Definition of molecular determinants of prostate cancer cell bone extravasation

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Abstract

Advanced prostate cancer (PCa) commonly metastasizes to bone, but transit of malignant cells across the bone marrow endothelium (BMEC) remains a poorly understood step in metastasis. PCa cells roll on E-selectin+ BMEC through E-selectin ligand-binding interactions under shear flow, and PCa cells exhibit firm adhesion to BMEC via β1, β4 and αvβ3 integrins in static assays. However, whether these discrete PCa cell-BMEC adhesive contacts culminate in cooperative, step-wise transendothelial migration into bone is not known. Herein, we describe how metastatic PCa cells breach BMEC monolayers in a step-wise fashion under physiologic hemodynamic flow. PCa cells tethered and rolled on BMEC and then firmly adhered to and traversed BMEC via sequential dependence on E-selectin ligands and β1 and αvβ3 integrins. Expression analysis in human metastatic PCa tissue revealed that β1 was markedly upregulated compared with expression of other β subunits. PCa cell breaching was regulated by Rac1 and Rap1 GTPases and, notably, did not require exogenous chemokines as β1, αvβ3, Rac1 and Rap1 were constitutively active. In homing studies, PCa cell trafficking to murine femurs was dependent on E-selectin ligand, β1 integrin and Rac1. Moreover, eliminating E-selectin ligand-synthesizing α1,3 fucosyltransferases (α1,3 FT) in transgenic adenoma of mouse prostate (TRAMP) mice dramatically reduced PCa incidence. These results unify the requirement for E-selectin ligands, α1,3 fucosyltransferases, β1 and αvβ3 integrins and Rac/Rap1 GTPases in mediating PCa cell homing and entry into bone and offer new insight on the role of α1,3 fucosylation in PCa development.

PRÉCIS: This study offers the first comprehensive mechanism of how metastatic prostate cancer cells traverse the vascular endothelium.
Introduction

Metastatic PCa killed 33,720 men in 2011, and the prognosis with bone metastasis is poor (1). Unfortunately, how PCa cells breach bone marrow (BM) microvessels is ill-defined. Accordingly, we established an adhesion paradigm wherein PCa cells roll on BMEC under physiologic hemodynamic flow by recognizing BMEC endothelial (E)-selectin (2-5). This led to the premise that bone metastasis is conferred by PCa cell E-selectin ligand-BMEC E-selectin binding and has been supported by several observations. First, human BM microvessels express E-selectin constitutively (6). Second, E-selectin-binding sLeX-membrane glycoproteins and glycolipids, defined operationally as E-selectin ligands, and regulators of E-selectin ligand synthesis, alpha 1,3 fucosyltransferases (α1,3 FT) 3, 6 and 7, are upregulated in bone-metastatic PCa cells (4) and PCa lesions in bone (5) and are associated with elevated PCa grade, progression and bone metastasis (2, 3, 7-9). Third, E-selectin ligand+ PCa cells home more efficiently than E-selectin ligand- cells to bone, and homing is neutralized with anti-E-selectin Ab (5).

The putative role of E-selectin ligands in PCa bone metastasis was first conjectured by earlier evidence in mice that E-selectin ligand+ hematopoietic stem cells (HSCs) bound and traversed BM microvessels via rolling on E-selectin+ BM microvessels (10-12). In fact, HSC extravasation into murine bone involved the E-selectin ligand, PSGL-1 (12, 13). Other evidence indicated that integrins α4β1 and α4β7 contributed to initial HSC rolling on VCAM-1 and MAdCAM-1 (12, 14) expressed constitutively on BMEC (6, 14). HSCs then firmly adhere and traverse BMEC via contributions from CD44, α4β1, α5β1, α6β1, αLβ2 and SDF-1-CXCR4 signaling (11, 15-18). Homing to and engraftment in BM also involves HSC Rho and Ras GTPases, Rac1 and Rap1 (19, 20). Accordingly, E-selectin ligands, integrins and GTPases are candidate molecules regulating PCa extravasation into bone.

