Clinical Cancer Research

Aberrant Presentation of HPA-Reactive Carbohydrates Implies Selectin-Independent Metastasis Formation in Human Prostate Cancer

Tobias Lange, Mareike Kupfernagel, Daniel Wicklein, et al.

Updated version	Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-13-2308
Supplementary Material	Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2014/02/14/1078-0432.CCR-13-2308.DC1.html

Cited Articles	This article cites by 46 articles, 17 of which you can access for free at: http://clincancerres.aacrjournals.org/content/20/7/1791.full.html#ref-list-1

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

Human Cancer Biology

Aberrant Presentation of HPA-Reactive Carbohydrates Implies Selectin-Independent Metastasis Formation in Human Prostate Cancer

Tobias Lange[1], Mareike Kupfernagel[1], Daniel Wicklein[1], Florian Gebauer[1,3], Hanna Maar[1], Kathrin Brügge[1], Imke Müller[1], Ronald Simon[2], Thorsten Schlomm[4], Guido Sauter[2], and Udo Schumacher[1]

Abstract

Purpose: To investigate the impact of prostate cancer cell surface glycosylation as part of the tumor cell–endothelial cell interaction in prostate cancer metastasis.

Experimental Design: Glycosyltransferase expression was profiled in metastasis-derived prostate cancer cell lines and compared with primary epithelium. Prostate cancer cells were examined for HPA- and selectin-binding and adhesion to endothelium. Spontaneous metastasis xenograft models were established to test the lectin HPA-binding sites as a marker of metastatic competence and to evaluate E-selectin-binding sites *in vivo*. The importance of selectins for metastasis formation was analyzed using $Sele^{-/-}/Selp^{-/-}$ mice. The clinical relevance of HPA- and E-selectin-binding sites in prostate cancer was determined.

Results: Glycosyltransferases involved in the synthesis of common HPA-binding sites are downregulated in prostate cancer cells. An absence of HPA-reactive carbohydrates specifically indicates spontaneous metastatic spread of prostate cancer xenografts *in vivo* and a poor prognosis of patients with prostate cancer. HPA-binding sites decrease in lymph node metastases compared with corresponding primary tumors. Common selectin ligands are absent on prostate cancer cells, which do not adhere to recombinant selectins or endothelium under shear stress *in vitro*. Spontaneous metastasis formation is largely independent of selectins *in vivo*. E-selectin-binding sites are detectable in only 2% of patients with prostate cancer without prognostic significance.

Conclusion: Prostate cancer is characterized by an inverse functional and prognostic importance of HPA-binding sites compared with other adenocarcinomas. Accordingly, this study surprisingly shows that the selectin–selectin ligand axis, which is essential for extravasation and thus metastasis formation in several malignancies, can be circumvented in prostate cancer. *Clin Cancer Res; 20(7); 1791–802. ©2014 AACR.*

Introduction

Prostate cancer is the predominant neoplasm in males and represents the second leading cause of cancer-related deaths in men (1). As with all other cancers, it is the development of distant metastases, which is responsible for the majority of prostate cancer–associated deaths. During the multistep process of metastatic spread, primary tumor cells are interacting with their microenvironment via their glycocalyx (2), which is commonly aberrantly composed in carcinoma cells compared with their normal counterparts (3). This altered cell surface glycosylation, which has widely been explained by an altered glycosyltransferase expression (4), is often associated with invasion and metastasis (5). In particular, increased cell surface presentation of terminal N-acetylgalactosamine- (GalNAc-) and N-acetylglucosamine- (GlcNAc)-residues correlate with progression and metastasis in breast and colorectal cancer as determined by the specific binding of the lectin *Helix pomatia* agglutinin (HPA) toward these terminal glycoconjugates. Hence, HPA has been shown to be a marker of metastatic competence in human breast and colorectal xenograft primary tumors in severe combined immunodeficient (SCID) mice (2) and of a poor patient prognosis in these malignancies (6, 7) as well as in adenocarcinoma of the lung (8), gastric cancer (9), and malignant melanoma (10). HPA-reactive carbohydrates are typical for two of the most prominent *O*-glycans in cancer, namely Tn antigen and core 2 *O*-glycans (4, 11), which are synthesized through

Authors' Affiliations: Institutes of [1]Anatomy and Experimental Morphology and [2]Pathology, University Cancer Center Hamburg, [3]Department of General, Visceral and Thoracic Surgery, and [4]Martini-Clinic, Prostate Cancer Center, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

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

T. Lange and M. Kupfernagel contributed equally to this work and share first authorship.

Corresponding Author: Tobias Lange, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, Hamburg 20246, Germany. Phone: 0049-40-7410-52591; Fax: 0049-40-7410-55427; E-mail: to.lange@uke.de

