

Disseminated Prostate Cancer Cells Can Instruct Hematopoietic Stem and Progenitor Cells to Regulate Bone Phenotype

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Abstract

Prostate cancer metastases and hematopoietic stem cells (HSC) frequently home to the bone marrow, where they compete to occupy the same HSC niche. We have also shown that under conditions of hematopoietic stress, HSCs secrete the bone morphogenetic proteins (BMP)-2 and BMP-6 that drives osteoblastic differentiation from mesenchymal precursors. As it is not known, we examined whether metastatic prostate cancer cells can alter regulation of normal bone formation by HSCs and hematopoietic progenitor cells (HPC). HSC/HPCs isolated from mice bearing nonmetastatic and metastatic tumor cells were isolated and their ability to influence osteoblastic and osteoclastic differentiation was evaluated. When the animals were inoculated with the LNCaP C4-2B cell line, which produces mixed osteoblastic and osteolytic lesions in bone, HPCs, but not HSCs, were able to induced stromal cells to differentiate down an osteoblastic phenotype. Part of the mechanism responsible for this activity was the production of BMP-2. On the other hand, when the animals were implanted with PC3 cells that exhibits predominantly osteolytic lesions in bone, HSCs derived from these animals were capable of directly differentiating into tartrate-resistant acid phosphatase-positive osteoclasts through an interleukin-6-mediated pathway. These studies for the first time identify HSC/HPCs as novel targets for future therapy involved in the bone abnormalities of prostate cancer. *Mol Cancer Res*; 10(3); 282–92. ©2012 AACR.

Introduction

Prostate cancer is the second leading cause of cancer-related death in men. Bone is the preferred metastatic site of advanced prostate cancer (1–3). Prostate cancer cells that reach the bone marrow can stimulate osteoblasts or osteoclasts, leading to either the production of poorly woven bone (osteoblastic lesions) or bone loss (osteolytic lesions; refs. 4, 5). Prostate cancer cells secrete many factors that directly or indirectly alter osteoblast and osteoclast activities that potentially may result in the skeletal phenotype of prostate cancer observed clinically (6, 7). Yet the precise mechanisms that lead to whether a lesion will be predominantly osteoblastic or osteolytic have not clearly been defined. Therefore understanding the mechanisms that control the differentiation and

activity of osteoblasts/osteoclasts is of central importance in the area of bone metastasis.

Work by our group and others have shown that the metastatic process is functionally similar to the homing behavior of hematopoietic stem cells (HSC) and hematopoietic progenitor cells (HPC) to the bone marrow (8, 9). A combination of markers c-kit and sca-1 has been usually used for identifying murine HSCs ($\text{Lin}^- \text{CD48}^- \text{CD41}^- \text{Sca-1}^+ \text{cKit}^+$ (LSK HSCs; ref. 10). Recently discovery of SLAM family protein (CD150) as a selection marker for HSCs provide a new strategy for HSC enrichment ($\text{CD150}^+ \text{Lin}^- \text{CD48}^- \text{CD41}^- \text{Sca-1}^+ \text{cKit}^+$ (SLAM HSCs; refs. 10, 11). It has been suggested that SLAM HSCs are more primitive HSCs compared with LSK HSCs (10, 11). SLAM and LSK HSCs reside in specialized compartments within the bone marrow termed the "stem cell niche" which control HSC quiescence and self-renewal (12, 13). More recently, we have shown that metastatic cells shed from a primary prostate cancer tumor directly target the HSC niche and compete with HSCs for occupancy of the niche (14). These disseminated tumor cells (DTC) may remain in a dormant/quiescent state for extended periods in the niche (15–17). In addition, we have shown that HSCs can regulate mesenchymal fate by inducing osteoblastic precursors to commit to the osteoblastic lineage (18). Once in the niche, whether disseminated prostate cancer disrupt the ability of HSCs to regulate normal bone formation is not known.

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To determine whether disseminated prostate cancer disrupts the ability of HSCs and HPCs to regulate the normal bone phenotype, HSCs/HPCs from the bone marrow of tumor-bearing animals were cocultured with mixed bone marrow stromal cells (BMSC). Our data suggest that HPCs derived from animals implanted with prostate cancer cell lines that form osteoblastic lesions in bone stimulated osteoblastic differentiation of BMSCs *in vitro* through the bone morphogenetic protein (BMP)-2 signaling. Interestingly, HSCs derived from animals implanted with prostate cancer cell lines that produce a predominantly osteolytic lesion in bone differentiated into tartrate-resistant acid phosphatase (TRAP)-positive osteoclastic cells through interleukin-6 (IL-6) signaling. These data showed that the disseminated prostate cancer cells indirectly regulate the bone phenotype at metastatic site by directing HSC/HPCs.

Materials and Methods

Cell culture

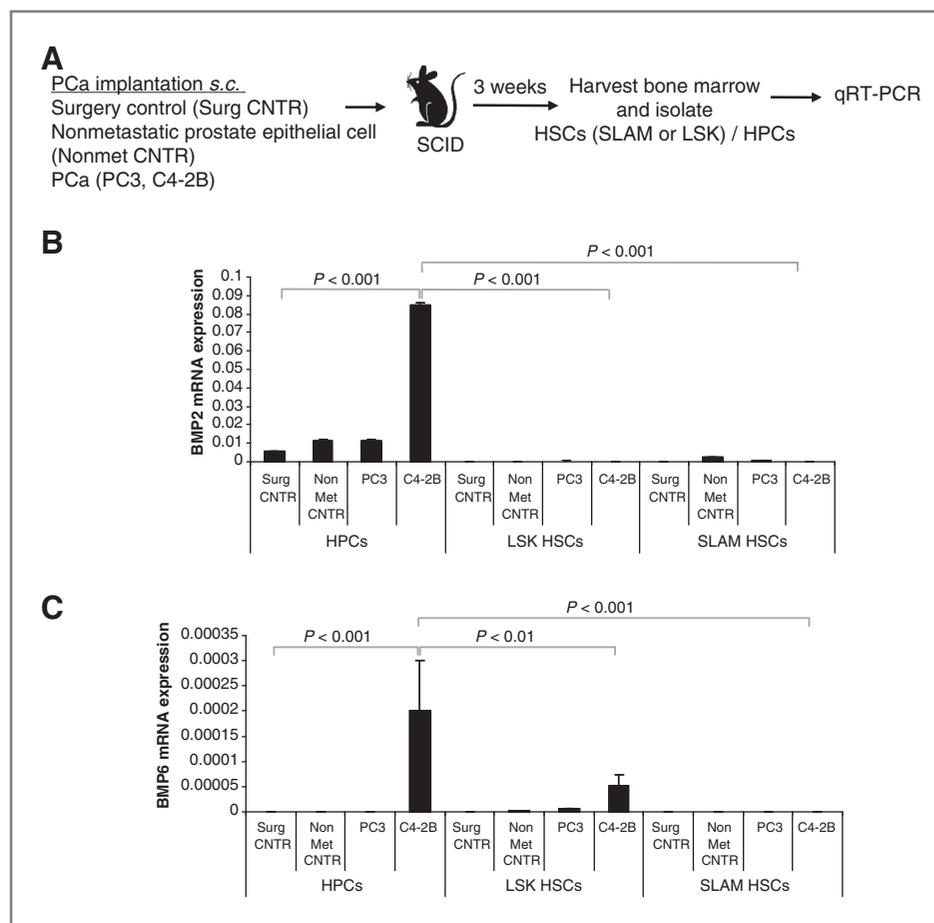
The PC3 (CRL-1435) human prostate cancer cell line and RAW 264.7 mouse monocytic cell line were obtained from the American Type Culture Collection. The metastatic human prostate cancer subline LNCaP C4-2B was originally isolated from a lymph node of a patient with disseminated