While α4β1, α4β7 and αLβ2 integrins have not been found on PCa cells, α2β1, α3β1, α5β1, α6β1, αVβ1, αVβ3, αIIbβ3 and α6β4 have been identified (21). All 8 integrins appear constitutively active, though can be regulated partly by chemokines SDF-1 or CCL2, which may enhance PCa cell adherence and migration/invasion on endothelial and basement membrane integrin ligands under static conditions (22-26). PCa cell adhesion to BMEC, BM stroma or endothelium under static
conditions, in fact, is blocked with anti-β1 or -β3 mAbs or with Arg-Gly-Asp (RGD) peptide (27-29), and PCa cell transendothelial migration (TEM) through vascular endothelium in static assays can be attenuated by anti-β3 mAb (30). Whether these PCa integrins cooperate with E-selectin ligands to elicit step-wise rolling, adhesion and TEM under hemodynamic conditions analogous to the HSC molecular paradigm is unknown.

Here, we describe a step-wise molecular dependency of PCa cells to adhere and breach BMEC monolayers under physiologic blood flow. Utilizing microfluidics models, we observed E-selectin ligand-dependent PCa cell tethering and rolling on E-selectin⁺ BMEC, transition to firm adhesion and breaching BMEC monolayers via dependency on β1 and αV/β3 integrins. However, PCa cell breaching did not require exogenous chemokines or G-protein receptor signaling, which was likely circumvented through constitutively active β1 and αV/β3 integrins and GTPases, Rac1 and Rap1. Moreover, αLβ2 and α4β1 integrins, characteristically involved in HSC TEM, were not required for PCa cell breaching. In addition, E-selectin ligand⁺ PCa cells homed more efficiently to murine femurs than E-selectin ligand⁻ cells in a β1 integrin- and Rac1-dependent manner. Finally, transgenic adenoma of mouse prostate (TRAMP) mice deficient in E-selectin ligand-synthesizing α1,3 FT genes, FT4 and FT7, exhibited reduced PCa incidence, implicating α1,3 fucosylation in transforming activity of prostate epithelia and/or in tumorigenic regulation via tumor – tumor or tumor - host/stroma interactions. These results unify requisite factors required for PCa cell extravasation and offer new insight on the role of α1,3 FTs in PCa development.
Materials and Methods

Cells and tissues. Human PCa cells, primary normal prostate epithelial cells and leukemic KG1a cells were cultured as described (2-4), and human bone-metastatic PC-E1 and PC-R1 cells were maintained in RPMI 1640/glutamine/10% fetal bovine serum (FBS)/1% pen/strep (Life Technologies, Grand Island, NY). All cell lines were tested and authenticated by morphological observation and by qRT-PCR, Western blotting and flow cytometry of glycosyltransferase genes/gene products as previously described (2-5).

Flow cytometry. Flow cytometry was performed as described (4), antibody concentrations were 10 μg/ml, and conformation anti-β1 Abs, N29, HUTS-21 and 9EG7 were incubated 1hr at 37°C.

Real-time PCR. Real-time PCR was performed (4), and primer sequences are shown in Table S1.

Western blotting. Western blots were conducted as described (4, 5).

PCa rolling and breaching in flow. Rolling frequency and velocity was measured as described (2, 5, 31). For breaching through ECs, PCa cells (1x10^6 in RPMI or endothelial growth media) were infused at 1.5 ml/min for 35s, and a physiologic shear stress of 0.5 Dynes/cm^2 was maintained 4 hrs. Photographs were taken every second the first 4 min to capture rolling and then every 5 min to capture adhesion and breaching. 37°C was maintained via heat fan and water bath. Breaching, defined as a PCa cell piercing and penetrating the EC layer, was also investigated with BioFlux microfluidics technologies (Fluxion Biosciences, San Francisco, CA). Where indicated, PCa cells and ECs were loaded with 0.5 μM CellTracker Green or CellTracker Red CMTPX (Life Technologies), respectively, and imaged by confocal fluorescence microscopy (Dana Farber Cancer Institute, Boston, MA).

Statistical analysis. Results were analyzed by two-tailed t-test, one-way ANOVA with Dunnett post test or contingency table on GraphPad Prism (GraphPad Software, La Jolla, CA).
Results

PCa PC-E1 and PC-R1 cells traverse BMEC and prominently express β1 and αVβ3 integrins. To identify PCa cells with robust transendothelial migration (TEM) activity for subsequent molecular analysis, we first examined the relative efficiency of TEM using a number of metastatic PCa cell lines. Using a static approach and a 16 hr incubation period, we found that bone metastatic PC-E1 and PC-R1 cells showed a higher capacity than other PCa cells to breach confluent HUVEC and HBMEC-60 monolayers, traverse 8 micron pores and attach to insert undersides (Fig. 1A-B).