Translational Relevance

This is the first study applying spontaneous metastasis xenograft models of human prostate cancer to test the functional relevance of aberrant cell surface glycans and downstream molecules of the leukocyte adhesion cascade for prostate cancer metastasis. Carbohydrate residues recognized by the lectin *Helix pomatia* agglutinin (HPA), which predict metastasis formation and a poor prognosis in other malignancies, are surprisingly absent in highly metastatic prostate cancer xenografts and decrease in clinical lymph node metastases. Corresponding to the fact that such carbohydrates are common intermediates for selectin ligand synthesis, prostate cancer metastasis formation is largely independent of selectins. These findings are also reflected by a beneficial prognosis of patients with HPA-positive prostate cancers and by a particularly low incidence of E-selectin-binding sites in prostatectomy specimens underlining the translational relevance of our model. Moreover, this study indicates a particular importance of adhesion partners other than selectins that accomplish the unique selectin-independent extravasation in this malignancy.

the sequential activity of polypeptide GalNAc-transferases (pp-GalNac-T's), core 1 synthase (C1GALT1) and core 2 synthases (C2GNT1,2,3), respectively (Fig. 1A). Importantly, as summarized in Fig. 1A, these glycans are common intermediates for the synthesis of sialylated Lewis X and A antigens (sLe^X and sLe^A; ref. 12), which are presented at the surface of human breast and gastrointestinal adenocarcinoma (13–15) as well as leukemia cells (16). sLeX and sLeA are the main ligands for E- and P-selectin and therefore crucially involved in the adhesion cascade of leukocytes into inflamed tissues (17). Our hypothesis is that circulating tumor cells (CTC) imitate this adhesion cascade involving selectin-, integrin- and chemokine-mediated interactions to migrate into host organs of distant metastases as well (refs. 18–20; Fig. 1A, bottom). Accordingly, spontaneous metastasis formation from xenograft primary tumors of human breast, colorectal (HT29), and pancreatic cancer (PaCa5061) drastically decreases in $Sele^{-/-}/Selp^{-/-}$ SCID and $Pfp^{-/-}/Rag2^{-/-}$ mice (13–15). Likewise, engraftment and organ invasion of human eosinophilic leukemia cells (EOL-1) are remarkably reduced in selectin-deficient mice (16). Furthermore, the importance of HPA-reactive glycoconjugates as intermediates of selectin ligand synthesis has also been shown previously, as preincubation of human breast cancer cells with HPA inhibits their adhesion toward vascular endothelium (7, 21).

In prostate cancer, we recently established spontaneous metastasis xenograft models and identified abnormally presented β (1, 6) branched oligosaccharides as a marker of metastatic behavior *in vivo* and elevated prostate specific antigen (PSA) values in patients (22). The impact of HPA- and selectin-binding sites for metastasis formation and

patient prognosis in prostate cancer, however, has so far not been analyzed in detail. We therefore aimed to determine glycosylation patterns in prostate cancer with a particular focus on HPA-reactive carbohydrates and selectin ligands (including their potential relevance for prostate cancer adhesion to selectins/endothelium), to test HPA as a marker of metastatic competence in prostate cancer xenograft models and as a prognostic factor in clinical prostate cancer, to analyze E-selectin binding sites in prostate cancer xenograft tumors, and in patients and to determine whether selectin binding is essential for metastasis formation in prostate cancer.

Materials and Methods

Cell lines and culture conditions

PC-3, DU-145 (prostate cancer), PPEC (human primary, nonmalignant prostate epithelial cells), HT29 (colon cancer), EOL-1 (eosinophilic leukemia), and PaCa5061 (pancreatic adenocarcinoma) were used as described before (refs. 14, 22; see also Supplementary Table S1; refs. 16, 23). VCaP (prostate cancer) cells were obtained from American Type Culture Collection and cultured in RPMI-1640 supplemented with 2 mmol/L L-glutamine, 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (all Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2 (24). Human pulmonary microvascular endothelial cells (HPMEC, Supplementary Table S1; refs. 25–27) were from PromoCell and cultured in endothelial cell growth medium MV supplemented with the corresponding supplement mix (PromoCell). All experiments with primary cells were performed during the first six passages.

Quantitative real-time-PCR glycosylation array

RNA isolation and cDNA synthesis from cell culture grown PC-3, DU-145, VCaP, and PPEC was performed as described (22); expression of glycosyltransferases was assessed using the Human Glycosylation RT^2 Profiler PCR array including 84 glycosyltransferase and glycosidase genes (Qiagen). All arrays were repeated twice with independently isolated RNAs.

HPA-binding flow cytometry and lectin histochemistry

Tumor cells were detached and incubated for 30 minutes at 4°C with fluorescein isothiocynate (FITC)-conjugated HPA (Sigma) diluted 1:100 in lectin buffer (0.05 mol/L TRIS-bufferd saline, pH 7.6, added with 1 mmol/L $CaCl_2$ and $MgCl_2$). Binding specificity was evaluated by inhibiting HPA with 100 mmol/L D-GalNAc (Sigma) before incubation. All samples were washed once, marked dead or alive by propidium iodide staining (Sigma), and subjected to flow cytometry (FACS) using a CyFlow Cube cytometer (Partec). Data were analyzed using CyView software (Partec).

