HSC homing, quiescence, and self-renewal depend on the bone marrow HSC niche. A large proportion of solid tumor metastases are bone metastases, known to usurp HSC homing pathways to establish footholds in the bone marrow. However, it is not clear whether tumors target the HSC niche during metastasis. Here we have shown in a mouse model of metastasis that human prostate cancer (PCa) cells directly compete with HSCs for occupancy of the mouse HSC niche. Importantly, increasing the niche size promoted metastasis, whereas decreasing the niche size compromised dissemination. Furthermore, disseminated PCa cells could be mobilized out of the niche and back into the circulation using HSC mobilization protocols. Finally, once in the niche, tumor cells reduced HSC numbers by driving their terminal differentiation. These data provide what we believe to be the first evidence that the HSC niche serves as a direct target for PCa during dissemination and plays a central role in bone metastases. Our work may lead to better understanding of the molecular events involved in bone metastases and new therapeutic avenues for an incurable disease.

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Human prostate cancer metastases target the hematopoietic stem cell niche to establish footholds in mouse bone marrow

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Introduction

Metastases represent the most common malignant tumors involving the skeleton: nearly 70% of patients with breast cancer or prostate cancer (PCa) — and approximately 15%–30% of patients with carcinomas of the lung, colon, stomach, bladder, uterus, rectum, thyroid, or kidney — have bone lesions (1). Several mechanisms are thought to account for the organ-specific nature of bone metastases, including direct tumor extensions, retrograde venous flow, and tumor embolization. It is also clear, however, that anatomy alone does not explain the organ-specific pattern of metastasis.

One hypothesis that has gained favor is that the metastatic process is functionally similar to the homing behavior of HSCs to the BM (2, 3). HSC homing, quiescence, and self-renewal in the BM are now known to depend on a region termed the HSC niche (4, 5). Recent studies identified cells of the osteoblastic and endothelial lineages as key components of the niche (6–11). Molecules that play critical roles in HSC niche selection are now thought to be used by metastases as key components of the niche (6–11). Molecules that play critical roles in HSC niche selection are now thought to be used by metastases as key components of the niche (6–11). Molecules that play critical roles in HSC niche selection are now thought to be used by metastases as key components of the niche (6–11). Molecules that play critical roles in HSC niche selection are now thought to be used by metastases as key components of the niche (6–11). Molecules that play critical roles in HSC niche selection are now thought to be used by metastases as key components of the niche (6–11). Molecules that play critical roles in HSC niche selection are now thought to be used by metastases as key components of the niche (6–11). Molecules that play critical roles in HSC niche selection are now thought to be used by metastases as key components of the niche (6–11). Molecules that play critical roles in HSC niche selection are now thought to be used by metastases as key components of the niche (6–11). Molecules that play critical roles in HSC niche selection are now thought to be used by metastases as key components of the niche (6–11). Molecules that play critical roles in HSC niche selection are now thought to be used by metastases as key components of the niche (6–11). Molecules that play critical roles in HSC niche selection are now thought to be used by metastases as key components of the niche (6–11).

HSC homing, quiescence, and self-renewal depend on the bone marrow HSC niche. A large proportion of solid tumor metastases are bone metastases, known to usurp HSC homing pathways to establish footholds in the bone marrow. However, it is not clear whether tumors target the HSC niche during metastasis. Here we have shown in a mouse model of metastasis that human prostate cancer (PCa) cells directly compete with HSCs for occupancy of the mouse HSC niche. Importantly, increasing the niche size promoted metastasis, whereas decreasing the niche size compromised dissemination. Furthermore, disseminated PCa cells could be mobilized out of the niche and back into the circulation using HSC mobilization protocols. Finally, once in the niche, tumor cells reduced HSC numbers by driving their terminal differentiation. These data provide what we believe to be the first evidence that the HSC niche serves as a direct target for PCa during dissemination and plays a central role in bone metastases. Our work may lead to better understanding of the molecular events involved in bone metastases and new therapeutic avenues for an incurable disease.

Results

PCa cells target the HSC niche during metastasis. To directly test whether metastatic cells compete with HSCs for occupancy of the endosteal HSC niche during dissemination, we have shown in a mouse model of metastasis that human prostate cancer (PCa) cells directly compete with HSCs for occupancy of the endosteal HSC niche during BM transplantation (BMT). Critically, HSCs colocalized with PCa cells to the endosteal bone surfaces in vivo and in vitro, suggestive of niche competition. Additional evidence suggesting that PCa cells target the HSC niche during metastases was obtained when the osteoblastic niche was ablated using conditional osteoblast knockout tissues (Col2.3A-TK; ref. 15) in metastatic assays. Our results showed that fewer metastatic cells homed to the BM when the HSC niche was compromised. Conversely, increasing the number of HSC niches with parathyroid hormone (PTH) promoted metastasis. Once in the niche, metastatic cells like HSCs could be mobilized back into the peripheral blood using agents that mobilize HSCs. Importantly, disseminated PCa cells reduced the number of HSCs in the BM by driving HSCs into progenitor pools and peripheral blood. These findings demonstrated that PCa cells and HSCs compete for the endosteal HSC niche and use the same mechanisms to access and egress the niche. These data provide direct evidence that the endosteal HSC niche plays a central role in bone metastases.