To identify integrin – integrin ligand interactions controlling TEM of PC-E1 and PC-R1 cells, we surveyed expression of β and α integrin subunits and found that β1 and β3 were most prominent (Fig. 1C). In fact, qRT-PCR analysis of β chains in PC-E1 and PC-R1 cells and in PCa tissue confirmed that β1 transcript level was markedly higher than all other β subunits and at minimum several hundred-fold higher than even the next most highly expressed β subunit (Fig. S1A-B). We also found that β3 was elevated on PC-E1 and PC-R1 cells (Fig. 1C) relative to normal prostate epithelial (NPE) cells (Fig. S1C). Candidate α partners for β1 on PC-E1 and PC-R1 included α2, α5, α6 and αV, and for β3, αV (Fig. 1C-D). αVβ3 heterodimer was present on PC-E1 and PC-R1 cells, while β2, β4, β5, β6, β7 and β8 were undetectable on PC-E1 and PC-R1 cells (Fig. 1C) despite prominent β4 and β6 expression on NPE (Fig. S1C). Of note, α4 was upregulated on PC-E1 cells, the first evidence of α4β1 on a PCa cell (Fig. 1C).

Data underscored four putative β1 heterodimers, α2β1, α5β1, α6β1 and αVβ1, and a β3 heterodimer, αVβ3, as most highly expressed on PC-E1 and PC-R1 cells (Fig. 1D). To strengthen these findings, we also assessed integrin expression on a number of other PCa cells and found that β1 and αVβ3 were prominently expressed on bone-metastatic PCa PC-3M LN4, PC-3 and MDA PCa 2b cell lines and on the brain-metastatic PCa DU-145 cell line, though reduced on lymph node-metastatic LNCaP and LNCaP C4-2b cells (Fig. S1C). These results suggested that high expression of β1 and upregulation of αVβ3 correlated with PCa bone and brain metastasis.
β1 and αVβ3 integrins on PCa PC-R1 cells are constitutively active. Using β1+ and αVβ3+ PC-R1 cells to conduct molecular modeling of TEM, we first validated β1- and αVβ3-dependent PC-R1 cell adhesion and found that PC-R1 cells bound to β1 and αVβ3 ligands, fibronectin (FN), laminin ((LN), collagen I (COL) and vitronectin (VN) without exogenous chemokine dependency (Fig. 2A). β1 and αVβ3 ligand binding was also observed with PC-3, PC-3M LN4, PC-E1, LNCaP, LNCaP C4-2b, DU-145 PCa cell lines (Fig. S2). Unexpectedly, PC-R1 and PC-E1 cells also adhered strongly to VCAM-1, which is the first demonstration of VCAM-1-dependent PCa cell adhesion (Fig. 2A and Fig. S2A). As expected, PC-R1 cells, including all other PCa cell lines, did not adhere to β2 ligand, ICAM-1 (Fig. 2A and Fig. S2A); and no adhesion to PECAM-1 or osteopontin was found (data not shown).

Given high avidity of PC-R1 cell adhesion to β1 and αVβ3 ligands independent of chemokine addition, we assessed the activation state of β1 and αVβ3 integrins using conformation-sensitive and blocking mAbs. All β1 conformation-sensitive mAbs recognizing increasingly active β1 structures, N29 < HUTS-21 < 9EG7 (32), reacted strongly with PC-R1 cells in buffer devoid of exogenous chemokines (Fig. 2B). These quiescent conditions typically do not permit for HUTS-21 and 9EG7 mAb reactivity to β1 on resting leukocytes (33). These data were confirmed with other PCa cell lines and their reactivity to these conformation-sensitive Abs closely correlated with their adhesiveness to β1 and αVβ3 integrin ligands (Fig. S2B). In agreement with this finding, PC-R1 cell adhesion to VCAM-1 and to COL was completely blocked by anti-β1 mAb, while adhesion to FN or VN was significantly blocked with both anti-β1 and anti-αVβ3 mAbs (Fig. 2C-F). Inhibition of adhesion to FN and VN was also evidenced by pretreating PC-E1 cells with anti-β1 and anti-αVβ3 mAbs (Fig. S2C-D). Similar to inhibitory efficacy on purified ligands, we found that PC-R1 and PC-E1 cell adhesion to HUVEC and HBMEC-60 cells was reduced by 20-75% with anti-β1 and/or anti-αVβ3 mAbs (Fig. 2G-H and Fig. S2E).