bony and lymph node involvement (19). Normal human nonmetastatic prostate epithelial control cell lines (Non Met CNTR) were obtained from patients undergoing prostatectomy in accordance with the Investigation Review Board of University of Michigan. The tissue was collected from a distal location from the tumor (within the prostate). This cell line is morphologically and pathologically distinct from the tumor. Prostate cancer cell lines and control cells were cultured in RPMI-1640 (Invitrogen) and Dulbecco's modified Eagle's medium (DMEM; Invitrogen), respectively. RAW 264.7 cells were cultured in low glucose DMEM (Invitrogen). All cultures were supplemented with 10% (v/v) FBS (Invitrogen) and 1% (v/v) penicillin–streptomycin (Invitrogen) and maintained at 37°C, 5% CO₂, and 100% humidity.

Tumor implantation

All experimental procedures were approved by the University of Michigan Committee for the Use and Care of Animals (UCUCA). Male 5- to 7-week-old severe combined immunodeficient (SCID) mice (CB.17. SCID; Taconic) were implanted subcutaneously with 2×10^5 cells (prostate cancer cell line or control cell) within sterile collagen scaffolds ($3 \times 3 \times 3$ mm³; Gel foam; Pharmacia and Upjohn) in the mid-dorsal region of each mouse ($n = 5$). Animals

Figure 1. Disseminated prostate cancer alters BMP expression in HSC/HPCs. A, experimental outline. HSCs (CD150⁺ Lin⁻ CD48⁻ CD41⁻ Sca-1⁺ cKit⁺ (SLAM HSCs) or Lin⁻ CD48⁻ CD41⁻ Sca-1⁺ cKit⁺ (LSK HSCs)/HPCs (Lin⁻ CD48⁻ CD41⁻ Sca-1⁺) from tumor-bearing animals were isolated. Total RNA was extracted and subjected to qRT-PCR for BMP-2 and BMP-6. Relative expression of mRNA was calculated for (B) BMP-2 and (C) BMP-6 and normalized to GAPDH. Data represent mean \pm SD carried out in triplicate in 3 independent experiments. Level of significance ($P < 0.001$ and $P < 0.01$) were calculated compared with the surgical control-treated group.



implanted with scaffolds alone during surgery were kept as negative controls (surgical control). After 3 weeks, animals were sacrificed and bone marrow cells from the mice were isolated.

Isolation of HSCs

Tumor-bearing animals were euthanized and the bone marrow cells were flushed from the femurs and tibias with Hanks buffered salt solution (Invitrogen) supplemented with 2% (v/v) FBS. HSCs were isolated as previously described (14, 18). The bone marrow cells were incubated first with a biotinylated anti-Lin [CD5, CD45R (B220), CD11b, Gr-1 (Ly- 6G/C), and Ter-119] antibody cocktail (Miltenyi Biotec) for 10 minutes at 4°C and then stained with an antibody cocktail of allophycocyanin-conjugated anti-Sca-1 (clone D7; eBioscience), PE/Cy7-conjugated anti-c-Kit (clone 2B8; BioLegend), PE-conjugated anti-CD150 (clone TC15-12F12.2; BioLegend), fluorescein isothiocyanate (FITC)-conjugated anti-CD41 (clone MWRReg30; BD Biosciences), FITC-conjugated anti-CD48 (clone BCM-1; BD Biosciences), and FITC-conjugated anti-biotin antibodies (Miltenyi Biotec) for another 20 minutes at 4°C. HSCs were sorted on a BD FACS Aria I flow cytometer by gating on HSCs (i.e., CD150⁺ Lin⁻ CD48⁻ CD41⁻ Sca-1⁺ cKit⁺ (SLAM HSCs) or Lin⁻ CD48⁻ CD41⁻ Sca-1⁺ cKit⁺ (LSK HSCs). Lin⁻ CD48⁻ CD41⁻ Sca-1⁺ progenitor cells (HPCs) were

isolated from mouse bone marrow after lineage depletion followed by magnetic cell sorting (Mouse Sca-1 selection Kit, EasySep; Stem cell Technologies Inc.). A representative fluorescence-activated cell sorting (FACS) plot to confirm the specificity of antibodies and a typical FACS plot of the recovered cells are presented in Supplementary Figs. S1 and S2.

Bone marrow stromal cells

Marrow obtained from the femur and tibia of C57BL/6 (Charles River Laboratories) animals was used to generate stromal cells. After flushing the marrow with α -MEM medium supplemented with 2% (v/v) FBS, cells were cultured in α -MEM containing 10% (v/v) FBS and 1% (v/v) penicillin–streptomycin. After 4 days, nonadherent cells were removed and fresh media were replaced. Once confluent, the cells were passaged 2 to 3 times with trypsin.