Conflict of interest: The authors have declared that no conflict of interest exists.

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static transformed prostate epithelial (NMPE) cell lines as controls (Figure 1A and Supplemental Methods). Later, the tumors were removed, and BMT was performed using BM cells derived from CD45.2 animals (Figure 1A). To preserve the integrity of the niche, preparative transplant regimens (e.g., radiation and chemotherapy) were not used, and engraftment was analyzed by FACS. In all cases, greater HSC engraftment was observed in control groups (NMPE or no tumor implanted) than in the tumor-bearing animals (Figure 1B), which suggests that the cells shed from a primary tumor prevent HSC engraftment by occupying the HSC niche.

Figure 1
PCa cells compete for the HSC niche and prevent HSC engraftment. (A) Experimental model of BMT in the presence or absence of disseminated PCa cells. (B) Fewer donor HSCs (CD45.2) were able to engraft into NOD/SCID mice (CD45.1) when disseminated tumor cells (PC3 or C4-2B PCa cells) were present in BM. *P < 0.05, **P < 0.01 versus NMPE, Student's t test (n = 10 per group). (C) At 4, 8, 12, and 16 weeks after transplantation, the establishment of metastases was followed by bioluminescent imaging. Data are representative of bioluminescent imaging of mice that had developed metastases at approximately 40 weeks. Dashed circles denote where the primary s.c. tumors were implanted and removed. Arrows show metastatic PCa. (D) Representative images of BM histology of mice that developed micrometastases at 16 weeks. Original magnification, ×60. Scale bars: 50 μm. (E) Osteoblast numbers were determined in the long bones. (F and G) mRNA levels of (F) CXCR4 and (G) CXCR7 in PCa cells at peripheral blood (PB) and BM. Significance of differences was determined by Kruskal-Wallis test.
To verify that the reduced HSC engraftment was in fact caused by the disseminated PCa cells, we used bioluminescent imaging and observed that tumors developed from the metastatic cells in these animals (4 weeks, 0 of 10; 8 weeks, 0 of 10; 12 weeks, 2 of 10; 16 weeks, 3 of 10; Figure 1C). Additionally, disseminated PCa cells in the BM were identified by immunohistochemistry; few or no disseminated cells were observed in either of the controls (Figure 1D and Supplemental Figure 1A). Furthermore, these results were not likely attributable to changes in the number or size of the niche (e.g., osteoblast number; Figure 1E) resulting from the primary tumor alone.
CXCL12 and its receptors, CXCR4 and CXCR7, are known to play major roles in HSC homing to the BM (17, 18) and in establishing PCa metastases in bone (3, 12, 19–21). To determine whether a similar mechanism regulates PCa cell targeting to the HSC niche, mRNA levels of CXCR4 and CXCR7 expressed by PCa cells in the niche were compared with those of circulating PCa cells in the blood. Compared with PCa cells in culture (Supplemental Figure 1, B and C) or in peripheral blood, CXCR4 expression was dramatically reduced in PCa cells isolated from the HSC niche, whereas no remarkable changes were observed in CXCR7 levels (Figure 1, F and G). Further time course studies tracking PCa cell dissemination into the niche along with CXCL12 BM levels (Supplemental Figure 2, A–E, and Supplemental Methods) — in conjunction with our previous work showing that CXCR4 and CXCR7 regulate metastasis (3, 12, 19–21) — further support the concept that CXCL12 plays an important mechanism whereby PCa cells target the HSC niche.

To directly test whether disseminated tumor cells compete with HSCs for the niche, we used a competitive engraftment assay. Lethally irradiated animals were transplanted with BM cells alone or with PCa cells or NMPE control cells, and survival was evaluated as a measure of HSC engraftment (Figure 2A). To ensure that only occupancy of the niche occurred, rather than tumor growth, PCa and NMPE cells were irradiated to prevent proliferation (Figure 2B). Significantly more of the PCa cell–injected animals failed to engraft and subsequently required euthanasia than the control animals (Figure 2C), yet this effect was dependent on the number of PCa cells (data not shown), which suggests that PCa cells are not as efficient as HSCs in targeting the niche. BM histology demonstrated significant delays in engraftment of the animals injected with tumor cells along with their transplants (Figure 2D and Supplemental Figure 3), demonstrating competition between HSCs and PCa cells for the niche.