Since Rap1 and Rac1 GTPases are known regulators of β1 and β3 integrin-mediated adhesion on bone-homing HSCs, leukocytes, mesenchymal stem cells (MSCs) and platelets (19, 20, 34), we examined their role in β1 and β3-dependent PCa cell adhesion. We found that β1-dependent PC-R1 cell adhesion to VCAM-1 was reduced by 75% with a Rap1 antagonist, whereas no change was observed.
using a Rac1 antagonist (**Fig. 2I**). Importantly, Rap1 and Rac1 inhibitors did not reduce PC-R1 cell adhesion to FN, COL or VN, nor change β1 or αVβ3 expression or β1 structure as determined by N29, HUTS-21 or 9EG7 mAb reactivity (data not shown). These data collectively indicated that PC-R1 cell adhesion involved constitutively active β1 and αVβ3 integrins, while α4β1 function appeared to be partially regulated by Rap1-GTPase activity.

**Development of a model for molecular analysis of PCa cell adhesion and migration under physiologic shear flow.** To examine the role of β1 and αVβ3 integrins in PCa cell adhesion in the more physiologic context, we needed to generate PC-R1 cells that could stably express E-selectin ligands for initiating adhesion under shear flow. In that PCa cells require E-selectin ligands to bind endothelium in blood flow and characteristically lose E-selectin ligand expression along with α1,3 fucosyltransferases required for synthesizing E-selectin-binding determinants (2-5), we first generated PC-R1 cells stably expressing FT3, 6 or 7. Our prior studies on FT3, 6 and 7 in PCa tissue and in E-selectin ligand+ MDA PCa 2b cells and PC-3 cell transfectants suggested that E-selectin ligand+ PC-R1 cells that express functionally-active integrins would be an ideal model for studying adhesion to and migration through vascular endothelium under physiologic shear stress (4, 5). As such, FT3, 6 or 7 were stably expressed in PC-R1 cells (PC-R1 FT) and resulted in upregulated sLeX levels (**Fig. 3A**). FT expression did not change adhesion to integrin ligands, integrin expression or E-selectin glycoprotein ligand transcription (**Fig. S3A-E**). Overexpression of FT3, FT6 or FT7 in PC-3M LN4 and DU-145 cells also resulted in high sLeX expression (**Fig. S4A**).

In cell binding assays, PC-R1 FT cells and control E-selectin ligand+ KG1a and MDA PCa 2b cells adhered strongly to E-selectin (**Fig. 3B**). Western blot and thin layer chromatographic (TLC) analysis revealed candidate E-selectin-binding determinants on PC-R1 FT glycoproteins and glycolipids (**Fig. S4B-C**). E-selectin-binding glycoproteins were identified previously in related PCa FT+ cells as CD44 (HCELL), carcinoembryonic antigen, melanoma cell adhesion molecule and podocalyxin-like protein (5). Moreover, there were distinct PC-R1 FT glycosphingolipids (GSLs) bearing sialofucosylated moieties reactive to E-selectin/Fc that co-migrated with GM1 and GD3 gangliosides (**Fig. S4C**). Of note, FT3 and
FT6 cell transductants also expressed an E-selectin-reactive GSL that co-migrated with GD1b, which was absent in FT7 cells (Fig. S4C). When glycoproteins were proteolytically removed as evidenced by complete loss of CD44 (Fig. S4D), remaining glycolipids bearing sLeX-E-selectin ligands were detected and accounted for roughly half of total E-selectin binding activity (Fig. S4D). In fact, protease-digested PC-R1 FT cells rolled in equal number and velocity on E-selectin (Fig. S4E-F). In all, these data affirm: (1) our intent to use PC-R1 FT cells as a model for adhesion and TEM analysis under blood flow conditions, (2) highlight the key roles of FT3, 6 and 7 in regulating E-selectin ligand expression and (3) provide direct evidence of the importance of GSLs in E-selectin-dependent adhesion.