Xenograft primary tumors of all prostate cancer cell lines, HT 29 and PaCa5061 as well as prostate cancer prognosis and heterogeneity tissue microarrays (see below) were evaluated for HPA-binding sites by a standard lectin histochemistry in accordance with several previous studies in
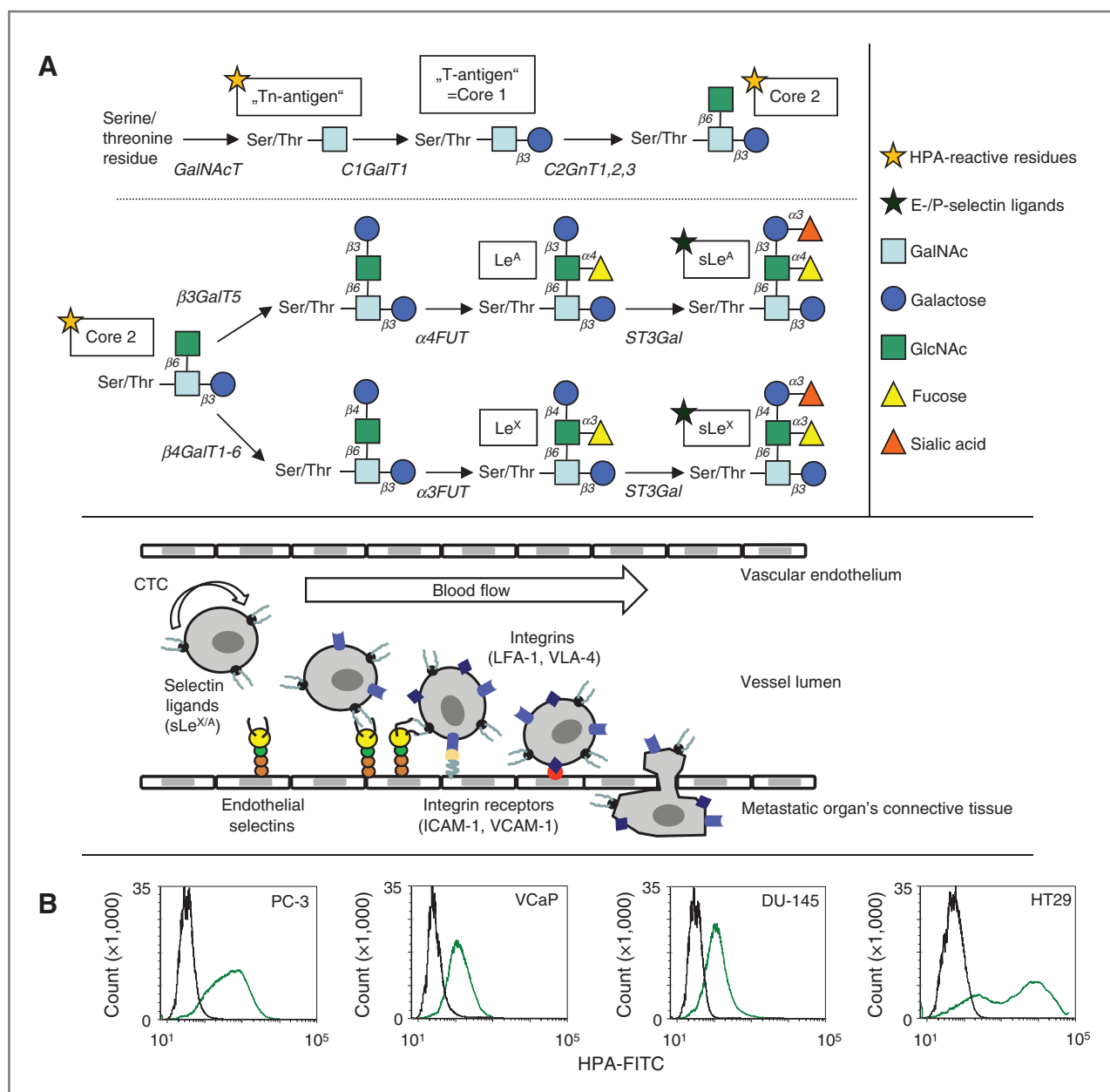
Figure 1. *O*-glycan biosynthesis, HPA-reactive sugar residues and synthesis of selectin ligands. A, representation of *O*-glycosylation pathways relevant to the study (35). Tn-antigen and core 2 *O*-glycans present terminal GalNAc or GlcNAc and are specifically recognized by the lectin *Helix pomatia* agglutinin (HPA; refs. 11, 46). The core 2 structure is a critical scaffold for the production of the main selectin ligands sLe^A and sLe^X (top; ref. 12). Selectin ligands are involved in the initiation of flow adhesion of leukocytes to vascular endothelium during inflammation. There is an increasing body of evidence indicating that these pathways are mimicked by CTCs for extravasation at a distant site during metastasis formation (17). Several molecules downstream of selectins in this cascade such as integrins and chemokines (not illustrated) are relevant as well (lower). B, in accordance with the glycosyltransferase expression changes summarized in Table 1, prostate cancer (PCa) cells show a moderate (PC-3) to weak (VCaP, DU-145) HPA binding compared with HT29 colon cancer cells (2). Black histograms represent HPA binding after inhibition with D-GalNAc. GalNAc: N-acetylgalactosamine; GlcNAc: N-acetylglucosamine; (s)Le$^{A/X}$: (sialylated) Lewis$^{A/X}$-antigen.

different human adenocarcinomas (2, 6–10). Briefly, tissue sections were treated overnight with xylol, deparaffinized, and pretreated with 0.1% trypsin (obtained as trypsin powder from Biochrom, substance activity 1512 USP U/mg) in lectin buffer for 15 minutes at 37°C. Afterward, sections were incubated with biotinylated HPA (Sigma) and

stained as described before (22). Again, 100 mmol/L D-GalNAc was used for inhibition of HPA on parallel sections.