Figure 3
HSCs and PCa cells colocalize to BM niches through Runx2. (A) To determine whether metastatic cells and HSC colocalize to the same niche, a confocal microscope was used to track prelabeled LSK HSCs (red) and prelabeled PCa cells (green) 24 hours after transplantation. Nuclei were stained with DAPI (blue). DIC, differential image contrast. (B–D) SCID mice were implanted with PCa cells. After 3 weeks, the long bones were collected. Representative elements of the BM were triple-stained with (B) anti-HLA antibodies, anti-Runx2 antibodies, and DAPI; (C) anti-CD150 antibodies, anti-lineage antibody cocktail, and anti-Runx2 antibodies; and (D) anti-CD150 antibodies, anti-lineage antibody cocktail, and anti-HLA antibodies. Arrows denote colocalization of HSCs and PCa cells (A and D), osteoblasts and PCa cells (B), or osteoblasts and HSCs (C). Original magnification, x60. Scale bars: 10 μm.
HSCs and PCa cells colocalize to BM niches, and alteration of niche size regulates tumor dissemination. (A and B) To determine whether metastatic cells and HSCs colocalize to the same niche, multiphoton imaging was used to track prelabeled LSK HSCs (red) and (A) prelabeled PCa cells (green) or (B) NMPE control cells 24 hours after transplantation. Nuclei were stained with DAPI (blue). Original magnification, ×200. (C) Statistical analyses of A. (D) SLAM HSCs and PC3 or C4-2B PCa cells colocalized to a single osteoblast in vitro, as imaged by confocal microscopy. (E) Statistical analyses of in vitro adhesion assays to Anxa2<sup>−/−</sup> versus Anxa2<sup>+/-</sup> osteoblasts (see D). (F) SLAM HSCs, but not NMPE cells, in vitro localized to a single osteoblast. NMPE cells were unable to bind to Anxa2<sup>−/−</sup> or Anxa2<sup>+/-</sup> osteoblasts. (G) To expand the osteoblast numbers, animals were pretreated with vehicle or PTH prior to establishing primary tumors, and the number of disseminated PC3 cells was determined at 3 weeks (n = 8 per group). *P < 0.05, **P < 0.01 versus vehicle. (H) Homing of PC3 cells to Col2.3Δ-TK versus control vossicles with or without ganciclovir (n = 8 per group). The number of disseminated PCa cells homed to vehicle-treated control vossicles was set as 100%. Significance of differences was determined by Student’s t test (C and E) or Kruskal-Wallis test (G and H). Scale bars: 10 μm (A and B); 50 μm (D and F).
Thus far, our findings demonstrated that human PCa cells compete with murine HSCs for occupancy of the niche. To determine whether human PCa cells and HSCs compete for the niche in vivo, direct competition assays were performed using human CD34+ BM cells and human PCa cells in sublethally irradiated mice. Under these conditions, engraftment of the human cells was low, as expected. Significantly fewer human CD34+ cells engrafted into PCa cell–injected animals (Figure 2E). These data suggest that disseminated tumor cells directly compete with HSCs for occupancy of the niche.

PCa cells and HSCs colocalize to the endosteal niche. At present, the precise cellular composition and location of the HSC niche remains controversial (5, 22). Recent reports have demonstrated...
that endosteal cells of the osteoblastic lineage contribute to the development of the HSC niche (6–10). To determine whether disseminated PCa cells and HSCs colocalize to the endosteal niche, prelabeled HSCs (Lin^−/CD41^−/CD48^−^) and prelabeled PCa cells were injected into animals simultaneously. After 24 hours, the long bones were recovered, and confocal microscopy was used to track PCa cells and HSCs after transplantation. Both cell types colocalized within a few microns of each other in the BM of recipient animals (Figure 3A).

To further characterize the interactions between PCa cells and HSCs, the long bones recovered at 3 weeks from animals implanted s.c. with human tumors and tissue sections were implanted. To ensure no direct effect of PTH on the tumor cells and HSCs compete for the HSC niche in BM. Anxa2^−/− osteoblasts (Figure 4F). Together, these findings, along with the competitive engraftment data, demonstrated that PCa cells and HSCs compete for the HSC niche in BM.

