Definition of Molecular Determinants of Prostate Cancer Cell Bone Extravasation

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Bone Extravasation
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Introduction
Metastatic prostate cancer killed 33,720 men in 2011, and the prognosis with bone metastasis is poor (1). Unfortunately, how prostate cancer cells breach bone marrow microvessels is ill-defined. Accordingly, we established an adhesion paradigm wherein prostate cancer cells roll on bone marrow endothelium (BMEC) under physiologic hemodynamic flow by recognizing BMEC endothelial (E)-selectin (2–5). This led to the premise that bone metastasis is conferred by prostate cancer cell E-selectin–BMEC E-selectin binding and has been supported by several observations. First, human bone marrow microvessels express E-selectin constitutively (6). Second, E-selectin-binding sLe\textsuperscript{x} membrane glycoproteins and glycolipids, defined operationally as E-selectin ligands, and regulators of E-selectin ligand synthesis, \(\alpha\)1.3 fucosyltransferases in transgenic adena of mouse prostate mice dramatically reduced prostate cancer incidence. These results unify the requirement for E-selectin ligands, \(\alpha\)1.3 fucosyltransferases, \(\beta1\) and \(\alpha\)V\(\beta\)3 integrins, and Rac1 GTPases in mediating prostate cancer cell homing and entry into bone and offer new insight into the role of \(\alpha\)1.3 fucosylation in prostate cancer development. Cancer Res; 73(2); 1–11. ©2012 AACR.

Abstract
Advanced prostate cancer commonly metastasizes to bone, but transit of malignant cells across the bone marrow endothelium (BMEC) remains a poorly understood step in metastasis. Prostate cancer cells roll on E-selectin–BMEC through E-selectin ligand-binding interactions under shear flow, and prostate cancer cells exhibit firm adhesion to BMEC via \(\beta1\), \(\beta4\), and \(\alpha\)V\(\beta\)3 integrins in static assays. However, whether these discrete prostate cancer cell–BMEC adhesive contacts culminate in cooperative, step-wise transendothelial migration into bone is not known. Here, we describe how metastatic prostate cancer cells breach BMEC monolayers in a step-wise fashion under physiologic hemodynamic flow. Prostate cancer cells tethered and rolled on BMEC and then firmly adhered to and traversed BMEC via sequential dependence on E-selectin ligands and \(\beta1\) and \(\alpha\)V\(\beta\)3 integrins. Expression analysis in human metastatic prostate cancer tissue revealed that \(\beta1\) was markedly upregulated compared with expression of other \(\beta\) subunits. Prostate cancer cell breaching was regulated by Rac1 and Rap1 GTPases and, notably, did not require exogenous chemokines as \(\beta1\), \(\alpha\)V\(\beta\)3, Rac1, and Rap1 were constitutively active. In homing studies, prostate cancer cell trafficking to murine femurs was dependent on E-selectin ligand, \(\beta1\) integrin, and Rac1. Moreover, eliminating E-selectin ligand-synthesizing \(\alpha\)1.3 fucosyltransferases in transgenic adena of mouse prostate mice dramatically reduced prostate cancer incidence. These results unify the requirement for E-selectin ligands, \(\alpha\)1.3 fucosyltransferases, \(\beta1\) and \(\alpha\)V\(\beta\)3 integrins, and Rac/Rap1 GTPases in mediating prostate cancer cell homing and entry into bone and offer new insight into the role of \(\alpha\)1.3 fucosylation in prostate cancer development.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-12-3264
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Flow cytometry

Flow cytometry was conducted as described (4), antibody concentrations were 10 μg/mL, and conformation anti-β1 antibodies, N29, HUTS-21, and 9E7 were incubated 1 hour at 37°C.

Real-time PCR

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

Western blotting

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

Prostate cancer rolling and breaching in flow

Rolling frequency and velocity was measured as described (2, 5, 31). For breaching through endothelial cells (EC), prostate cancer cells (1 × 10⁶ cells in RPMI or endothelial growth media) were infused at 1.5 mL/min for 35 seconds, and a physiologic shear stress of 0.5 Dynes/cm² was maintained for 4 hours. Photographs were taken every second for the first 4 minutes to capture rolling and then every 5 minutes to capture adhesion and breaching. Temperature was maintained at 37°C via heat fan and water bath. Breaching, defined as a prostate cancer cell piercing and penetrating the EC layer, was also investigated with BioFlux microfluidics technologies (Fluxion Biosciences). Where indicated, prostate cancer cells and ECs were loaded with 0.5 μmol/L 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 2-tailed t test, one-way ANOVA with Dunnett posttest, or contingency table on GraphPad Prism (GraphPad Software).