Subcutaneous xenograft mouse models

Male *Pfp/Rag2*$^{-/-}$ double-knockout mice (8–12 weeks, 20–25 g) from Taconic were used as described (22).

Animals were maintained under pathogen-free conditions in individually ventilated cages and fed with sterile standard food and water *ad libitum*. All animal experiments were approved by the local animal experiment approval committee (project No. G08/75). PC-3 and DU-145 cells were xenotransplanted as described (22). This study firstly describes the use of VCaP cells as a suitable model of metastatic prostate cancer. For VCaP tumor growth, it was necessary to mix 1×10^6 cells 1:2 with Matrigel (BD Biosciences) immediately before injection. $Pfp/Rag2^{-/-}$ mice were crossbred with E- and P-selectin–deficient mice (Jackson Laboratory, stock 002916) and selectin deficiency was verified as described (13).

When primary tumors exceeded 2 cm^3 or ulcerated the mouse skin, the mice were terminally narcotized and sacrificed by cardiocentesis. Right lungs were excised *en bloc* and prepared for histologic analyses as described (28). Three representative lung sections from 3 animals of the PC-3 group were subjected to HPA lectin histochemistry to determine the presence of HPA-reactive carbohydrates in spontaneous lung metastases. The left lungs were homogenized in a sample disruptor (TissueLyser II, Qiagen) and subjected to DNA-isolation (QIAamp DNA Mini Kit, Qiagen). Bone marrow was collected by flushing the left femora with 1 mL NaCl 0.9%. Two hundred microliters of blood and the bone marrow suspensions were subjected to DNA isolation using the QIAamp DNA Blood Mini Kit. Finally, primary tumors were removed, weighed, and processed for histologic examinations.

Quantification of disseminated tumor cells and CTC by Alu-PCR

DNA concentrations of all samples were quantified using a NanoDrop spectrophotometer (Peqlab). As the content of detectable *Alu*-sequences in the following qPCR would have been affected simply by varying DNA concentrations, all lung and bone marrow DNA samples were normalized to 30 ng/µL using AE buffer (Qiagen). The concentrations of blood-DNA were quite similar in all samples (∼10 ng/µL) and were therefore not normalized. Quantitative PCR (qPCR) was performed with established human-specific *Alu* primers (29). Two microliters total DNA (i.e., 60 ng lung/bone marrow-DNA, 20 ng blood-DNA) were used for each qPCR. Numerical data were determined against a standard curve as described (22). The detection limit for specific human *Alu*-sequence signals was determined for each tissue type by testing DNA from 5 healthy (noninjected) $Pfp/Rrag2^{-/-}$ mice of similar sex and age. For each sample, analyses were performed in duplicates and as independent experiments at least twice.

Morphological and immunohistochemical analysis of spontaneous lung metastases

Pulmonary metastases were examined histologically in 10 standardized hematoxylin and eosin (H&E)-stained lung sections per mouse (28). Human cancer cells were recognized by their characteristically large, basophilic,

and polymorphic nuclei, which were clearly distinguishable from the smaller nuclei of mouse cells (Figs. 2 and 4). To evaluate potential differences between wild-type and E-/P-selectin-deficient $Pfp/Rag2^{-/-}$ mice, the lungs of 10 mice per group were analyzed by two blinded investigators with a particular focus on the differentiation between intrastromal metastases and intravascular tumor cells. Tumor cell location was considered as to be intravascular, when erythrocytes or blood plasma were adjacent to cancer cells or a surrounding layer of vascular endothelium was morphologically present. In addition, immunostainings for S1P$_1$ (polyclonal rabbit, Santa Cruz #25489) were performed on consecutive lung tissue slides to ascertain the presence of intrastromal PC-3 cells in selectin-deficient mice.

Detection of E-selectin-binding sites on tissue sections by immunofluorescence

Cell surface E-selectin-binding sites were assessed in prostate cancer xenograft tumors and prostatectomy cancer epithelium using a rh-E-selectin/IgG1-Fc chimera or IgG-Fc (isotype control, both from R&D Systems) on xylol-treated, deparaffinized tissue, or microarray sections as described before (15). Human pancreatic adenocarcinoma grown in $Pfp/Rag2^{-/-}$ mice served as a positive control (15). The use of anonymized human tissue microarrays and clinical follow-up data was permitted by the local ethical review committee (Project No.WF-060/12).