**Figure 6**

PCa cells target the HSC niche, and disseminated PCa cells can be mobilized from the BM niche via CXCR4/CXCL12 axis. (A) Experimental model of HSC mobilization out of the niche via AMD3100 treatment to open the HSC niche. (B) PCa cell number in BM after i.c. injection of 1 × 10^6 cells after AMD3100 mobilization of HSCs. *P < 0.05, **P < 0.01 versus vehicle. (C) Experimental model to determine whether AMD3100 mobilizes disseminated PCa cells from BM (n = 8 per group). (D) Peripheral blood levels of PC3 cells mobilized with AMD3100 or vehicle, evaluated by QPCR. (E) SLAM HSC numbers in the BM after AMD3100 treatment were enumerated by FACS. (F) and (G) mRNA levels of (F) CXCR4 and (G) CXCR7 in HSCs at peripheral blood and BM with or without AMD3100 treatment. (H) Peripheral blood levels of PC3 cells mobilized with G-CSF or vehicle, evaluated by QPCR. (I) Number of HSCs in BM after G-CSF treatment, enumerated by FACS. (J) BM mRNA levels of MMP2 and MMP9 after G-CSF treatment, determined by QPCR. *P < 0.05, **P < 0.01 versus vehicle. (K) CXCL12-immunostained BM. Original magnification, ×60. Scale bars: 50 μm. Significance of differences was determined by Kruskal-Wallis test (B, D, F–H, and J) or Student’s t test (E and I).
Figure 7
Mechanisms regulating niche competition between PCa cells and HSCs: competition for binding to osteoblasts. (A) Competition binding assays to murine osteoblasts between $10^4$ LSK HSCs and $0–10^5$ PCa cells or NMPE control cells. (B) A fixed number of labeled NMPE control cells and PCa cells ($10^4$ cells) and $0–10^5$ HSCs were layered onto murine osteoblasts. The binding ability of NMPE control cells and PCa cells to osteoblasts in the presence of HSCs was evaluated using a fluorescent plate reader. (C) A fixed number of fluorescently labeled HSCs ($10^4$ cells) was layered onto murine osteoblasts. At the same time, cultures were treated with $0–1 \mu g/\mu l$ of medium alone or conditioned medium (CM) derived from NMPE control cells and PCa cells. The binding ability of HSCs was measured by fluorescent plate reader. (D) Competition binding assays between $10^4$ HSCs and $10^4$ CD133-CD44+ or CD133+CD44+ PCa cells. Data are from 3 independent experiments. (E–H) mRNA levels of (E) CXCR4, (F) CXCR7, (G) CCND1, and (H) CCNA1 in CD133-CD44+ or CD133+CD44+ PCa cells. Significance of differences was determined by Student's t test (A–D) or Kruskal-Wallis test (E–H).
ref. 25) and when CXCR4/CXCL12 signaling is interrupted (18). If PCa cells and HSCs compete for the niche, then it should be possible to increase the number of metastatic cells in the niche by vacating HSCs from the niche. To explore this possibility, mechanistic studies were designed to explore whether disseminated PCa cells use the CXCR4/CXCL12 pathway to gain entrance and egress of the HSC niche (18). Here, experimental animals were pretreated for 5 days with 5 mg/kg AMD3100, an antagonist of CXCR4 that mobilizes HSCs into the peripheral blood to “open” the HSC niche (18). Subsequently, PCa cells were inoculated into the animals by intracardiac (i.c.) injection to establish disseminated tumor cells (Figure 6A). More PCa cells had homed to the BM 24 hours later in the bones of the AMD3100-pretreated animals than in the vehicle-treated animals (Figure 6B and Supplemental Figure 6A). These data suggest that HSC occupancy of the niche limits metastasis.

PCa cells can be mobilized out of the HSC niche and into the blood using HSC mobilizing agents. If PCa cells target the HSC niche, then it should also be possible to induce PCa cells to reenter the peripheral circulation by interfering with CXCR4/CXCL12 signaling, as has been shown for HSCs. To explore this possibility, disseminated PCa cells were first established in bone after tumor implantation (Figure 6C). After removal of the primary tumors, the animals were rested and then treated with AMD3100 or vehicle. Blood was collected 24 hours after the last AMD3100 injection, and the number of circulating PCa cells determined (Figure 6C). More circulating PCa cells were found in blood after treatment with AMD3100 versus vehicle (Figure 6D and Supplemental Figure 6B). AMD3100 mobilized the HSCs from the BM by inhibiting mRNA expression of both CXCR4 and CXCL12 in the HSCs (Figure 6, E–G). These data suggest that disseminated PCa cells target the HSC niche through the CXCR4/CXCL12 pathway.

G-CSF is another agent that is used clinically to mobilize HSCs out of the niche and into the peripheral blood to improve stem cell collection prior to BMT (25). Here, the mobilization studies were repeated using recombinant G-CSF. G-CSF mobilized PCa cells from the HSC niche and into the peripheral blood with higher frequency than in vehicle-treated control animals (Figure 6H and Supplemental Figure 6C). To exclude a direct effect of G-CSF on PCa cells, we also confirmed that PCa cells did not express G-CSF receptors, nor did G-CSF induce PCa cell proliferation (Supplemental Figure 6, D–G). As expected, G-CSF mobilized the HSCs from the BM (Figure 6I), which suggests that PCa cells use the same mechanisms as HSCs and HPCs to enter the peripheral circulation.

Mobilization of HSCs and HPCs by G-CSF is thought to occur through the induction of 2 pathways. The first is through the production of enzymes (e.g., CD26, cathepsin G, elastase, MMP2, and MMP9) that degrade CXCL12 (25). To determine whether loss of CXCL12 could be responsible for the mobilization of PCa cells into the peripheral blood once in the HSC niche, levels of MMP2, MMP9, and CXCL12 were examined after G-CSF treatment. As expected, G-CSF induced increases in the expression of MMP2 and MMP9 in the BM of G-CSF–treated tumor-bearing mice and resulted in a substantial decrease in the levels of CXCL12 (Figure 6, J and K). A second major pathway believed to regulate HSC mobilization is through the induction of osteoclastic bone resorption (26). Therefore, we next explored the role of osteoclasts in mobilization of PCa cells from the niche. Interestingly, G-CSF induced osteoclastogenesis in the presence of disseminated PCa cells (Supplemental Figure 6, H and I). Conversely, AMD3100— which does not mobilize HSCs by activating osteoclastogenesis, but rather by interfering directly with CXCR4/CXCL12 binding — did not active osteoclasts to mobilize PCa cells, as predicted (Supplemental Figure 6, J and K; and ref. 27). Together, these data suggest that disseminated PCa cells compete with HSCs for the niche using the same molecular mechanisms as do HSCs to gain access and egress of the niche (e.g., CXCR4/CXCL12).

