migrate toward conditioned medium from cells with osteoblastic potential. Plating controls revealed similar number of cells plated into different conditions and similar rates of C4-2 growth in calvaria- or lung fibroblast-conditioned medium in up to 120 h time period (Figure 1D) were observed. This indicates that the difference in C4-2 cell migration toward different conditioned medium was not caused by differential growth effects.
To evaluate whether the preferential migration of cancer cells toward calvaria-conditioned medium is reflective of metastatic bone tropism in vivo, we compared cell migration of a panel of cancer cell lines toward calvaria- and lung-conditioned medium. LnCaP is a human prostate cancer cell line that does not metastasize to bone (33). PC-3 is a prostate cancer cell line that can metastasize to multiple organs including lung, bone and liver (34–36). The human bladder cancer cell line, T24 is a highly metastatic derivative of T24 (37), which is known to metastasize to bone (38) and other organs such as lung and liver and UMUC-3, a human bladder cancer cell line, is able to metastasize to lung and bone (D. Theodorescu, unpublished observations). Here show that LnCaP or C4-2 cells migrated 9- and 29-fold more, respectively, toward calvaria-conditioned medium compared with lung fibroblast-conditioned medium (Figure 2A and B). In contrast, cells that did not have bone tropism in vivo had only a 3- to 4-fold preferential migration toward calvaria-conditioned medium (Figure 2A and B). This result suggests that the pattern of cell migration toward calvaria-conditioned medium versus lung-conditioned medium reflects the degree of metastatic osteotropism in prostate cancer and is thus an effective tool to discover the mechanisms underlying this process. However, the preferential migration of LnCaP, a non-metastatic cell line, to calvaria-conditioned medium also suggests that cell migration is not the only mechanism associated with organ-specific metastasis.

PTEN induction specifically inhibits cell migration to bone-conditioned medium and this requires its lipid phosphatase activity. To explore the role of PTEN in prostate cancer metastasis to bone, we used previously generated cell lines conditional expressing WT or G129E mutant PTEN. Previous work demonstrated that under similar expression levels with 1 µg/ml doxycycline treatment, expression of WT PTEN inhibited the level of pAkt, whereas induction of G129E mutant PTEN had no effect on pAkt level (24), consistent with its lack of lipid phosphatase activity. PTEN can inhibit cell migration (23,30), and this is confirmed by our results (Figure 3A). In addition, we found that the lipid phosphatase activity of PTEN was required for this inhibitory effect (Figure 3A).

Since the data presented above (Figure 2B) suggest that migration to bone-conditioned media closely relates to the bone tropism of cancer cell metastasis, we sought to evaluate the effect of PTEN reconstitution on C4-2 cell migration toward calvaria- and lung fibroblast-conditioned medium. While C4-2 cell migration toward lung fibroblast-conditioned medium was not inhibited (P = 0.1) after the induction of WT PTEN, cell migration toward calvaria-conditioned medium was inhibited 80% (P = 0.01) (Figure 3B). In contrast, induction of G129E mutant PTEN had no effect on C4-2 cell migration toward either lung fibroblast- or calvaria-conditioned medium (Figure 3C), suggesting that the inhibition of PTEN in cell migration toward calvaria-conditioned medium was dependent on its lipid phosphatase activity. Importantly, plating controls revealed no significant decrease of cell number after WT PTEN expression during the 24 h time period of migration assay (Figure 3D), indicating that the inhibition of cell migration by PTEN expression was not due to its growth inhibitory effect.

The specific inhibition of cell motility by PTEN in response to bone-conditioned medium is mediated through Rac1 but not Cdc42 or FAK. The inhibitory effect of PTEN on cell motility has been related to decreased phosphor-FAK (pFAK) level presumably through its protein phosphatase activity (41). Since FAK plays an essential role in cell migration (42,43), we sought to determine whether the decreased cell migration after PTEN induction was associated with alterations of pFAK. Expression of PTEN in C4-2 cells decreased pFAK level (Figure 4A) by ~30% during cell reattachment (P = 0.03) (Figure 4B). However, over-expression of FAK failed to rescue the decreased cell migration toward calvaria-conditioned medium after PTEN expression (Figure 4C), indicating that FAK was unlikely involved in the inhibitory effect of PTEN on C4-2 cell migration toward calvaria-conditioned medium. This is consistent with the result from Figure 3C where G129E mutant PTEN showed no effect on cell migration toward calvaria-derived conditioned medium.