The collection of prostate cancer–conditioned medium

For preparing conditioned media, prostate cancer cells were cultured in RPMI-1640 (Invitrogen) supplemented with 10% (v/v) FBS and 1% (v/v) penicillin–streptomycin under a humidified atmosphere of 5% CO₂ at 37°C to 90% confluency. Cells were then washed with PBS and cultured in serum-free media for 24 hours. The conditioned media was concentrated 10 \times using centrifugal filters (Ultracel-3K,

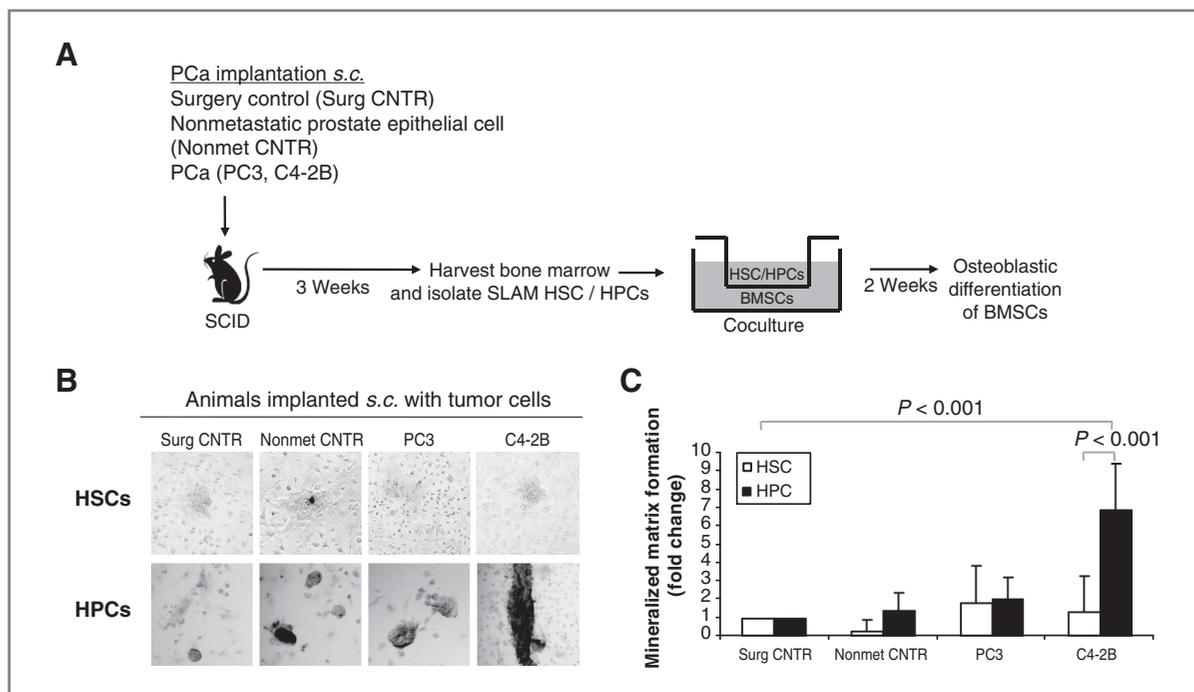


Figure 2. HPCs from osteoblastic tumor-bearing animals increase mineralization of BMSCs. A, HSC/HPCs were isolated from tumor-bearing animals and cocultured with BMSCs in osteogenic media. B, images of monolayer cultures incubated in osteogenic media for 2 weeks stained with alizarin red, showing substantial staining in the C4-2B (osteoblastic) HPC group compared with other tumor groups and compared with HSCs derived from prostate cancer tumor-bearing groups. C, the amount of alizarin staining in the mineralized matrix was quantified using NIS Elements software and normalized to cell number. Fold change was calculated compared with surgical control-treated group.

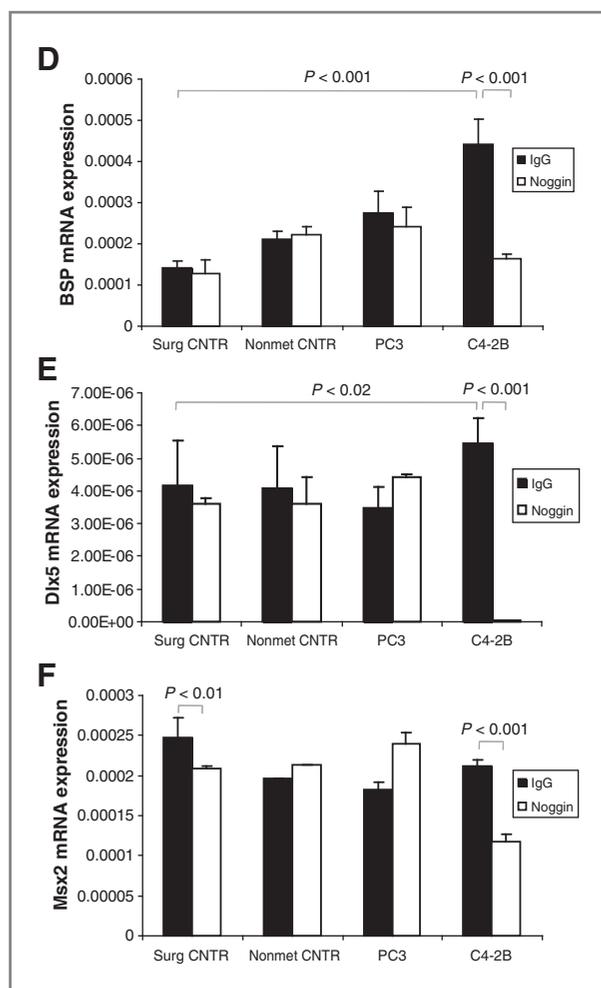


Figure 2. (Continued) D–F, HPCs from osteoblastic tumor-bearing animals regulate mesenchymal fate through BMPs. Coculture investigations were established by placing HPCs derived from tumor-bearing animals in the top chamber of a dual culture plate and BMSCs in the bottom well in the presence of neutralizing antibody to BMPs or an isotype-matched IgG control (5 ng/mL). Osteoblast phenotype was examined by RT-PCR for osteoblast-specific markers, (D) BSP, (E) Dlx5, and (F) Msx2. Data represent mean \pm SD fold change carried out in triplicate in 2 independent cultures. The data indicate that HPCs from osteoblastic tumor-bearing animals directed the formation of osteoblast differentiation and BMP neutralizing antibody significantly reduced the osteoblast differentiation from HPCs isolated from osteoblastic tumor-bearing animals ($P < 0.02$, $P < 0.01$, and $P < 0.001$).

Amicon Ultra; Millipore) and stored in -80°C until use. For standardization, total protein in the conditioned medium was measured by a Bradford microassay (Bio-Rad Laboratories).

Osteoblastic differentiation assays

HPCs (2×10^3 cells) or HSCs (200 cells) isolated from tumor-bearing mice were placed in the top chambers of 24-well Transwell plates (0.4 μm pore size, polycarbonate; Corning Life Sciences). BMSCs were plated in the bottom chambers at a final density of approximately $2 \times 10^4/\text{cm}^2$ in α -MEM containing 10% heat-inactivated FBS, antibiotics,

10 mmol/L β -glycerol phosphate, and 10 $\mu\text{g}/\text{mL}$ L-ascorbic acid (Sigma-Aldrich). After 2 weeks, the cell matrix were fixed with 10% (v/v) normal buffered formalin and stained with 2% (v/v) Alizarin red (Sigma-Aldrich). Quantification of the staining density was analyzed using NIS Elements software (Nikon Instruments Inc.) as an index of mineralization. In some cases, monoclonal anti-mouse Noggin antibodies or corresponding IgG (5 ng/mL) were added every other day to the cocultures (R&D Systems). After 2 weeks, osteoblastic differentiation from BMSCs was evaluated by real-time quantitative reverse transcription PCR (qRT-PCR).