Prostate cancer prognosis and heterogeneity tissue microarrays

The clinical impact of glycoconjugates terminating in GalNAc and/or GlcNAc and of E-selectin-binding sites was analyzed using HPA lectin histochemistry and E-selectin immunofluorescence on TMA slides containing primary tumor samples from 1,285 or 1,600 patients with prostate cancer, respectively. All patients underwent radical prostatectomy at the Department of Urology at our Medical Center (1992–2005). PSA values were measured quarterly in the first year, followed by biannual measurements in the second and annual measurements after the third year following surgery. Biochemical relapse (BCR) was defined as a postoperative PSA of 0.2 ng/mL and rising thereafter; patients without evidence of recurrence were censored at last follow-up. Prostatectomy specimens were transferred onto a TMA format as described before (30–33). HPA- and E-selectin–binding toward prostate cancer epithelium was evaluated (negative *vs.* positive; positivity was considered when >50% of tumor cells were stained) and correlated with histopathologic and clinical follow-up data. Next, prostate cancer heterogeneity TMAs were analyzed to investigate whether HPA binding is heterogeneous in prostate cancer and whether the binding status differs between primary tumors and lymph node metastases. This additional microarray included a total of 1,727 tissue punches, taken from 20 different remote areas of each primary tumor and one tissue punch each from 1–8 matched lymph node metastases ($n = 76$).

Figure 2. HPA binding is absent in metastatic prostate cancer xenograft tumors and lung metastases; E-selectin-binding sites are not detectable in prostate cancer xenografts. A–E, tumor growth, growth period and spontaneous metastasis formation after subcutaneous xenotransplantation of PC-3, VCaP, and DU-145 into *Pfp/Rag2*$^{-/-}$ mice. DTCs in lungs (C) and bone marrow (D) as well as CTCs in the blood (E) were quantified by *Alu*-PCR (detection limits are indicated by red lines). Photomicrographs show representative samples of HPA-binding histochemistry and E-selectin-binding immunofluorescence on xenograft tumors. HT29 colon cancer and PaCa5061 pancreatic adenocarcinoma xenografts served as positive controls (2, 6, 14, 15; see also Supplementary Table S1). Inserts demonstrate HPA binding after inhibition with D-GalNAc. F, HPA-reactive carbohydrates are detectable in only 16.2% of spontaneous prostate cancer lung metastases [bars represent means ± SD of three mice (10 lung sections each)]. *, $P < 0.05$ versus DU-145; ***, $P < 0.0001$ versus VCaP and DU-145; #, $P < 0.05$.
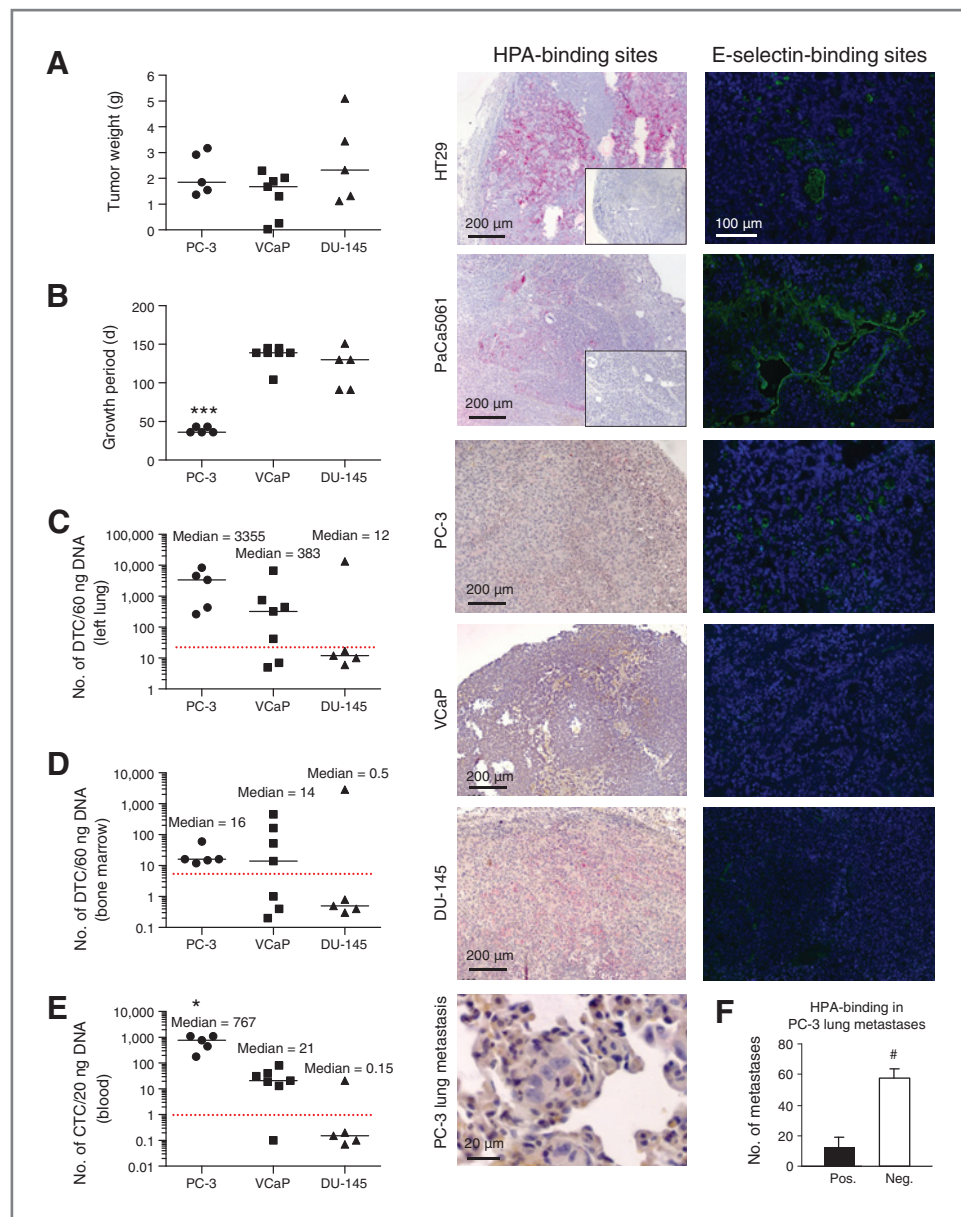
These clinical studies were approved by the local ethics committee (WF-049/09).