It has been shown that Rac1 and Cdc42 are associated with PI3K (phosphoinositide 3-kinase) signaling (44–46). Since our data (Figure 3C) indicate that the inhibitory effect of PTEN on cell motility is dependent on its lipid phosphatase activity that is related to PI3K-Akt signaling, we decided to examine whether Rac1 or Cdc42 is involved in PTEN inhibition of cell migration toward calvaria-conditioned medium. Western blotting demonstrated similar expression levels of FAK, Rac1 and Cdc42 transgenes (Figure 4D) and thus excludes the possibility that our results were due to different expression levels between these proteins. Expression of constitutively active Rac1 or Cdc42 alone had no significant effect on cell migration toward calvaria-conditioned medium. Interestingly, expression of constitutively active Rac1 but not Cdc42 could partially restore the cell migration toward calvaria-conditioned medium upon PTEN induction (Figure 4E), suggesting that Rac1 might be involved in PTEN inhibition of cell migration toward calvaria-conditioned medium. This led us to assess the Rac1 activity in the context of lung- and calvaria-conditioned medium with and without PTEN expression. Whereas lung-conditioned medium marginally increased Rac1 activity in C4-2 cells, calvaria-conditioned medium markedly increased Rac1 activity of cells. Furthermore, PTEN expression inhibited the increased Rac1 activity in response to calvaria-conditioned medium (Figure 4F and G). This result indicates that preferential C4-2 cell migration toward calvaria-conditioned medium was mediated through the activation of Rac1 and PTEN could decrease this migration by inhibiting Rac1 activity.

**Discussion**

To overcome the therapeutic limitations in cancer treatment that largely target cancer cells, researchers have begun to focus on specific interactions between cancer cells and their host cells. For metastatic lesion, understanding the interaction between cancer cells and the host environment at sites of metastasis is critical to the development of new treatments (1,3,6,8). The osteoblastic nature of prostate cancer bone metastasis suggests that the interaction of prostate cancer cells with bone involves cells of osteoblastic lineage (3). The classical 'seed and soil' hypothesis proposes that neoplastic cells prefer to colonize an organ that may serve as fertile soil (47). One explanation of this organ tropism hypothesis is that prostate cancer cells may be specifically attracted by factors released from bone and thus, migrate preferentially to it (48). This is supported by previous studies showing that osteoblast-conditioned medium could stimulates PC-3 prostate cancer cell migration (49,50). However, it is not clear whether this migration stimulatory effect of osteoblast-conditioned medium reflects in vivo tropism to bone and whether conditioned medium of cells from other organ would also stimulate prostate cancer cell migration. To explore this, we used primary mouse calvaria cell-conditioned medium as a surrogate to bone microenvironment and used conditioned medium from primary mouse lung fibroblast as a control for lung metastasis. In this system, we found that specific migratory responses to calvaria-conditioned media were reflective of the cancer cells' bone tropism in vivo. This finding suggests that the current assay system is a reasonable surrogate platform for dissecting the molecular mechanisms underlying bone tropism of prostate cancer metastasis. Furthermore, we found that conditioned medium from the established osteoblastic cell line, MC3T3-4, had a similar stimulatory effect on C4-2 cell migration whereas the non-osteoblastic cell line, MC3T3-24, had minimal effect. This suggests that cancer cell migration is strongly influenced by the osteoblastic characteristics of the bone microenvironment.

Despite their non-metastatic characteristic in animal models (33), we found that LnCaP cells migrated to the bone-conditioned medium preferentially compared with metastatic C4-2 cells and to other cells that do not have bone tropism. This finding may be explained by the fact that...
cancer metastasis is a process involving multiple steps. Cell migration is a necessary but not the only mechanism associated with this organ-specific metastasis. To reach the bone site, prostate cancer cells need to extravasate through the bone marrow endothelial cells and this process is correlated directly with cell invasion. In an in vitro study, Sikes et al. (6) demonstrated that LnCaP cells traversed poorly through human bone marrow endothelial cell monolayers whereas the naturally bone metastatic C4-2 cells could traverse very well. Upon arrival to the distant organ, tumor cell survival and growth in the new environment are critical to the development of metastatic lesions (51,52). Although LnCaP cells could be attracted by bone-specific factors as our current study suggests, this phenotype is clearly not explanatory of the entire bone metastatic phenotype in vivo. In support of this, Pinski et al. (53) have shown that the coculture of LnCaP cells with human osteoblasts could increase p53, p27 and p21 expression in LnCaP cells, leading to an enhanced cell cycle G0/G1 block. Therefore, although LnCaP cells could be attracted to bone site by bone-specific factors, they might not be able to proliferate in the bone environment.