Osteoclastic differentiation assays

Three types of differentiation assays were done. First, mixed bone marrow mononuclear cells (BMMC) were evaluated for their ability to differentiate into osteoclasts following treatment with HSCs derived from tumor-bearing animals in a Transwell assay. After 2 weeks, the cell matrix were fixed with 10% (v/v) normal buffered formalin and stained for TRAP (K-ASSAY; Kamiya Biomedical Company). Colonies (CFU-OC) of more than 50 cells were counted under a light microscope (10 \times). Secondly, the ability of test conditions to influence osteoclast differentiation from preosteoclasts was examined. Here, HSCs from tumor-bearing animals were cultured in direct contact with murine RAW264.7 cells in the presence of receptor activator of NF- κB ligand (RANKL) for 4 days and stained for TRAP. In the final assay, cells of hematopoietic origin, recovered from tumor-bearing animals (SLAM HSCs/LSK HSCs), were cultured in the presence or absence of prostate cancer-conditioned medium (standardized by total protein, 20 μg), RANKL (50 ng/mL, Peprotech Inc.), macrophage colony-stimulating factor (M-CSF) (50 ng/mL; Peprotech Inc.) or in combination. After 9 days the cells were fixed in 10% formalin and stained for TRAP. In some cases, the cultures were treated with anti-IL-6 monoclonal antibody or corresponding IgG (1 $\mu\text{g}/\text{mL}$; BD BioScience).

RNA analysis and real-time reverse transcription PCR

Total RNA was harvested from cells using RNeasy Mini or Micro Kit (Qiagen). First strand cDNA synthesis and real-time qRT-PCR were carried out following the manufacturer's directions (Applied Biosystems) or using Message Booster cDNA synthesis kit when evaluating mRNA levels from isolated HSCs (Epicenter Biotechnologies). TaqMan predeveloped assay reagents (FAM/MGB probe; Applied Biosystems) were used for detection of mouse BMP-2, BMP-6, IL-6, BSP, Dlx5, Msx2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Universal mouse reference RNA (Stratagene) was used to generate a relative standard curve. Real-time detection of the PCR products was done using an ABI PRISM 7700 sequence detector (Applied Biosystems). Expression levels were calculated on the basis of a standard curve and normalized to GAPDH. Cycle numbers of analyzed genes are presented in Supplementary Table S1.

Statistical analysis

Results were presented as mean \pm SEM. Significance of the difference between 2 measurements was determined by unpaired Student *t* test. Statistical differences between the means for the different groups were evaluated with InStat 4.0 (GraphPAD software) using 1-ANOVA. Values of *P* < 0.01 were considered significant.

Results

Disseminated osteoblastic prostate cancer cells target HPCs to drive osteoblast phenotype of BMSCs by modulating BMP-2 response

In previous work we showed that under conditions of stress (20% blood volume loss and 5-fluorouracil), HSCs and HPCs produce BMP-2 and BMP-6, which are able to activate osteoblastic differentiation from mesenchymal stem cells (18). To determine whether disseminated prostate cancer are also capable of inducing changes in the bone marrow microenvironment, perhaps through HSC or HPC intermediaries, we used a micro-metastasis model in which DTCs from primary tumors can be established in the marrow (20). Thereafter, HSCs and HPCs were isolated from the bone marrow of tumor-bearing mice and analyzed for the expression of BMP-2 and BMP-6 by qRT-PCR (Fig. 1A). BMP-2 and BMP-6 mRNA levels were significantly higher in the HPCs isolated from animals implanted with the C4-2B cell line compared with HPCs isolated from the control treated (surgical control and nonmetastatic control cells implanted) or PC3-implanted mice (Fig. 1B and C). However, BMP-2 mRNA expression was virtually unchanged under any conditions in which HSCs (SLAM or LSK) were examined (Fig. 1B and C).

To validate that BMPs produced by HPCs can regulate mesenchymal fate, cocultures were established by placing the HSC/HPCs in the top chamber and mixed BMSCs in the bottom chamber of a dual chamber plates under osteogenic conditions. Following a 2-week culture period, the osteoblastic differentiation of BMSCs was analyzed (Fig. 2A). To determine the mineralization of BMSCs, the extracellular matrices were stained with alizarin red. HPCs obtained from animals implanted with C4-2B cells enhanced mineralized matrix formation compared with HPCs isolated from animals implanted with PC3 or controls, whereas HSCs did not enhance any mineralization (Fig. 2B and C). A control study was done by coculturing PC3 or C4-2B cells with BMSCs. Prostate cancer cells could not induce mineralization when cocultured with BMSCs (Supplementary Fig. S3A). To further substantiate whether BMPs derived from HPCs regulate BMSC fate, coculture investigations were treated with neutralizing antibody to BMPs (Noggin). After 2 weeks, the osteoblastic phenotype was examined by qRT-PCR for the expression of osteoblast-specific markers *Dlx5*, *BSP*, and *Msx2*. As expected, HPCs derived from osteoblastic C4-2B tumor-bearing animals expressed enhanced levels of osteoblast-specific markers compared with HPCs

derived from controls or PC3 tumor-bearing animals (Fig. 2D–F). Interestingly, Noggin prevented the increase in the expression of osteoblastic markers when HPCs were cocultured with BMSCs isolated from C4-2B tumor-bearing animals (Fig. 2D–F). These data suggested that prostate cancer may target HPCs to induce osteoblastic differentiation of BMSCs.