Direct cell-to-cell competition for niche binding favors PCa cells. A second molecular mechanism for the competition between HSCs and PCa cells for the niche may be that disseminated PCa cells can displace or outcompete HSCs for adhesion of niche-binding elements. Accordingly, we performed competitive binding assays between HSCs and PCa cells. PCa cells blocked HSC binding to osteoblasts, whereas NMPE control cells were less able to prevent HSC binding to osteoblasts (Figure 7A). Recently, we demonstrated that Anxa2 expressed by osteoblasts is a critical molecule used by both HSCs and PCa cells for binding to osteoblasts (13, 24). The competitive binding assays were therefore repeated using Anxa2 as the binding target. As expected, PCa cells blocked the binding of HSCs to Anxa2 better than did the NMPE control cells (Supplemental Figure 7A). Conversely, HSCs more efficiently prevented the binding of NMPE cells to osteoblasts and Anxa2 than they blocked PCa cell binding (Figure 7B and Supplemental Figure 7B). These effects required direct cell-to-cell interactions, as soluble factors present in the conditioned medium of PCa cells alone were unable to alter HSC binding to osteoblasts (Figure 7C).

When PCa cells expressing a putative PCa stem cell phenotype (CD133$^{+}$CD44$^{-}$) were isolated from culture and used in competitive adhesion assays with HSCs, they were better able to block osteo- clasts in mobilization of PCa cells from the niche. Interestingly, G-CSF induced osteoclastogenesis in the presence of disseminated PCa cells (Supplemental Figure 6, H and I). Conversely, AMD3100— which does not mobilize HSCs by activating osteoclastogenesis, but rather by interfering directly with CXCR4/CXCL12 binding — did not active osteoclasts to mobilize PCa cells, as predicted (Supplemental Figure 6, J and K; and ref. 27). Together, these data suggest that disseminated PCa cells compete with HSCs for the niche using the same molecular mechanisms as do HSCs to gain access and egress of the niche (e.g., CXCR4/CXCL12).

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When PCa cells expressing a putative PCa stem cell phenotype (CD133$^{+}$CD44$^{-}$) were isolated from culture and used in competitive adhesion assays with HSCs, they were better able to block HSC binding to osteoblasts compared with CD133$^{+}$CD44$^{-}$ cells (Figure 7D). These CD133$^{+}$CD44$^{-}$ cells expressed higher levels of CXCR4, but lower CXCR7 (Figure 7, E and F), and progressed through the cell cycle slower than did PCa cells expressing a CD133$^{+}$CD44$^{-}$ phenotype, based on expression of CCNA1 and CCND1 (encoding cyclin A1 and cyclin D1, respectively; Figure 7, G and H). Intriguingly, although the CD133$^{+}$CD44$^{-}$ cells represented a very small fraction of the total cells found in culture (0.86% ± 0.52% of PC3 and 1.47% ± 0.74% of C4-2B in vitro), the frequency of this population was significant when CD133$^{+}$CD44$^{-}$ cells were evaluated for CD133 and CD44 expression. Thereafter, 1 × 10$^6$ PCa cells were injected i.c. into SCID mice to establish disseminated cells in the BM. After 24 hours, BM was recovered from the long bones, and hematopoietic lineage cells were depleted using magnetic beads. CD133 and CD44 frequency in disseminated PCa cells (shown as percentages) was evaluated with FACS by gating on HLA-ABC (>99% PCa positive).

### Table 1

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<tr>
<td>In vitro</td>
<td>0.86 ± 0.52</td>
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<td>In vivo BM recovered</td>
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PCa cells recovered from culture were evaluated for CD133 and CD44 expression. Thereafter, 1 × 10$^6$ PCa cells were injected i.c. into SCID mice to establish disseminated cells in the BM. After 24 hours, BM was recovered from the long bones, and hematopoietic lineage cells were depleted using magnetic beads. CD133 and CD44 frequency in disseminated PCa cells (shown as percentages) was evaluated with FACS by gating on HLA-ABC (>99% PCa positive).
be able to drive HSCs to maturity and into HPC populations so that they eventually vacate the niche. To explore this possibility, mice were implanted with metastatic PCa cells or NMPE control cells to establish disseminated tumors in the BM. HSCs isolated from animals with disseminated PCa cells expressed lower levels of the niche adhesion molecules (\textit{NOTCH1}, ref. 7; \textit{TIE2}, ref. 9) and transcription factors known to regulate HSC self-renewal and proliferation (\textit{BMI1} and \textit{INK4A}; Figure 8A and refs. 28, 29). These results suggest that disseminated PCa cells reduce HSC numbers by altering HSC self-renewal. Consistent with these observations, fewer HSCs were found in the tumor-bearing animals (Figure 8B). One mechanism to explain this reduction is that HSCs are driven into HPCs pools. Indeed, more HPCs were recovered from the BM of tumor-bearing animals than from that of controls (Figure 8C).