Results

Prostate cancer PC-E1 and PC-R1 cells traverse BMEC and prominently express β1 and αvβ3 integrins

To identify prostate cancer cells with robust TEM activity for subsequent molecular analysis, we first examined the relative efficiency of TEM using a number of metastatic prostate cancer cell lines. Using a static approach and a 16-hour incubation period, we found that bone metastatic PC-E1 and PC-R1 cells showed a higher capacity than other prostate cancer cells to breach confluent human umbilical vein endothelial cell (HUVEC) and HBMEC-60 monolayers, traverse 8 micron pores, and attach to insert undersides (Fig. 1A and 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 prostate cancer tissue confirmed that β1 transcript level was markedly higher than all other β subunits and, at minimum, several 100-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.
Figure 1. Prostate cancer 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 posttest). C, anti-integrin antibody (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.

Candidate α partners for β1 on PC-E1 and PC-R1 included α2, α5, α6, and αV, and for β3, αV (Fig. 1C and D). αVβ3 Heterodimer was present on PC-E1 and PC-R1 cells, whereas 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 (Supplementary Fig. S1C). Of note, α4 was upregulated on PC-E1 cells, the first evidence of α4β1 on a prostate cancer cell (Fig. 1C). Data underscored 4 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 prostate cancer cell lines and found that β1 and αVβ3 were prominently expressed on bone-metastatic prostate cancer PC-3M LN4, PC-3, and MDA prostate cancer 2b cell lines and on the brain-metastatic prostate cancer DU-145 cell line, though reduced on lymph node-metastatic LNCaP and LNCaP C4-2b cells (Supplementary Fig. S1C). These results suggested that high expression of β1 and upregulation of αVβ3 correlated with prostate cancer bone and brain metastasis.

β1 and αVβ3 integrins on prostate cancer 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 prostate cancer cell lines (Supplementary Fig. S2). Unexpectedly, PC-R1 and PC-E1 cells also adhered strongly to VCAM-1, which is the first demonstration of VCAM-1–dependent prostate cancer cell adhesion (Fig. 2A and Fig. S2A). As expected, PC-R1 cells, including all other prostate cancer cell lines, did not adhere to β2 ligand, ICAM-1 (Fig. 2A and Supplementary 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 prostate cancer cell lines and their reactivity to these conformation-sensitive antibodies closely correlated with their adhesiveness to β1 and αVβ3 integrin ligands (Supplementary Fig. S2B). In agreement with this finding, PC-R1 cell adhesion to VCAM-1 and to COL was completely blocked by anti-β1 mAb, whereas adhesion to FN or VN was significantly blocked with both anti-β1 and anti-αVβ3 mAbs.
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 (Supplementary Fig. S2C and 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% to 75% with anti-β1 and/or anti-αVβ3 mAbs (Fig. 2G and H and Supplementary Fig. S2E).

Because Rap1 and Rac1 GTPases are known regulators of β1 and β3 integrin-mediated adhesion on bone-homing HSCs, leukocytes, mesenchymal stem cells (MSC), and platelets (19, 20, 34), we examined their role in β1 and β3-dependent prostate cancer 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, whereas α4β1 function seemed to be partially regulated by Rap1-GTPase activity.

Development of a model for molecular analysis of prostate cancer cell adhesion and migration under physiologic shear flow

To examine the role of β1 and αVβ3 integrins in prostate cancer 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, prostate cancer cells require E-selectin ligands to bind endothelium in blood flow and characteristically lose E-selectin ligand expression along with α1,3 FTs 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 prostate cancer tissue and in E-selectin ligand+ MDA prostate cancer 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 (Supplementary Fig. S3A–E). Overexpression of FT3, FT6, or FT7 in PC-3M LN4 and DU-145 cells also resulted in high sLeX expression (Supplementary Fig. S4A).

In cell-binding assays, PC-R1 FT cells and control E-selectin ligand+ KG1a and MDA prostate cancer 2b cells adhered strongly to E-selectin (Fig. 3B). Western blot and thin layer chromatographic analysis revealed candidate E-selectin–binding determinants on PC-R1 FT glycoproteins and glycolipids (Supplementary Fig. S4B–C). E-selectin–binding glycoproteins were identified previously in related prostate cancer FT+ cells.