E-selectin ligand+ PCa cells breach E-selectin+ HBMEC in a β1, αVβ3 and Rac/Rap1-dependent manner. Using E-selectin ligand+ PC-R1 FT7 cells, we first analyzed whether they could traverse HUVEC or HBMEC-60 cell monolayers stimulated with IL-1β, which induces E-selectin expression (Fig. S4G). We found that TEM of PC-R1 FT7 cell pretreated with neutralizing anti-β1 or anti-αVβ3 mAbs was blocked by 40-80% (Fig. 3C-D). Moreover, RNA silencing of β1 protein (Fig. 3E) reduced TEM of PC-R1 FT7 cells by 50% (Fig. 3F). Inhibitors of Rac1 and Rap1 blocked TEM through HBMEC-60 cells by 30% and 80%, respectively, in contrast to antagonists of CXCR4 and CCR2 or pertussis toxin pretreatment, which did not have any inhibitory effects (Fig. 3G). In agreement with lack of inhibitory effect, expression of CXCR4 and related chemokine receptor, CXCR7, along with CCR2 was undetected or minimal in PC-R1 cells and in a number of PCa cell lines (Fig. S5A), and mRNA levels of CXCR4, CXCR7 and CCR2 along with another known PCa chemokine receptor, CCR4, were variable and/or downregulated in primary and metastatic PCa tissue when compared with normal prostate tissue (Fig. S5B-E). Thus, PC-R1 FT cell TEM under static conditions was dependent on β1 and αVβ3 and Rac1 and Rap1 GTPases though not on G-protein signaling through CXCR4 or CCR2.

We then examined the role of β1 and αVβ3 integrins in adhesion and TEM of PC-R1 FT cells under flow conditions in the parallel-plate flow chamber. In studies using IL-1β-stimulated HBMEC-60 monolayers, flow data revealed that PC-R1 FT3, 6 and 7 tethered and rolled on E-selectin+ HBMEC-60 cell monolayers while E-selectin ligand+ PC-R1 empty cells did not (Fig. 4A-B). This rolling activity was
completely blocked with anti-E-selectin mAb, indicating that E-selectin ligand – E-selectin interactions are critical for initiating PCa cell adhesion under shear flow (Fig. 4A). Following the rolling activity, we then observed a transition to firm adhesion, then cellular piercing of tight EC junctions and incorporation into the endothelial plane, which peaked after a 4hr period (Fig. S6A). This activity is defined here as a ‘Breaching’ process. Phase contrast and confocal fluorescence imaging in 3-dimensions confirmed breached PCa cells (green) adjacent to endothelial cells ((red), indicating paracellular PCa-endothelial breaching (Fig. S6B). Interestingly, PC-R1 FT7 – EC breaching occurred in RPMI medium without serum or exogenous growth factors/chemokines. Notably, all E-selectin ligand+ PC-R1 FT and control MDA PCa 2b cells tethered, rolled, firmly adhered and breached E-selectin+ HBMEC-60 monolayers (Fig. 4C and Movie S1). Conversely, E-selectin ligand− PC-R1 empty or E-selectin+ HBMEC-60 cells neutralized with anti-E-selectin mAb showed no breaching, which underscored E-selectin ligand dependency (Fig. 4C and Movie S2). When anti-β1 and αVβ3 mAbs were added to control MDA PCa 2b and PCR1 FT7 cells in these breaching experiments, there was a significant reduction in breaching activity (p<0.01) (Fig. 4D-E). Furthermore, incubating Rap1 or Rac1 inhibitors with PC-R1 FT7 cells also significantly blocked breaching activity (p<0.01) (Fig. 4F). These results suggested that PCa cells sequentially rolled on HBMEC, transitioned to firm adhesion and breached EC monolayers in an E-selectin ligand-, β1 and αVβ3 integrin-, and Rac1 and Rap1-dependent manner.

**E-selectin ligand+ PC-R1 FT cells traffic to BM via dependency on E-selectin, β1 integrin and Rac1.**

To analyze the role of β1 and αVβ3 integrins and Rap1 and Rac1 GTPases in PCa homing to bone, we generated PC-R1 FT cells stably expressing luciferase and injected them into Rag2/Janus kinase(Jak)-3 null mice deficient in T, B, and NK cells (5). Following PCa cell injection, we assayed for presence of luciferase+ PCa cells in normalizing control tissues (blood and spleen) and in bone by qPCR (5). After 4hr post-injection, we found that all mice injected with E-selectin ligand+ PC-R1 FT3, 6 or 7 cells contained luciferase+ cells in bone, whereas only 25% of mice contained E-selectin ligand− PC-R1 empty cells (Fig. 5A). By pretreating mice with neutralizing anti-E-selectin mAb, PC-R1 FT cell homing to bone was reduced by 40-88% (Fig. 5A). Moreover, after 24 hr post-injection, 95% of femurs contained PC-R1 FT7,
67% PC-R1 FT6, 30% PC-R1 FT3 and only 13% PC-R1 empty cells; and bone retention was ablated by pretreating mice with anti-E-selectin mAb (Fig. 5B). We also found that pretreating PC-R1 cells with anti-β1 mAb blocked PC-R1 FT7 cell retention in bone by 88%, whereas anti-αVβ3 mAb blocked retention by 20% (Fig. 5C). Pretreatment with a Rac1 inhibitor blocked bone retention by 78%, while Rap1 inhibitor blocked by 25% (Fig. 5D). These data showed that E-selectin ligand+ PC-R1 FT cells homed to bone, which was dependent on E-selectin ligands, β1 integrin and Rac1 with minor contributions from αVβ3 integrin and Rap1. Also, FT7 was most influential of all α1,3 FTs in bone homing activity of PCa cells.