HSCs derived from prostate cancer-bearing animals may regulate osteoclastic differentiation from mixed marrow mononuclear cells but not from preosteoclasts

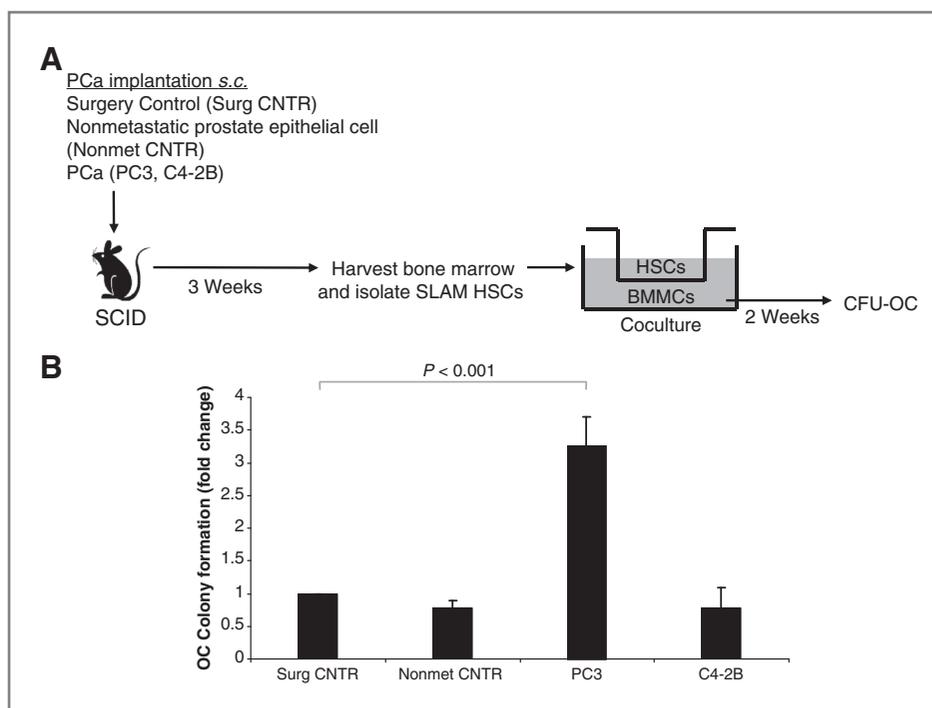
Thus far, the results showed that DTCs from osteoblastic tumor cell lines could stimulate BMP-2 production by HPCs (but not HSCs) and stimulate osteoblastic induction of BMSCs. Because osteoblastic and osteoclastic activities are often coupled, we next determined the ability of DTCs to influence osteoclastic activities. For these studies cocultures were established by placing the recovered SLAM HSCs or HPCs in the top chamber of a dual culture well plate, with mixed marrow mononuclear cells in the bottom well. After 2 weeks the phenotype of the mixed marrow mononuclear cells were evaluated for the ability to generate osteoclast colonies (Fig. 3A). A significant increase in the number of progenitors with osteoclastic potential were detected in SLAM HSCs isolated from animals harboring PC3 tumors compared with SLAM HSCs isolated from animals implanted with C4-2B tumors (Fig. 3B). HPCs isolated from tumor-bearing animals were, however, unable to influence osteoclast development of mixed marrow cells (Data not shown). A control study was done by coculturing PC3 or C4-2B cells with preosteoclastic RAW264.7 cells in the presence of RANKL. Prostate cancer cells could not induce TRAP-positive cells when cocultured with RAW264.7 cells (Supplementary Fig. S3B). These data suggested that HSCs respond to the presence of different DTCs in a way that reflects the bone phenotype of the parental prostate cancer cells implanted.

To determine whether SLAM HSCs isolated from tumor-bearing animals could stimulate preosteoclasts to differentiate into osteoclasts, direct cell-to-cell cocultures were established between SLAM HSCs and the murine preosteoclastic RAW264.7 cells (Fig. 4A). SLAM HSCs isolated from nonmetastatic control, PC3 and C4-2B cell-implanted animals, but not from surgical control animals, stimulated multinucleation (>3 nuclei) of TRAP-positive cells from RAW264.7 cells (Fig. 4B). There was however no differences in number of osteoclasts produced (Fig. 4B). These data suggested that HSCs may not be primed to regulate cells already committed to osteoclast lineages.

Disseminated osteolytic prostate cancer cells directly differentiate HSCs into osteoclasts

The ability of HPCs to differentiate into osteoclastic cells has been reported in previous studies (21). Our finding that SLAM HSCs influence osteoclastic differentiation from mixed marrow populations was intriguing

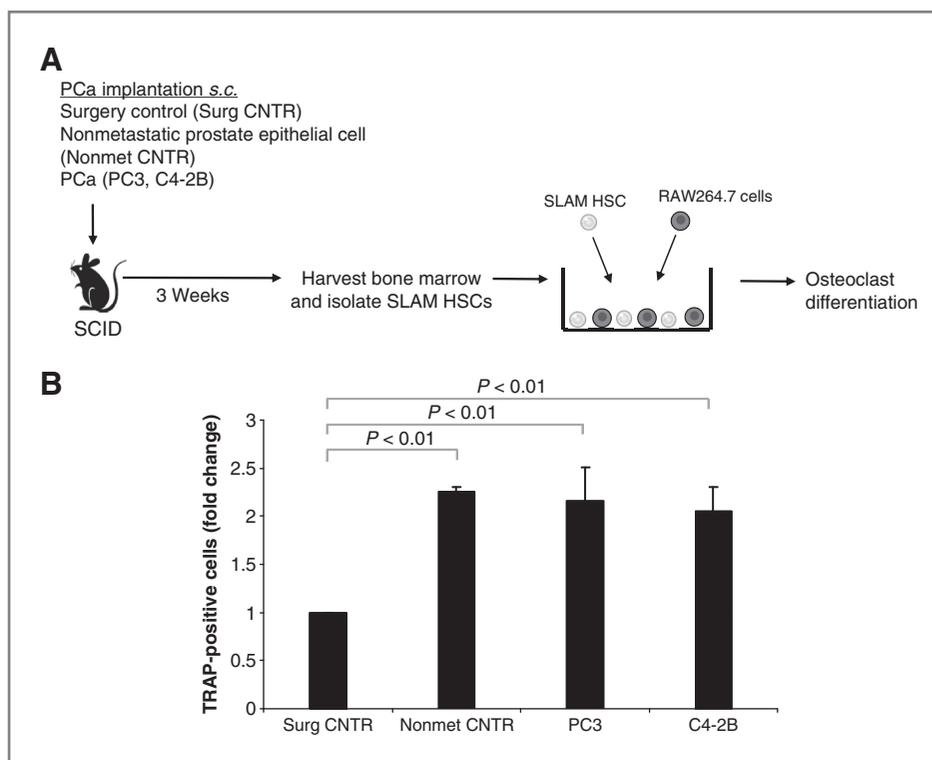
Figure 3. HSCs from osteolytic prostate cancer–implanted animals induce osteoclastic colony forming units from BMMCs. A, experimental outline. Cocultures were established by placing SLAM HSCs ($CD150^+$ $Lin^- CD48^- CD41^- Sca-1^+ cKit^+$) derived from animals bearing subcutaneous tumors or control animals in the top chamber of a dual culture well plate, and mixed mononuclear bone marrow cells in the bottom well. After 2 weeks the phenotype of the resulting colonies were stained for TRAP to visualize osteoclast colonies (CFU-OC). B, number of TRAP stained cells per culture. Significant increase in CFU-OCs was noted in the presence of HSCs derived from PC3 tumor-bearing animals versus surgical control animals. Data represent mean \pm SD fold change conducted in triplicate in 3 independent cultures. Significance ($P < 0.001$) were calculated compared with surgical control-treated group.