Figure 3. β1 and αvβ3 integrins and Rac/Rap1 GTPases regulate TEM of E-selectin ligand+ PC-R1 cells through BMEC. A, anti-sLeX antibody (open histogram) or isotype (shaded histogram); representative of n = 3. B, adhesion of PC-R1 empty or FT cells along with positive control prostate cancer (PCa) MDA PCa 2b cells and KG1a cells to E- or P-selectin chimeras or to Fc control (n = 9 ± SEM). C and D, TEM in the presence or absence of blocking anti-β1 or αvβ3 mAbs or of isotype control antibodies (n = 16 ± SEM, 4 experiments; ***, P < 0.01; ****, P < 0.001, vs. isotype; one-way ANOVA with Dunnett posttest). E, fluorescence-activated cell sorting 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 antibody (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 posttest).
as CD44 (HCELL), carcinoembryonic antigen, melanoma cell adhesion molecule, and podocalyxin-like protein (5). Moreover, there were distinct PC-R1 FT glycosphingolipids (GSL) bearing sialofucosylated moieties reactive to E-selectin/Fc that comigrated with GM1 and GD3 gangliosides (Supplementary Fig. S4C). Of note, FT3 and FT6 cell transductants also expressed an E-selectin–reactive GSL that comigrated with GD1b, which was absent in FT7 cells (Supplementary Fig. S4C). When glycoproteins were proteolytically removed as evidenced by complete loss of CD44 (Supplementary Fig. S4D), remaining glycolipids bearing sLeX-E-selectin ligands were detected and accounted for roughly half of total E-selectin binding activity (Supplementary Fig. S4D). In fact, protease-digested PC-R1 FT cells rolled in equal number and velocity on E-selectin (Supplementary Fig. S4E and S4F). In all, these data affirm: (i) our intent to use PC-R1 FT cells as a model for adhesion and TEM analysis under blood flow conditions, (ii) highlight the key roles of FT3, 6, and 7 in regulating E-selectin ligand expression, and (iii) provide direct evidence of the importance of GSLs in E-selectin–dependent adhesion.

**E-selectin ligand** prostate cancer 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 (Supplementary Fig. S4G). We found that TEM of PC-R1 FT7 cell pretreated with neutralizing anti-β1 or anti-αvβ3 mAbs was blocked by 40% to 80% (Fig. 3C and 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 prostate cancer cell lines (Supplementary Fig. S5A), and mRNA levels of CXCR4, CXCR7, and CCR2 along with another known prostate cancer chemokine receptor, CCR4, were variable and/or downregulated in primary and metastatic prostate cancer tissue when compared with normal prostate tissue (Supplementary Fig. S5B–S5E). Thus, PC-R1 FT cell TEM under static conditions was dependent on β1 and αvβ3 and Rac1 and Rap1 GTPases, although 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, whereas E-selectin ligand** PC-R1 empty cells did not (Fig. 4A and B). This rolling activity was completely blocked...
with anti-E-selectin mAb, indicating that E-selectin ligand–E-selectin interactions are critical for initiating prostate cancer 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 4-hour period (Supplementary Fig. S6A). This activity is defined here as a "Breaching" process. Phase contrast and confocal fluorescence imaging in 3 dimensions confirmed breached prostate cancer cells (green) adjacent to endothelial cells [red, indicating paracellular prostate cancer-endothelial breaching (Supplementary 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 prostate cancer 2b cells tethered, rolled, firmly adhered, and breached E-selectin⁺ HBMEC-60 monolayers (Fig. 4C and Supplementary 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 Supplementary Movie S2). When anti-β1 and αVβ3 mAbs were added to control MDA prostate cancer 2b and PCRI FT7 cells in these breaching experiments, there was a significant reduction in breaching activity (P < 0.01; Fig. 4D and 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 prostate cancer 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 bone marrow via dependency on E-selectin, β1 integrin, and Rac1**

To analyze the role of β1 and αVβ3 integrins and Rap1 and Rac1 GTPases in prostate cancer 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 natural killer (NK) cells (5). Following prostate cancer cell injection, we assayed for presence of luciferase⁺ prostate cancer cells in normalizing control tissues (blood and spleen) and in bone by quantitative PCR (5). After 4 hours postinjection, 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% to 88% (Fig. 5A). Moreover, after 24 hours postinjection, 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%, whereas 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

![Figure 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 hours postintracardiac injection as determined by PCR analysis of luciferase; *, P < 0.05; **, P < 0.01; ***, P < 0.001, contingency table with 2-tailed Fisher test.](cancerres.aacrjournals.org)
αVβ3 integrin and Rap1. Also, FT7 was most influential of all α1,3 FTs in bone homing activity of prostate cancer cells.