Figure 8
Mechanisms regulating niche competition between PCa cells and HSCs: PCa cells drive HSCs out from the HSC niche. (A–G) SCID mice were implanted either NMPE cells or PCa cells (PC3 or C4-2B) (\(n = 5\) per group). After 3 weeks, the BM cells were collected. (A) Expression of stem cell survival and cell-to-cell adhesion genes in SLAM HSCs was evaluated by quantitative real-time RT-PCR (\(n = 5\) per group). \(* P < 0.05, \# P < 0.01\) versus NMPE. (B) SLAM HSC numbers in BM were counted by FACS, and (C) HPC numbers were determined using colony-forming assays. (D and E) mRNA levels of (D) \textit{CCND1} and (E) \textit{CCNA2} in HSCs. (F) Cell cycle (Ki-67–positive cells) and (G) apoptotic state (PE-conjugated annexin V/7-ADD) of HPCs were analyzed by gating on Lin−Sca-1+ populations. (H) Peripheral blood was collected from subjects with local PCa disease (\(n = 18\), 61.6 ± 9.2 years of age) and disseminated PCa disease (\(n = 39\), 68.1 ± 11.1 years of age). The number of hematopoietic colonies was compared with that in healthy controls (young age, \(n = 13\), 34.3 ± 5.6 years; age-matched, \(n = 11\), 62.4 ± 7.1 years). Significance of differences was determined by Kruskal-Wallis test (A, D, and E) or Student’s \(t\) test (B, C, and F–H); \(P\) values in B–F are versus NMPE.
was no effect of PCa cells on HPC apoptosis (Figure 8G). Although it is not presently clear what molecules are responsible for these activities, HSCs were chemotactic toward PCa cells themselves, but PCa cells did not have similar effects on HSCs, and HSCs were able to enhance the growth of PCa cells (Supplemental Figure 8, A–C).

If PCa cells compete for the niche with HSCs and HPCs and drive these cells into the circulation, then it would be expected that more HSCs or HPCs would be present in the circulation of men with metastatic bone disease than in age-matched controls. Therefore, HPC assays were performed on peripheral blood collected from subjects with disseminated PCa bone disease. As expected, more circulating HPCs were found in subjects with disseminated disease compared with local disease (defined as PCa with no imaging evidence of metastases and PSA < 15 ng/ml) and age-matched controls (Figure 8H). Although other explanations are possible (e.g., inflammatory cytokines or cachexia), these data are consistent with the hypothesis that disseminated tumor cells target the HSC niche and displace HPCs into the peripheral blood. Together, these data strongly suggest that PCa cells target the HSC niche during metastasis.

Discussion

Our data suggest that the HSC niche serves as a specific site where disseminated cells from PCa gain footholds in the BM (Figure 9). These studies showed, for the first time to our knowledge, that disseminated cells from solid tumors are able to compete with HSCs for occupancy of the HSC niche and colocalize with HSCs in the BM. In fact, by mobilizing HSCs out of the niche, or by altering the size and/or number of the endosteal HSC niches, we were able to alter the number of metastatic cells present in the BM. We observed at least 3 molecular mechanisms that may be operative during metastasis and facilitate disseminated tumor cells' ability to gain footholds in the HSC niche. First, PCa cells use the CXCR4/CXCL12 pathway to gain access and egress from the HSC niche area, as do HSCs themselves. In fact, we previously demonstrated that blocking this pathway, or blocking the alternative CXCL12 receptor CXCR7, prevents metastasis and PCa growth in bone (12, 19, 20). A second mechanism that may provide PCa cells an advantage over HSCs in establishing themselves in the niche is a superior ability to bind to and/or engage receptors that HSCs use to localize to the niche. Indeed, we recently demonstrated that both HSCs and PCa cells both bind to Anxa2 and that blocking the receptor for Anxa2 prevents homing to the BM (13, 24). Finally, we demonstrated that HSCs themselves were targets of metastatic cells, as they were driven into progenitor pools, into the peripheral blood, or both, and may be able to support the growth of the cancer within the niche. Together, these data suggest that metastatic PCa cells, and perhaps cells from other tumors, serve as molecular parasites of the HSC niche.

Little is known about the postdissemination and niche engagement events activated by PCa cells to coopt the HSC niche. Based on the average length of time for relapse to occur after primary therapy (surgery or radiation; refs. 30, 31), it is possible that the HSC niche is able to regulate dormancy of tumor cells for extended periods. In fact, clinically relevant metastases are relatively rare compared with the number of disseminated tumor cells (32). Because the endosteal HSC niche plays a crucial role in maintaining stemness, the ability of tumor cells to engage the endosteal HSC niche may facilitate the ability of tumor cells to establish metastatic foci in bone while remaining dormant over long time periods (33, 34). Moreover, it has been appreciated that preosteoblasts are critical compartment of the HSC niche rather than mature osteoblasts (35, 36). However, further studies are needed to determine whether preosteoblasts serve as the solid tumor niche.