and prompted us to explore whether DTCs from prostate cancer can induce osteoclastic differentiation directly from HSCs (LSK or SLAM). Therefore, we first isolated LSK HSCs from animals bearing tumor cells and were treated

in vitro with prostate cancer conditioned medium for 9 days (Fig. 5A). Initial studies suggested that conditioned medium from PC3 or C4-2B cells alone was insufficient to drive osteoclast differentiation from LSK HSCs. Hence,

Figure 4. HSCs from prostate cancer tumor-bearing animals induce same extent of osteoclast differentiation from existing preosteoclasts. A, experimental conditions. SLAM HSCs were isolated from tumor-bearing animals and cocultured with preosteoclastic RAW 264.7 cells. After the culture conditions, the cultures were stained with TRAP and evaluated for multinucleation. B, number of osteoclasts per culture. The data indicate that culturing HSCs derived from prostate cancer–implanted animals with preosteoclastic cells increased the multinucleated TRAP-positive cells (>3 nuclei) from RAW 264.7 cells compared with surgical control ($P < 0.01$). Data represent mean \pm SD conducted in triplicate in 2 independent experiments. No significant differences in osteoclast formation from HSCs derived from osteoblastic versus osteolytic tumors were obtained.



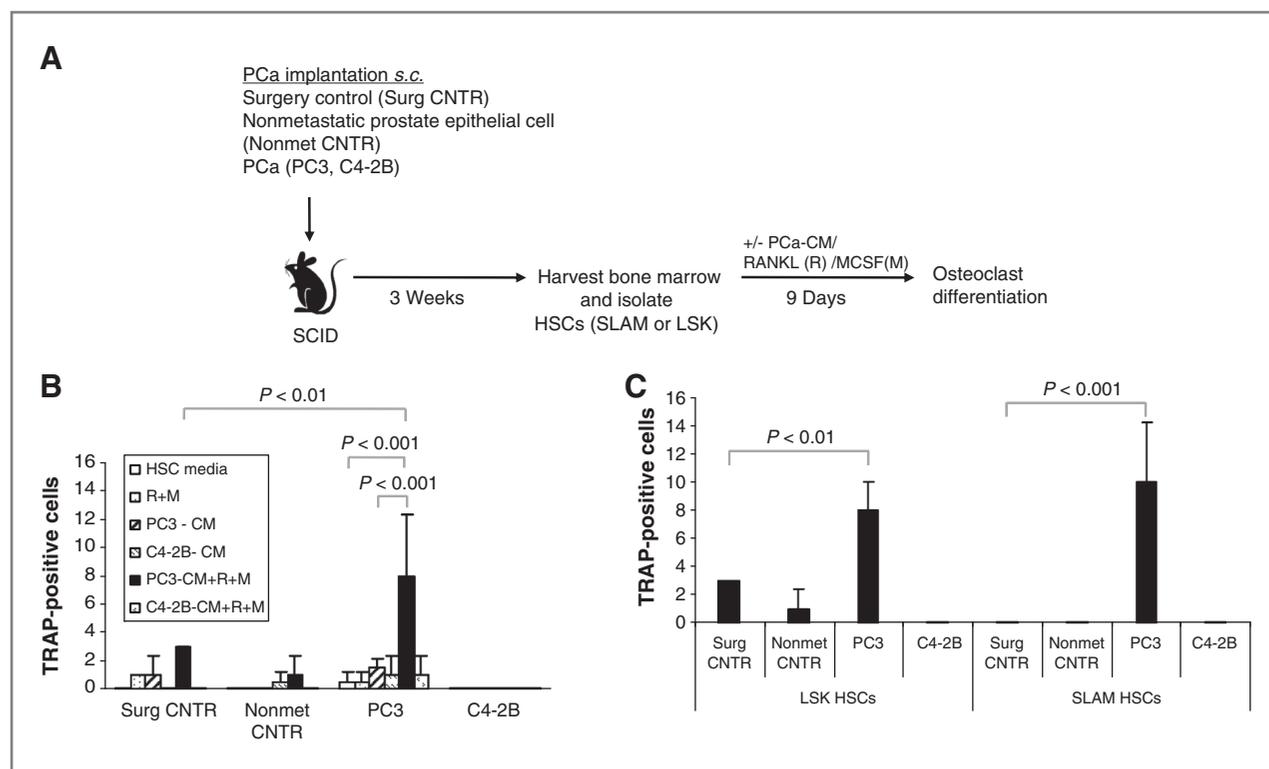


Figure 5. HSCs from osteolytic PC3 bearing animals differentiated directly into osteoclasts. A, experimental outline. HSCs ($CD150^+ Lin^- CD48^- CD41^- Sca-1^+ cKit^+$) (SLAM) and $Lin^- CD48^- CD41^- Sca-1^+ cKit^+$ (LSK) were isolated from tumor-bearing animals and differentiated into osteoclast in the presence or absence of prostate cancer-conditioned medium (PC3-CM and C4-2B-CM), RANKL/M-CSF or in combination. B, number of TRAP⁺ cells after culture. LSK HSCs from osteolytic PC3 tumor-bearing animals could direct the formation of osteoclast differentiation in the presence of PC3-conditioned medium (PC3-CM), M-CSF (M), and RANKL (R). Significant differences from the surgical control group ($P < 0.01$) or HSCs derived from PC3-implanted animals treated with either vehicle alone or PC3-conditioned medium alone ($P < 0.001$) were calculated. C, evaluation of the ability of different HSC populations to differentiate into osteoclasts. Osteoclast differentiation ability of SLAM HSCs and LSK HSCs from osteolytic PC3 tumor-bearing animals were compared in the presence of PC3-conditioned medium with RANKL and M-CSF. Data represent mean \pm SD conducted in triplicate in 3 independent experiments. Significant differences from surgical controls were established at $P < 0.01$ and $P < 0.001$.

the osteoclastic differentiation factors RANKL and M-CSF were included in the studies (Fig. 5B). RANKL and M-CSF alone were not sufficient to generate osteoclasts from LSK HSCs under our culture conditions (Fig. 5B). However, conditioned medium derived from PC3 cells in the presence of RANKL and M-CSF activated osteoclast formation directly from LSK HSCs that were isolated from PC3 tumor-bearing animals, but not from controls or C4-2B-implanted animals (Fig. 5B). To further dissect the stem cell population that could differentiate into osteoclasts in an environment with DTCs, bone marrow cells were isolated from tumor-bearing animals by FACS by gating on $Lin^- CD48^- CD41^- Sca1^+ cKit^+$ (LSK HSCs) and $CD150^+ Lin^- CD48^- CD41^- Sca-1^+ cKit^+$ (SLAM HSCs), which constitute 0.01% and 0.001% of total bone marrow cells, respectively. Each of these populations was cultured in the presence of conditioned medium derived from PC3 cells as well as RANKL and M-CSF. The numbers of osteoclasts generated by the SLAM HSCs were same as those produced by LSK HSCs (Fig. 5C). These observations suggested that DTCs from PC3-implanted animals are

able to instruct or prime HSCs to differentiate into TRAP-positive osteoclasts.