**α1,3 FTs promote prostate cancer progression**

We and others have shown a key role for α1,3 FT expression in prostate cancer growth and bone homing activity in vivo (2, 5). To explore the role of α1,3 FTs in spontaneous prostate cancer 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 sLeα or E-selectin ligand formation in prostate cancer cells, analysis of these mutant mice in terms of sLeα 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 prostate cancer formation (Fig. 6A and B) and lower rate of tumor progression as evidenced by significantly smaller prostate weights (Fig. 6C and 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 prostate cancer 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). Here, we identified functional regulators of prostate cancer extravasation, including tethering, firm adhesion, and movement into bone marrow endothelium under physiologic blood flow conditions. We described key mechanistic roles for prostate cancer cell α1,3 FT activity and related E-selectin ligand expression, for β1 and αVβ3 integrins, and for Rac1/Rap1 GTPases in prostate cancer cell extravasation (Fig. 7A). We also identified a new role for α1,3 FT activity in prostate cancer formation (Fig. 7B). Interestingly, contrary to evidence on the hallmark role of chemokine receptors in integrin activation, we found that integrin-mediated prostate cancer 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 sLeα and corresponding E-selectin ligands and bone-homing activity of metastatic prostate cancer cells (5). Considering our observation that α1,3 FTs, FT4 and FT7, promoted prostate cancer formation in TRAMP mice and FT3 promotion of human prostate cancer growth (40), the collective role of α1,3 FTs, FT3, 6, and 7, may be to aid the exit of prostate cancer 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 prostate cancer bone metastasis beyond a 24-hour assessment still needs to be conducted to...
further address the role of α1,3 FTs in prostate cancer growth in bone. This is the first report describing pleotropic roles of α1,3 fucosylation in malignant progression and metastasis of prostate cancer.

In all, our data parallel the molecular circuitry required for osteotropic activity of HSCs and MSCs, wherein E-selectin ligand-dependent cells display a greater osteotropism than E-selectin ligand-independent 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 mechanosignaling circuit for robust prostate cancer cell firm adhesion and vascular breaching. Moreover, prostate cancer cell lines capable of extravasating BMEC, and metastatic prostate cancer tissues, in general, showed variably low or downregulated levels of chemokine receptors thought involved in prostate cancer metastasis, CXCR4, CXCR7, CCR2 and CCR4. Extravasating prostate cancer 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 binding or Step-2 bypass pathway, our findings show that seeding and exiting of circulating prostate cancer 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 coassociate on prostate cancer 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 prostate cancer cells from patients to help rationalize pharmacologic targeting and treatment strategies (34, 43). Moreover, as these studies were conducted in...
immunocompromised mice, the role of immune cells in prostate cancer 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 prostate cancer had low levels of FT3, 6, and 7 and corresponding sLeX structures compared with prostate cancer in other tissues, notably bone and liver (5), indicating that β1 and αvβ3 integrins and E-selectin ligands may be less important in lymph node metastasis. Our molecular paradigm outlined here may be more applicable for bone metastasis. Surprisingly, while dermal postcapillary venules express E-selectin, prostate cancer metastasis in skin is rare, suggesting that E-selectin ligand 1 prostate cancer cells may not survive or proliferate within skin (44). Thus, while E-selectin ligand 1 circulating prostate cancer cells efficiently bind and break 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 prostate cancer cells into distant tissues. Antagonizing E-selectin ligands, αv13 FT, β1 and αvβ3 integrins and/or Rap1/Rac1 GTPases could curtail prostate cancer 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 prostate cancer cells. Because FT4 and FT7 are predominant αv13 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, α3, α6, and αvβ3 structures, which would be selectively expressed on circulating prostate cancer cells not on circulating leukocytes.

Sampling and assaying for α13FT, E-selectin ligand, and β1 expression on localized prostate cancer or circulating prostate cancer cells might help prognosticate metastatic potential and guide treatment intervention.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors thank Dr. Ronald Schnaar for supporting efforts in prostate cancer glycolipid analyses (Johns Hopkins University, Baltimore, MD).

Grant Support
This work was supported by NIH/NCI grant (R01 CA118124; to C. Dimitroff), NIH/NCI grant (R01 AT004260; to C. Dimitroff), American Cancer Society Postdoctoral Fellowship (10–227 to S. Barthel), NIH Kirschstein-NRSA Postdoctoral Fellowship (F32 CA144219-01A1; to S. Barthel), Dermatology Foundation Research Grant (A05022 to S. Barthel), Fulbright/CAPES and CNPq to L. Nimrichter, NIH/NCI grant (R01 CA96357; to B. Foster), and Roswell Park Cancer Institute and NIH/NCI grant (P30 CA016506). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 16, 2012; revised October 4, 2012; accepted October 24, 2012; published OnlineFirst November 13, 2012.

References


Definition of Molecular Determinants of Prostate Cancer Cell Bone Extravasation


Cancer Res  Published OnlineFirst November 13, 2012.