Similarly, what leads to the loss of tumor dormancy is unknown. One prospect worth considering is that there are substantial niche reserves in the BM for HSCs. However, a recent study demonstrated that HSC replacement of the niche constantly occurs under normal physiological condition (37). Perhaps as disseminated tumor cells proliferate slowly over time, the capacity of the niche is overwhelmed, leading to clinically relevant disease. In fact, peripheral blood changes — including infection, anemia, and thrombocytopenia, indicative of HSC failure — are late-stage events, but are commonly observed at death in individuals with metastatic disease (38). Other possibilities are that the tumor cells themselves become resistant to the dormancy signals provided by the niche over time (38).

In 1889, Paget proposed a "seed and soil" metaphor to explain the marked affinity of cancer cells for different tissues (39). It has previously been reported that VEGFR1-expressing BM cells participate in the process by establishing premetastatic niches (14). Although inflammatory cytokines released by tumors might affect HSCs directly, our data suggest that there may be other biologic targets that tumors use to establish disseminated metastases in bone. Our work has the potential to facilitate better understanding of the molecular events involved in bone metastases and to lead to new therapeutic avenues for an incurable disease.

Methods

Further information can be found in Supplemental Methods.

Cell culture. The PC3 (CRL-1435) PCa cell line was obtained from the American Type Culture Collection. The metastatic subline LNCaP C4-2B was originally isolated from a lymph node of a patient with disseminated bony and lymph node involvement (40). Normal human NMPE control
cell lines 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. PCa cell lines and NMPE cell lines were cultured in RPMI 1640 (Invitrogen) and DMEM (Invitrogen), respectively. 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.

In vitro PCa metastasis assays. Male 5- to 7-week-old C57BL/6 SCID mice were implanted with 2 × 10⁶ cells (PCa) within sterile collagen scaffolds (3 × 3 × 3 mm³; Gelifoam; Pharmacia and Upjohn) in the mid-dorsal region of each mouse. In some cases, NMPE cells (2 × 10⁶ cells) were used. When the animals were sacrificed, tissue samples from the animals’ left organ/tissue (original scaffold, calvaria, mandible, humeri, femur, tibia, pelvis, spine, and peripheral blood) were dissected and stored at -80°C until genomic DNA extraction. The number of disseminated cells was assessed by QPCR (16). Further normalization was performed for differences in mouse tissue density using murine β-actin primers.

BMT. NOD/SCID mice (CD45.1) were implanted s.c. with 2 × 10⁶ PCa cells or control cells. At 3 weeks, s.c. tumors were removed. After 5 days, the NOD/SCID mice were injected with 2 × 10⁶ mononuclear BM donor cells (CD45.2 C57BL/6 mice) into the left retro-orbital sinus without irradiation. At 4, 8, 12, and 16 weeks after BMT, the percentage of blood cells bearing the CD45.2 and CD45.1 phenotypes was determined in peripheral blood obtained from the tail veins of individual recipient mice stained together with Mac1 (macrophage), Gr-1 (Ly-6G/C), and Ter-119) antibody cocktail (Miltenyi Biotec) for 10 minutes at 4°C, with a biotinylated anti-Lin (CD5, CD45R [B220], CD11b, Gr-1 [Ly-6G/C], and Ter-119) and anti-biotin MicroBeads (Miltenyi Biotec). Positive immunoselection was performed with PE-conjugated anti–Sca-1 and FITC-conjugated anti–c-Kit (BD Biosciences — Pharmingen) and sorted on a FACS Vantage dual laser flow cytometer.

Murine osteoblasts. Calvariae of mice (1–4 days old) were dissected, isolated from periosteum, and subjected to sequential digestions of 20, 40, and 90 minutes in collagenase A (2 mg/ml; Roche Molecular Biochemicals) with 0.25% trypsin (Invitrogen), as previously described (13, 41). Cells from the third digestion were plated in -MEM (Invitrogen) with 10% (v/v) FBS and 1% (v/v) penicillin and streptomycin.

Vossicle transplant. Lumbar vertebrae were isolated from 4- to 7-day-old mice. The vertebrae were sectioned into single vertebral bodies (i.e., vossicles). SCID mice were used as transplant recipients. 2 vossicles per mouse were implanted into s.c. pouches as previously described (13, 16).

In vitro cocolocalization assays. Murine osteoblasts labeled with CellTracker Blue CMAC (Invitrogen) were cultured in Lab-Tek II 4-chamber slides (Nalge Nunc International) at 1 × 10⁶ cells/chamber. After 24 hours, SLAM HSCs labeled with CellTracker Red CMTPX (Invitrogen) and PC3 and C4-2B PCa cells labeled with CellTracker Green CMFDA (Invitrogen) were added to this culture system (200 cells/chamber). Thereafter, the cultures were incubated in an atmosphere of 3% CO₂ and 95% O₂ at 37°C for 2 hours. Fluorescence was observed under a FV500 confocal laser-scanning microscope (Olympus).

In vivo cocolocalization assays. Vossicles were implanted into SCID mice. After 3 weeks, a total of 1 × 10⁶ PCa cells expressing green fluorescent protein were injected i.c. After 24 hours, animals received i.v. injection of LSK HSCs (1 × 10⁶ cells) into the left retro-orbital sinus labeled with Qtracker 655-nm nontargeted quantum dots (Quantum Dot Corp.). Vossicles were subsequently harvested and imaged with multiphoton microscopy or confocal microscopy, as previously described (16).