Here we report that C4-2 cell migration toward calvaria-conditioned medium could be specifically inhibited by PTEN expression. The importance of the lipid phosphatase activity of PTEN in inhibition of cell motility has been reported previously. Gao et al. (54) demonstrated that the lipid phosphatase activity of PTEN was essential to its negative regulation on CXCR-4-mediated chemotaxis. PTEN (−/−) deficient mouse fibroblasts display increased cell motility and this enhanced motility was due to the loss of PTEN lipid phosphatase activity (55). In addition, it has been shown that PI3K signals play an important role in the regulation of cell migration and particularly in controlling the directionality of chemotaxis (56,57). Current results are consistent with our previous data in bladder cancer where we showed that PI3K activity is an essential component of an EGF (epidermal growth factor)-mediated chemotaxis pathway in human bladder cancer (31,58).

It has been shown that over-expression of PTEN inhibits cell migration and this inhibitory effect is mediated by the down-regulation of pFAK level (41). In addition, over-expression of FAK or its downstream factor p130Cas can antagonize the inhibition of cell migration by PTEN, suggesting the involvement of protein phosphatase activity of PTEN in cell migration (40). In view of these results, we evaluated the role of FAK in bone-specific migration process. Although we observed a slight reduction of pFAK level after the PTEN expression in C4-2 cells, overexpression of FAK or its degradation was not able to reverse the inhibitory effect of PTEN on cell migration toward calvaria-conditioned medium suggesting that FAK is not responsible for the bone-specific migratory block associated with PTEN induction.

Activation of both Cdc42 and Rac1 has been implicated in promoting cell migration (59) and their GDP–GTP exchange factors can be activated in PI(3,4,5)P3-dependent manner (44,45). In PTEN-deficient cells, Akt plays an essential role in promoting cell motility downstream of Rac–Cdc42 (60). Interestingly, here we show that active Rac1 but not Cdc42 could partially abrogate the inhibitory effect of PTEN on C4-2 cell migration toward calvaria-conditioned medium. Consistent with this, we also found that calvaria-conditioned medium activated Rac1 in C4-2 cells and PTEN inhibited Rac1 activation in response to calvaria-conditioned medium. Our results suggest an important role of Rac1 in mediating the inhibition of cell migration by PTEN. These results support prior work in PTEN (−/−)-deficient fibroblasts that are observed to have higher Rac1 activation levels (55). While this work also indicated that PTEN (−/−) fibroblasts have increased Cdc42 activity, our results suggest that in prostate cancer, Cdc42 activity cannot rescue the migration inhibitory effect of PTEN. The potential differential effect of PTEN on Rac1 and Cdc42 is interesting since Rac1 and Cdc42 do have different roles in promoting cell migration. For example, Rac initiates actin polymerization at the cell membrane and is responsible for the generation of lamellipodia and membrane ruffles whereas Cdc42 promotes the formation of filopodia and microspikes at the cell periphery (61). Activation of Rac1 but not Cdc42 has been shown to be required for PDGF (platelet–derived growth factor)-stimulated cell migration in Boyden chamber assay (60,62). Furthermore, Rac1 but not Cdc42 can be activated by pro-inflammatory mediator leukotriene D4 and the inhibition of PI3K and Rac1-signaling pathway also blocked intestinal cell migration mediated by leukotriene D4 (63).

Specific bone factors have been implicated in the stimulation of cancer cell migration. For instance, Das et al. (64) have shown that osteopontin can stimulate breast cancer cell motility through the PI3K-signaling pathway. The increased breast cancer cell migration was also observed in response to bone sialoprotein and thrombospondin (65,66). In prostate cancer, bone has been shown to be able to provide chemo-tactic factors for the tumor cells (8). For example, collagen products appear to be one component of the bone that induces tumor chemotaxis (67). The integrins subunit alpha 2 beta 1 (α2β1) is expressed on prostate cancer cells and this α2β1 integrin can stimulate breast cancer cell motility through the PI3K-mediated by leukotriene D4 (63).

In summary, we find that prostate cancer cells specifically migrate toward calvaria-conditioned medium compared with lung fibroblast-conditioned medium and this preferential migration parallels the osteotropism of cancer metastasis and is dependent on the osteoblastic characteristics of the calvaria cells. In addition, conditional expression of PTEN in C4-2 cells specifically inhibits cell migration toward calvaria-conditioned medium and this inhibitory effect is dependent on its lipid phosphatase activity. Furthermore, we find that the inhibitory effect of PTEN on cell migration toward calvaria-conditioned medium is associated with PTEN inhibition of Rac1 activity of C4-2 cell in response to calvaria-conditioned medium and is partially rescued by the active small GTPase Rac1. Our studies implicate loss of PTEN as a key mediator of bone tropism in prostate cancer metastasis and provide a useful in vitro model to dissect the molecular pathways responsible for this tropism.

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References