**α1,3 FT promote PCa progression.** We and others have demonstrated a key role for α1,3 FTs expression in PCa growth and bone homing activity in vivo (2, 5). To explore the role of α1,3 FTs in spontaneous PCa formation and progression within the prostate gland, we generated TRAMP mice, which develop prostate adenocarcinoma, that were deficient in α1,3 FTs, FT4 and FT7 by targeted gene disruption. In that mice do not express FT3 and FT6 (35) and FT4 does not contribute to sLeX or E-selectin ligand formation in PCa cells, analysis of these mutant mice in terms of sLeX or E-selectin ligand formation was reliant on FT7. We found that TRAMP mice deficient in α1,3 FT activity exhibited a lower incidence of PCa formation (Fig. 6A-B) and lower rate of tumor progression as evidenced by significantly smaller prostate weights (Fig. 6C-D). Unfortunately, observations on metastatic activity in FT4 and 7-deficient TRAMP mice were not possible due to lack of primary tumor formation. As such, data indicated a key role for α1,3 FT in primary PCa development in the prostate gland.
Discussion

Dissemination, entry and growth of cancer cells in distal tissues causes 90% of cancer-related deaths and remains a major unsolved problem in prostate cancer mortality (36). Herein, we identified functional regulators of PCa extravasation, including tethering, firm adhesion and movement into BM endothelium under physiologic blood flow conditions. We described key mechanistic roles for PCa cell α1,3 FT activity and related E-selectin ligand expression, for β1 and αVβ3 integrins, and for Rac1/Rap1 GTPases in PCa cell extravasation (Fig. 7A). We also identified a new role for α1,3 FT activity in PCa formation (Fig. 7B). Interestingly, contrary to evidence on the hallmark role of chemokine receptors in integrin activation, we found that integrin-mediated PCa cell adhesion and migration across BMEC monolayers did not require chemokine(s) as β1 and αVβ3 and GTPases were constitutively active (23-25, 37-39). Our data also confirmed earlier reports whereby α1,3 FT3, 6 and 7 were critical for forming sLeX and corresponding E-selectin ligands and bone-homing activity of metastatic PCa cells (5). Considering our observation that α1,3 FTs, FT4 and FT7, promoted PCa formation in TRAMP mice and FT3 promotion of human PCa growth (40), the collective role of α1,3 FTs, FT3, 6 and 7, may be to aid the exit of PCa cells from circulation through E-selectin ligands and also to generate α1,3 fucose residues that may play a role in intrinsic transforming activity and/or tumor cell – host/stroma interactions promoting tumorigenicity. Analysis of PCa bone metastasis beyond a 24 hr assessment still needs to be conducted to further address α1,3 FTs role in PCa growth in bone. This is the first report describing pleotropic roles of α1,3 fucosylation in malignant progression and metastasis of PCa.

In all, our data parallel the molecular circuitry required for osteotropic activity of HSCs and MSCs, wherein E-selectin ligand+ cells display a greater osteotropism than E-selectin ligand− cells (34, 41, 42). In fact, considering recent data that bone-homing MSCs exhibit a Step-2 bypass pathway whereby chemokine-mediated integrin activation is not required for optimal integrin avidity and TEM activity (34, 41), our data indicated a similar chemokine receptor-independent mechano-signaling circuit for robust PCa cell firm adhesion and vascular breaching. Moreover, PCa cell lines capable of extravasating BMEC, and metastatic PCa tissues, in general, showed variably, low or downregulated levels of chemokine receptors thought involved in PCa metastasis, CXCR4, CXCR7, CCR2 and CCR4.
Extravasating PCa cells relied on E-selectin ligands, β1 and αVβ3 integrins and Rac1/Rap1 GTPase activity without chemokine-mediated integrin activation. While we did not find evidence of E-selectin ligand-dependent activation of β1 and/or αVβ3 integrins or Step-2 bypass pathway, our findings demonstrate that seeding and exiting of circulating PCa cells into bone require the cooperation of α1,3 fucosylation-dependent E-selectin ligands and β1 and αVβ3 integrins for efficient rolling, firm adhesion and TEM activity. Future studies need to address whether these adhesion molecules co-associate on PCa cells to regulate adhesion and movement as observed for α4β1 and CD44 on T cells and MSCs and also whether such molecules regulate extravasation of fresh-isolated, native circulating PCa cells from patients to help rationalize pharmacologic targeting and treatment strategies (34, 43). Moreover, as these studies were performed in immunocompromised mice, the role of immune cells in PCa trafficking requires further investigation.