Osteoclast differentiation of HSCs is driven by IL-6 secreted from osteolytic prostate cancer

Many cancer cell types that metastasize to the bone secrete high levels of IL-6 (22). Likewise, other cancer cell types stimulate the surrounding stromal cells to release copious amounts of this cytokine (23, 24). Deregulation of cytokine/growth factor production is also implicated in disorders of bone homeostasis such as osteoporosis and osteopetrosis (25). We therefore postulated that IL-6 may play a significant role in regulating the ability of DTCs to mediate HSC differentiation into osteoclasts. Accordingly, qRT-PCR was used to examine the extent to which HSCs isolated from tumor-implanted animals express mRNA for IL-6. IL-6 mRNA was significantly expressed by HSCs (SLAM or LSK) isolated from animals implanted with PC3 tumor cells, but not controls or C4-2B-implanted animals (Fig. 6A). To confirm and extend these findings, LSK HSCs from tumor-bearing animals were isolated and differentiated in the presence and absence of IL-6 blocking or control IgG

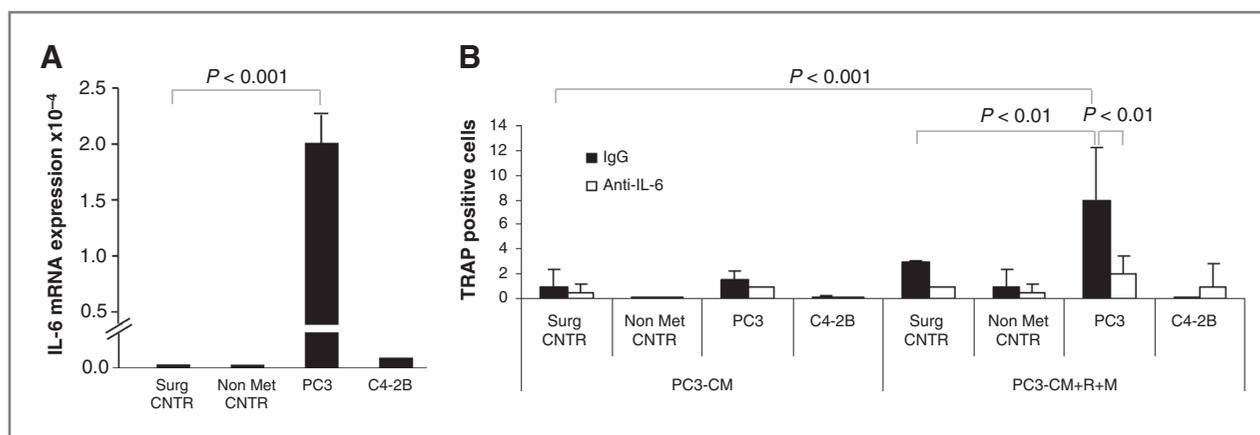


Figure 6. Osteoclast differentiation is mediated partly through IL-6 secreted by osteolytic prostate cancer. A, HSCs derived from osteolytic PC3-bearing animals express endogenous IL-6. HSCs (SLAM or LSK) isolated from tumor-bearing animals were analyzed by qRT-PCR to evaluate IL-6 mRNA levels. B, effect of IL-6 on osteoclast differentiation. LSK HSCs were isolated from tumor-bearing animals and differentiated into osteoclast in the presence or absence of PC3-conditioned medium (PC3-CM) or combination of PC3-CM, RANKL, and M-CSF. For blocking studies IL-6-neutralizing antibody (or corresponding control IgG) was added to the treatment groups. Data represent mean \pm SD conducted in triplicate in 2 independent experiments. The data indicate that IL-6-neutralizing antibody significantly reduced the osteoclast differentiation from HSCs isolated from osteolytic PC3 tumor-bearing animals ($P < 0.01$ and $P < 0.001$).

antibodies to neutralize the activity of IL-6. A significant reduction in the production of TRAP-positive colonies was observed under these experimental conditions (Fig. 6B). These data suggested that IL-6 participates in the osteoclastic differentiation of HSCs driven by DTCs.

Discussion

Our data suggest that metastatic prostate cancer can disrupt the ability of HSCs to regulate normal bone formation (Fig. 7). HSCs isolated from PC3-implanted animals differentiated directly into TRAP-expressing osteoclasts in the presence of PC3-CM, MCSF, and RANKL, but not HSCs isolated from animals implanted with C4-2B cells or control-implanted animals. In part, autocrine production of IL-6 possibly by the HSCs themselves is central to facilitating HSCs ability to form osteoclasts. On the other hand, more mature hematopoietic cells including HPCs isolated from animals implanted with C4-2B cells, which generate a predominantly osteoblastic bone phenotype, showed enhancement of osteoblastic differentiation, possibly through the production of BMP-2.

Due to the complexity and diversity of factors produced by the prostate cancer cells in marrow, the heterogeneity of bone metastases in a given patient is dramatic (26, 27). Prostate cancer cells express several factors associated with bone remodeling which are closely linked to an osteoblastic response or osteoclastic response (28). BMPs are osteoinductive morphogens critical for skeletal development (29, 30). Prostate cancer cells are known to produce BMPs which are believed to play a major role in the osteoblastic bone response to tumor. Furthermore, prostate cancer cells express receptors for the BMPs, which may serve in an autocrine fashion to induce SMAD1 signaling and over-expression of osteoprotegerin (31). Recent studies have

shown that BMP-2 and BMP-6 stimulate the invasive capability of prostate cancer cells (32). Therefore, inhibition of the BMPs may have clinical relevance (33, 34). In our experiments we observed that the osteogenic capability of HPCs could be mediated by production of BMP-2. HSCs derived from animals with disseminated prostate cancer cells in the marrow formed osteoblastic colonies, but there was not a significant difference between osteoblastic colonies formed from osteoblastic (C4-2B) or osteolytic (PC3) tumors. These results suggest a critical role of BMP-2 and HPCs in the context of osteoblastic phenotype in prostate cancer.