PTH treatment. Animals were administered human PTH (hPTH1–34; Bachem) or vehicle (0.9% saline) by i.p. injection at 50–80 μg/kg (100 μl) per day for 3 weeks (41).

Ganciclovir treatment. Vossicle-implanted animals were administered ganciclovir (Cytovene-JV; Roche) or vehicle (0.9% saline) by i.p. injection at 3–8 mg/kg (100 μl) per day for 3 weeks.

AMD3100 treatment. AMD3100 (Sigma-Aldrich) or vehicle (0.9% saline) was administered by i.p. injection at 5 mg/kg (100 μl) per day for 5 days. In some cases, animals were injected with 1 × 10⁶ PC3 and C4-2B PCa cells i.c. 24 hours after the last AMD3100 injections. After 24 hours, animals were sacrificed, and PCa cells in the BM were assessed by QPCR.

G-CSF treatment. Animals were administered G-CSF (Neupogen; Amgen) or vehicle (0.9% saline) i.p. at 250–300 μg/kg (100 μl) per day for 5 days.

Immunohistochemistry. Vessicles and murine long bones were stained with anti-cytokeratin antibody (diluted 1:250, rabbit polyclonal; Abcam) and anti-cyto-keratin antibody (diluted 1:250, rabbit polyclonal). Hematoxylin and eosin as previously described (41).

Biodistribution imaging. Biodistribution imaging was performed as previously described through the University of Michigan Small Animal Imaging Resource facility (13).

Methylocellulos cultures. BM cells were collected from control or experimentally treated SCID mice (n = 5). Peripheral blood was collected from subjects with disseminated bone PCa disease, subjects with local PCa disease, and healthy volunteers. Peripheral blood mononuclear cells were isolated from whole blood by density gradient centrifugation using Ficoll-Paque PLUS (StemCell Technologies). Approval was obtained from the University of Michigan’s Investigation Review Board, and written informed consent was obtained from participating subjects.

BM or blood cells (2 × 10⁶ cells) were plated onto 35-mm tissue culture dishes in methylocellulose with recombinant cytokines for colony assays of murine cells (Methocult GF M3434; StemCell Technologies) or human cell lines.
Transwell chemotaxis assays. Cell invasion assays were performed in dual-chambered Transwell plates (Costar Corp). PC3 and C4-2B PCa cells or NMPE control cells (2.5 × 10^6 cells) were seeded onto the top well of an 8-μm dual chamber, and LSK HSCs (2.5 × 10^5 cells) were placed in the bottom well of the chamber as chemotaxis targets. In some cases, LSK HSCs (2.5 × 10^6 cells) were seeded onto the top well of 5-μm dual chambers, and PC3 or C4-2B PCa cells or NMPE control cells (2.5 × 10^5 cells) were placed in the bottom well. Cells seeded on the top well were labeled with 2.5 mg/ml of the lipophilic dye carboxyfluorescein diacetate (Invitrogen) prior to assays. Spontaneous invasion was compared with invasion supported by cells at bottom wells. The plates were incubated at 37°C in 95% humidity and 5% CO₂. At the termination of the assay (4 hours), chambers were removed, and fluorescence was quantified by fluorescent plate reader ( Molecular Devices).

Proliferation assays. Proliferation assays were performed as previously described (13). Luciferase-labeled PCa cells (5,000 cells/well) were plated onto 96-well plates in growth medium with 0.1% FBS. The next day, LSK HSCs (0–5,000 cells/well) were added to the wells. Thereafter, cultures were incubated for 3 days. Proliferation was determined using a CCK IVIS system with a 50-mm lens ( Xenogen Corp.), and the results were analyzed using LivingImage software ( Xenogen Corp.).

In vitro binding assays. Cell-to-cell binding assays were performed as previously described (24). Murine calvarial osteoblasts were plated onto 96-well plates at a concentration of 1 × 10^4 cells/well (100 μl/well) in growth medium, and the cultures were incubated for 2 days. A fixed number of LSK HSCs (10^4 cells) labeled with fluorescent dyes (CFDA; Invitrogen) and increasing numbers of PCa cells (0–10^5 cells) or NMPE control cells (0–10^5 cells) were layered on murine osteoblasts. Binding assays were performed in PBS containing Ca²⁺/Mg²⁺, where the cells were added to a final reaction volume of 100 μl at 4°C. After washing, remaining fluorescence was quantified as a measure of HSC binding by fluorescent plate reader ( Molecular Devices). In some cases, PCa cells were first sorted into CD133(CD44+) and CD133(CD44-) fractions, and binding assays were performed.

Statistics. All numerical data are expressed as mean ± SEM. Statistical analysis was performed by ANOVA or unpaired 2-tailed Student’s t test using GraphPad Instat (GraphPad). For QPCR assays, Kruskal-Wallis test and Dunn multiple-comparisons test was used. For survival assays, Kaplan-Meier survival analysis was used, and log-rank test was performed using GraphPad Prism (GraphPad) to determine differences between survival curves. For all analyses, a P value less than 0.05 was considered significant.

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