Interestingly, lymph node-metastatic LNCaP and LNCaP C4-2b cells were least migratory and adhesive due to less active and lower β1 and αVβ3 levels and lacked sialo-LacNAc, a precursor for sLeX and E-selectin ligand formation. Prior data, in fact, show that lymph node PCa had low levels of FT3, 6 and 7 and corresponding sLeX structures compared with PCa in other tissues, notably bone and liver (5), indicating that β1 and αVβ3 integrins and E-selectin ligands may be less important in LN metastasis. Our molecular paradigm outlined here may be more applicable for bone metastasis. Surprisingly, while dermal post-capillary venules express E-selectin, PCa metastasis in skin is rare, suggesting that E-selectin ligand+ PCa cells may not survive or proliferate within skin (44). Thus, while E-selectin ligand+ circulating PCa cells efficiently bind and breach BMEC, growth-related events needed for colonization in bone may be a more critical and less efficient step of bone metastasis that could be investigated over time periods longer than assessed here.

Collectively, we identified multiple adhesion molecules for preventing extravasation of circulating PCa cells into distant tissues. Antagonizing E-selectin ligands, α1,3 FT, β1 and αVβ3 integrins and/or Rap1/Rac1 GTPases could curtail PCa cell homing and extravasation into bone (45). To avert ancillary alteration of homeostatic trafficking activity of HSCs or MSCs, strategic pharmacologic efforts could focus on antagonizing FT3 and FT6 in epithelial-derived tumors or selective blockade of hyperactive β1 variants
on PCa cells. Since FT4 and FT7 are predominant α1,3 FTs in leukocytes, targeting FT3 and 6 would spare altered leukocytic trafficking and tissue distribution patterns. Such a regimen could be further fine-tuned by inclusion of antagonists against activated α2, α5, α6 and αVβ3 structures, which would be selectively expressed on circulating PCa cells not on circulating leukocytes. Sampling and assaying for α1,3FT, E-selectin ligand and β1 expression on localized PCa or circulating PCa cells might help prognosticate metastatic potential and guide treatment intervention.

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References


Figure Legends

**Fig. 1.** PCa PC-E1 and PC-R1 cells traverse BMEC and prominently express β1 and αVβ3. (A-B) Number of cells that underwent TEM (n=9 ± SEM. *, P<0.05; **, P<0.01, vs. PC-3M LN4; one-way ANOVA with Dunnett post test). (C) Anti-integrin Ab (Open histogram) or isotype (Shaded histogram); representative of n=3. (D) Predicted integrin heterodimers on PC-E1 and PC-R1 cells based on flow cytometric data of integrin subunits.

**Fig. 2.** β1 and αVβ3 integrins are constitutively active and mediate PC-R1 cell adhesion to BMEC. (A) % PC-R1 cell adhesion to integrin ligands (n=9 ± SEM). (B) Anti-β1 activation-sensitive Abs (Open histogram) or isotype (Shaded histogram); (n=3). (C-I) PC-R1 cell adhesion to integrin ligands or EC monolayers in the presence of blocking or isotype Abs (n=9 ± SEM. *, P<0.05; **, P<0.01, vs. isotype or untreated; one-way ANOVA with Dunnett post test).