Results

Glycosyltransferases involved in the biosynthesis of HPA-reactive sugar residues are downregulated in prostate cancer cells

Significant changes of glycosyltransferase expression involved in the synthesis of HPA-reactive carbohydrates are summarized in Table 1. Note that several polypeptide GalNAc-transferases, core 1 and 2 synthases are downregulated in prostate cancer cells. Accordingly, all tested prostate cancer cell lines bind GalNAc/GlcNAc-specific HPA at a low (VCaP, DU-145) to moderate (PC-3) level compared with HT29 colon cancer cells (Fig. 1B).

Metastatic prostate cancer xenograft primary tumors and spontaneous lung metastases are HPA-negative; E-selectin-binding sites are absent in prostate cancer xenograft tumors

Xenograft tumors developed in 5 of 5 PC-3- and DU-145-bearing mice and 7 of 9 mice injected with VCaP in Matrigel. The median tumor weights are 1.85, 1.68, and 2.32 grams (Fig. 2A) after a mean growth period of 39 ± 3.8, 136 ± 12, and 119 ± 26.6 days ($P < 0.0001$; Fig. 2B) for PC-3, VCaP, and DU-145, respectively. The rates and median numbers of detected disseminated tumor cells (DTC) and CTC are depicted in Fig. 2C–E. This is the first description of VCaP as a suitable spontaneous metastasis model of human prostate cancer. Histology confirmed the presence of spontaneous lung metastases in the PC-3 model (Fig. 2). The

Table 1. Several glycosyltransferases involved in the synthesis of HPA-reactive glycoconjugates are downregulated in metastasis-derived prostate cancer cells compared with primary nonmalignant prostate epithelium (PPEC)

Glycosyltransferases	Gene	Ct-value PPEC	Fold up-/downregulation vs. PPEC		
			PC-3	VCaP	DU-145
ppGalNAc-T's (polypeptide	GALNT3	23.76	−2.95	−1.34	−31.25[b]
N-Acetyl-galactosaminyltransferases)	GALNT6	27.03	1.45	−119.91[a]	−6.05[a]
	(GALNT8	34.1	−3.1[a]	−2.8[a]	−4.01[a])
	GALNT12	29.92	7.47[a]	−1.51	9.03[a]
	GALNT14	27.63	−56.95[a]	−2.26	6.29[a]
O-glycan core structure	C1GALT1	23.75	−2.87[b]	−6.43[c]	−1.32
glycosyltransferases	C2GNT1	28.05	−5.45[a]	6.04[a]	1.01
	C2GNT2	27.64	−15.47[a]	−22.51[a]	2.9
	C2GNT3	27.97	−29.96[b]	−89.63[b]	−2.27[a]

[a]$P < 0.05$.
[b]$P < 0.001$.
[c]$P < 0.0001$.

detection limits for specific *Alu* sequences were 20, 5, and 1 tumor cells per used DNA in the lung, bone marrow, and blood, respectively (red dotted lines shown in Fig. 2C–E).

HPA binding is completely absent in VCaP primary tumors and in more than 80% of PC-3 primary tumors, whereas DU-145 tumors show a weak, homogeneous staining pattern throughout the samples. An average of 57 of 68 (83.8%) lung metastases per mouse is HPA-negative ($P < 0.05$). E-selectin-binding sites are only marginally detectable in PC-3 tumors and are absent in VCaP and DU-145 xenografts. In contrast, HT29 colon and PaCa5061 pancreatic adenocarcinoma xenograft primary tumors present increased levels of HPA- and E-selectin-binding sites *in vivo* (Fig. 2).

HPA-negative patients have an unfavorable prognosis and HPA-binding decreases in lymph node metastases

Six hundred and ninety six of 1,285 patients with prostate cancer show no detectable HPA binding, when one representative tissue spot is analyzed per patient, indicating the absence of carbohydrates terminating in GalNAc or GlcNAc at the cancer epithelium cell surface in the majority of cases. Importantly, HPA-negative patients have a decreased biochemical relapse (BCR)-free survival in comparison with the HPA-positive cohort ($P = 0.009$; Fig. 3A). The adverse prognostic effect of HPA negativity is more pronounced in the subset of R1-resected patients ($P = 0.003$; Fig. 3B). Accordingly, tumor stages ($P<0.0001$) and grades ($P = 0.002$) are increased in the HPA-negative cohort (Fig. 3C and D; Table 2). The percentage of overall biochemical relapses is increased in the HPA-negative patient group ($P = 0.006$, Table 2; Fig. 3E). In addition, HPA-negative patients have elevated PSA values ($P = 0.02$; Table 2). However, loss of HPA-binding sites is not an independent predictive biomarker ($P = 0.562$, multivariate Cox analysis including Gleason score, pT stage, pN and R status).