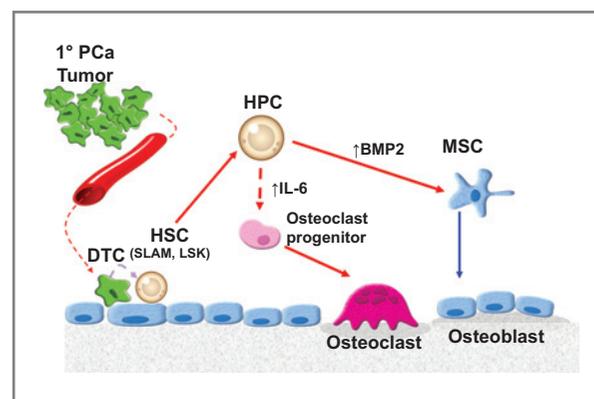


Figure 7. Experimental model: disseminated tumor cells (DTC) alter hematopoietic stem cell function to regulate bone phenotype. At early stage of dissemination process, osteoblastic prostate cancer activate BMP production by HPCs that induce osteoblastic differentiation from mixed BMSCs. At the same time, osteolytic prostate cancer activate HSCs to direct the differentiation of osteoclasts through IL-6 pathway. Although further study to exclude the paracrine effects or the effects from DTCs will be needed, the activation of HSCs/HPCs by prostate cancer may be a molecular mechanism for the prostate cancer bone phenotype.

Part of the mechanism whereby prostate cancer may direct osteoclastic differentiation by HSCs is through IL-6. Several investigators have observed increases in serum markers reflective of bone resorption in men with prostate cancer with bone lesions that are predominately osteoblastic in nature, and often several times the levels observed in individuals with predominantly osteolytic bone metastases, such as breast cancer (35–37). IL-6 has been shown to stimulate osteoclastogenesis in RANKL-dependent and RANKL-independent mechanism (38–40). Recently, interest in using serum IL-6 as a specific prognostic factor for prostate cancer has emerged (41, 42). In addition to serum IL-6 levels, the concentration of soluble receptor to IL-6 (sIL-6R) in the serum may also help to predict the aggressiveness of cancer metastasis and the level of bone destruction (43, 44). When cancer cells metastasize to the bone, increased IL-6 may be endogenously expressed or produced by both the cancer cells and the osteoblasts, as an inflammatory response to the cancer cells. IL-6 then stimulates various types of stromal cells in the bone in the area of the metastasis, to increase the expression of RANKL and M-CSF by osteoblasts which, in turn, activate the osteoclast differentiation cascade. Once this occurs, osteoclast activity becomes deregulated and reduces bone integrity. In our experiments we observed that PC3-conditioned medium alone was not sufficient to induce osteoclastic differentiation from HSCs. At the same time PC3-conditioned medium in the presence of RANKL and M-CSF potentiated the osteoclast formation from HSCs isolated from osteolytic PC3 tumor-bearing animals. Moreover, HSCs isolated from PC3-implanted animals express endogenous IL-6 and the subsequent activation of autocrine mechanism of IL-6 may lead to osteoclast differentiation.

We have shown that prostate cancer compete with HSCs for the stem cell niche and occupy the niche to facilitate metastasis (14). The major role of the niche is to limit cell division and therefore maintain stem cells with pluripotent potential (12, 45). Once stem cells or their immediate progeny leave the niche, they begin to proliferate and ultimately differentiate, and, in fact, we observed that in men with metastatic prostate cancer bone disease, there is a higher frequency of HPCs in their blood than in age matched or men newly diagnosed with localized prostate cancer (14). We determined the number of osteoblasts and osteoclasts in the long bone obtained from tumor-bearing animals. No significant differences between the treatment groups were observed (Supplementary Fig. S4), indicating that our studies represent early events during metastasis. Although further study will be needed, this study suggests that once the HSCs are out of the niche, it may affect mesenchymal fate. The concept that once HSCs are evicted by prostate cancer out of the niche, where they may be primed to differentiate into osteoclasts, may have immediate and long-term consequences. First, the generation of osteoclasts in the local bone environment may release a number of growth factors sequestered in bone matrix, most notably TGF- β , which stimulate tumor growth. Second, by expanding the marrow space within bone by activating endosteal resorption, addi-

tional room for tumor growth may occur within the confines of a tightly restricted tissue compartment. Combined, the process of expanding the space for growth, as well as the liberation of factors that may feed cancer growth directly, is often called a "vicious cycle" (46, 47).

In this study, we also compared osteoclastic differentiation potential of early hematopoietic cells expressing Lin⁻CD48⁻CD41⁻Sca1⁺cKit⁺ (LSKHSCs) or CD150⁺Lin⁻CD48⁻CD41⁻Sca-1⁺cKit⁺ (SLAM HSCs) and found a similar extent of osteoclast differentiation in the presence of PC3-conditioned medium, RANKL, and M-CSF. To date, the earliest identified osteoclast progenitor in bone marrow has been shown to express c-Kit⁺/c-Fms⁻/CD11b⁻/RANK⁻ cell (21). Interestingly, the CD150⁺Lin⁻CD48⁻CD41⁻Sca-1⁺cKit⁺ cells isolated from surgical control or control tumor-implanted animals did not generate osteoclasts even in the presence of RANKL and M-CSF and/or prostate cancer-conditioned media. Yet when isolated from animals with disseminated PC3 in their marrow, differentiation into osteoclasts was possible. This raises the possibility that the CD150⁺Lin⁻CD48⁻CD41⁻Sca-1⁺cKit⁺ cells may express RANKL or receptors that make these cells responsive to osteoclastic induction.

Bone is one of the most common sites of prostate cancer metastases with approximately 80% of men with metastatic disease having bone involvement. Typically, these lesions result in increased but uncontrolled bone formation. Clinically, pain, compression of the spinal cord, fracture, and in effective hematopoiesis result from disseminated prostate cancer cells in the marrow (48). Current paradigms suggest that the osteosclerosis of prostate cancer is the result of soluble factors produced by prostate cancer cells that induce osteoblastic differentiation of mesenchymal stem cells, prevent apoptosis of osteoblasts or their precursors, and inhibit osteoclastic differentiation or activation. Others have suggested that prostate cancer may mimic osteoblasts and may produce bone-like tissues directly by a process termed osteomimicry (28, 32). Data from our group suggest an alternative and important hypothesis that metastatic prostate cancer disrupts the ability of HSCs and/or HPCs to regulate normal bone metabolism. The concept that HSCs or HPCs directly participate in the generation of the skeletal phenotype of prostate cancer directly challenges the existing paradigms but suggest that these cells may serve as new therapeutic targets of the bone microenvironment that may be worth considering to ameliorate the effects of bone metastases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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