**Fig. 3.** β1 and αVβ3 integrins and Rac/Rap1 GTPases regulate TEM of E-selectin ligand+ PC-R1 cells through BMEC. (A) Anti-sLeX Ab (Open histogram) or isotype (Shaded histogram); representative of n=3. (B) Adhesion of PC-R1 empty or FT cells along with positive control PCa MDA PCa 2b cells and KG1a cells to E- or P-selectin chimeras or to Fc control (n=9 ± SEM). (C-D) TEM in the presence or absence of blocking anti-β1 or αVβ3 mAbs or of isotype control Abs (n=16 ± SEM, 4 experiments. **, P<0.01; ***, P<0.001, vs. isotype; one-way ANOVA with Dunnett post test). (E) FACS analysis of β1 in Scrambled (Scr) or β1 knockdown (KD) PC-R1 FT7 cells. Scr (Open histogram, bold), β1 KD (Open histogram, dotted) and isotype Ab (Shaded histogram). Mean fluorescence (n=3 ± SEM. ***, P<0.001, t-test). (F) TEM of PC-R1 FT7 (Scr) or β1 KD (n=3 ± SEM. ***, P<0.001, t-test). (G) TEM of untreated or inhibitor-treated PC-R1 FT7 cells (n=9 ± SEM. **, P<0.01; ***, P<0.001, one-way ANOVA with Dunnett post test).

**Fig. 4.** E-selectin-E-selectin ligands, β1 and αVβ3 integrins, and Rac1/Rap1 GTPases cooperate in PC-R1 cell breaching of BMEC under flow conditions. (A) Mean PC-R1 FT cell rolling events and (B) rolling velocity on IL-1β stimulated HBMEC-60 cells at 1 Dyne/cm² +/- anti-E-selectin mAb from 4-fields at 100X magnification (n=9 ± SEM. ND, not detected. ***, P<0.001 vs. isotype, one-way ANOVA with Dunnett post test).
ANOVA with Dunnett post test). (C-F) % PC-R1 FT and MDA PCa 2b cell breaching of HBMEC-60 cell monolayers +/- anti-E-selectin mAb, anti-integrin mAb, Rap1 inh. or Rac1 inh. normalized to isotype or untreated control (n=4 ± SEM, **, P<0.01; ***, P<0.001, t-test).

**Fig. 5.** E-selectin ligand+ PC-R1 FT cell dissemination to bone is dependent on E-selectin ligands, β1 and Rac1. (A-D) % incidence of Luciferase+ PC-R1 cells in bone at 4 or 24 hrs post-intracardiac injection as determined by PCR analysis of luciferase; *, P<0.05; **P<0.01; ***P<0.001, contingency table with two-tailed Fisher’s test.

**Fig. 6.** α1,3 FT4 and 7 are pro-tumorigenic in TRAMP mice. TRAMP mice wt (+/+), heterozygous (+/-) homozygous null (-/-) for FT4 and FT7 expression were generated and evaluated for primary tumor incidence and size (prostate weight) at 18 and 23 weeks. A and B; *, P=0.0361; **, P= 0.0051, contingency table with two-tailed Fisher’s test. C and D; *, P<0.05; **, P<0.01, one-way ANOVA with Dunnett post test.

**Fig. 7.** Model of PCa progression and extravasation to bone. (A) Model of PCa cell extravasation into bone. (STEP 1) α1,3 FTs, FT3, 6 and 7, catalyze the synthesis of sLeX on membrane glycoproteins and neolactosphingolipids to promote corresponding E-selectin ligand activity on PCa cells. E-selectin ligand+ PCa cells roll on BMEC E-selectin. Constitutively active β1 due partly to Rap1-GTPase activity and active αVβ3 integrins mediate (STEP 2) firm adhesion to putative integrin ligands FN, VN and VCAM-1 on BMEC. (STEP 3) PCa cells traverse BMEC junctions driven by Rap1 and Rac1 GTPase activity along with β1 and αVβ3 binding to putative surface and basement membrane integrin ligands, FN, VN, VCAM-1, LN and COL. (B) Development of PCa lesions in the prostate secretory and neuroendocrine cell layer is promoted by FT4 and FT7 expression.
Figure 1

A. HUVEC

B. HBMEC-60

C. Potential $\alpha/\beta$ pairings

D. $\alpha_2$, $\alpha_5$, $\alpha_6$, $\alpha_V$, $\alpha_V\beta_3$
Figure 2

A. PC-R1

B. β1 activation state

C. PC-R1

D. PC-R1

E. PC-R1

F. PC-R1

G. PC-R1

H. PC-R1

I. PC-R1

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Figure 6

A. 18 weeks

% Tumor Incidence

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B. 23 weeks

% Tumor Incidence

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C. 18 weeks

Prostate Weight (mg)

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D. 23 weeks

Prostate Weight (mg)

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Steven R. Barthel, Danielle Hays, Erika M. Yazawa, et al.

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