On the basis of our analysis of up to 20 different primary tumor spots taken from different remote areas of each primary tumor ($n = 76$), we report a heterogeneous HPA-binding pattern in 89.5% of all patients with prostate cancer (Fig. 3F). Only one patient is homogeneously HPA-positive, whereas 7 patients (9%) are homogeneously HPA negative. An average of 26.3% of tumor spots is HPA-positive per patient. Eighty-eight percent of all patients have at least one HPA-positive primary tumor spot. Interestingly, this number decreases to 50% in the corresponding lymph node metastases of the same patients ($P <0.0001$; Fig. 3F), indicating a decrease of HPA-reactive sugar residues during prostate cancer progression and metastatic spread. Lymph node spots were lost in 6 cases during sample processing. Representative pictures of positive and negative HPA binding on primary tumors (top) and lymph node metastases (bottom) are shown in Fig. 3.

E-/P-selectin are not essential for metastasis formation and E-selectin-binding sites are seldom presented in prostate cancer tumors

After engraftment of highly metastatic PC-3 cells into E- and P-selectin–deficient $Pfp/Rag2^{-/-}$ mice, the number of DTCs in the lungs (Fig. 4A) and CTCs in the blood (Fig. 4B) remains unchanged. Because the numbers of DTC in the lungs detected by *Alu*-PCR might at least partially be caused by intravascular DTCs and may not necessarily represent true metastases, the contralateral lungs were examined morphologically. By this approach, we demonstrate an increase of the median number of intravascular DTCs from 88 ± 543.6 in wild-type to $1,305 \pm 1,645.5$ in E-/P-selectin–deficient mice ($P = 0.038$; Fig. 4C), suggesting a disturbed extravasation in selectin deficiency. Nevertheless, the median number of intrastromal metastases is almost similar in both groups (Fig. 4D). Immunohistochemical staining of vascular lung endothelium (S1P$_1$) clearly demonstrates the
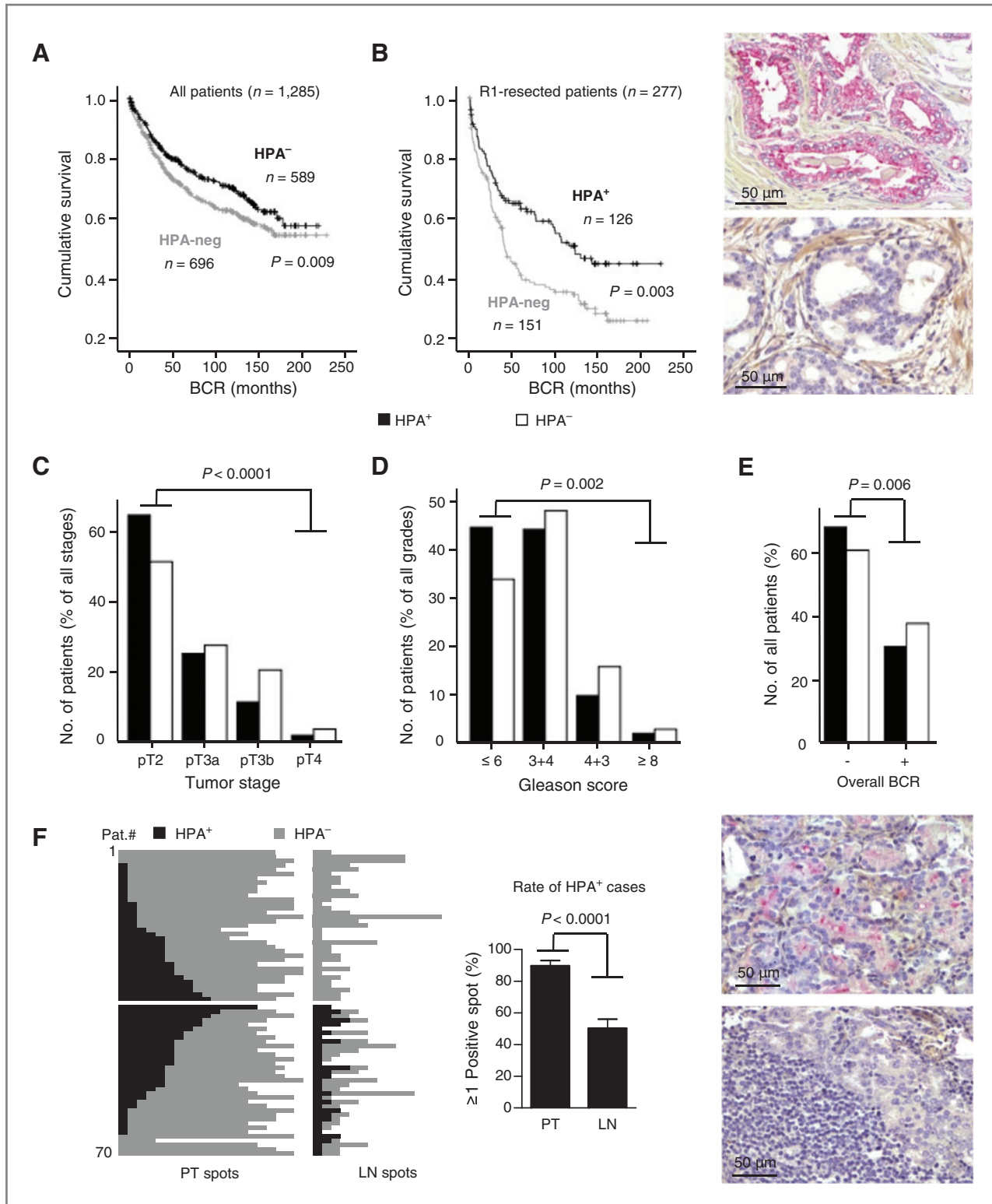
Figure 3. Adverse prognosis and lymph node metastases are accompanied by decreased HPA-reactive glycoconjugates. A and B, reduced BCR-free survival in HPA-negative prostate cancer patients. C and D, HPA-negativity correlates with higher tumor stages and increased Gleason scores. E, the percentage of patients suffering from BCRs is increased in the HPA-negative cohort. F, the number of cases with at least one HPA-reactive specimen decreases from 88% in primary tumors (PT) to 50% in lymph node metastases (LN). Photomicrographs show representative samples of HPA-positive and -negative PT (top) and LN (bottom).

Table 2. Clinicopathologic features of the study populations (completed follow-up ≥ 1 month)

	HPA-positive patients (*n* = 589)	HPA-negative patients (*n* = 696)	*P*
Patient age, y			
Mean (median)	62.5 (62.7)	62.24 (62.6)	
Range	43.2–76.1	40.6–76.3	
Follow-up, months			
Mean (median)	96.9 (120.4)	93.1 (105.3)	
Range	1.6–219.2	1.2–228.7	
Preoperative PSA, ng/mL			
Mean (median)	10.5 (7.6)	11.9 (8.0)	0.023
Range	0.0–74.6	0.0–102.8	
Missing data	13 pat. (2.2%)	21 pat. (3%)	
	No. of patients (%)		
pT Stage			
pT2	367 (62.3%)	345 (49.6%)	
pT3a	144 (24.4%)	186 (26.7%)	
pT3b	66 (11.2%)	139 (20%)	
pT4	12 (2%)	26 (3.7%)	
			<0.0001
Prostatectomy Gleason score			
≤6	262 (44.4%)	235 (33.8%)	
3+4	259 (44%)	333 (47.8%)	
4+3	57 (9.7%)	109 (15.7%)	
≥8	11 (1.9%)	19 (2.7%)	
			0.002
pN Stage			
pN0	495 (84%	590 (84.8%)	
pN1–3	23 (3.9%)	40 (5.7%)	
pNx	71 (12.1%)	66 (9.5%)	
Surgical margin status			
R0	463 (78.6%)	544 (78.2%)	
R1	126 (21.4%)	151 (21.7%)	
Rx	0 (0%)	1 (0.1%)	
Overall biochemical recurrences	182 (30.9%)	266 (38.2%)	
			0.006

presence of intrastromal PC-3 cells in selectin-deficient mice (Fig. 4, middle). The growth period and tumor weight at necropsy are not affected by selectin deficiency (not shown).

E-selectin binding is detectable in only 32 of 1,600 prostatectomy samples demonstrating an incidence of E-selectin ligands of 1:50 in clinical prostate cancer. Interestingly, this small subset of E-selectin–positive patients (as depicted in Fig. 4E) tends to have a decreased BCR-free survival after surgery (63.1 vs. 101.5 months; $P = 0.15$). The clinicopathologic features and outcomes of both groups, however, do not differ in a significant manner (Fig. 4E).

Discussion

This study demonstrates for the first time that prostate cancer progression is accompanied by decreased cell surface glycoconjugates terminating in GalNAc and GlcNAc

(= HPA-reactive carbohydrates) as (i) the corresponding glycosyltransferases are downregulated in metastasis-derived prostate cancer cell lines compared with nonmalignant prostate epithelium; (ii) HPA-negativity is associated with metastasis formation in xenograft mouse models; (iii) HPA-negativity indicates an unfavourable prognosis of prostate cancer patients; and (iv) the incidence of HPA-reactive carbohydrates decreases in lymph node metastases compared with primary tumors; (v) E- and P-selectin are not essential for spontaneous pulmonary metastasis formation *in vivo*; and (vi) selectin-binding sites are only rarely present in prostatectomy specimens (incidence 1:50).

Taken together, we demonstrate an inverse functional and prognostic relevance of HPA-binding sites in prostate cancer when compared with several other human adenocarcinomas as outlined in the Introduction (2, 6–10). Likewise, in accordance with our hypothesis that such glycoconjugates (e.g., Tn antigen and core 2 O-glycans;
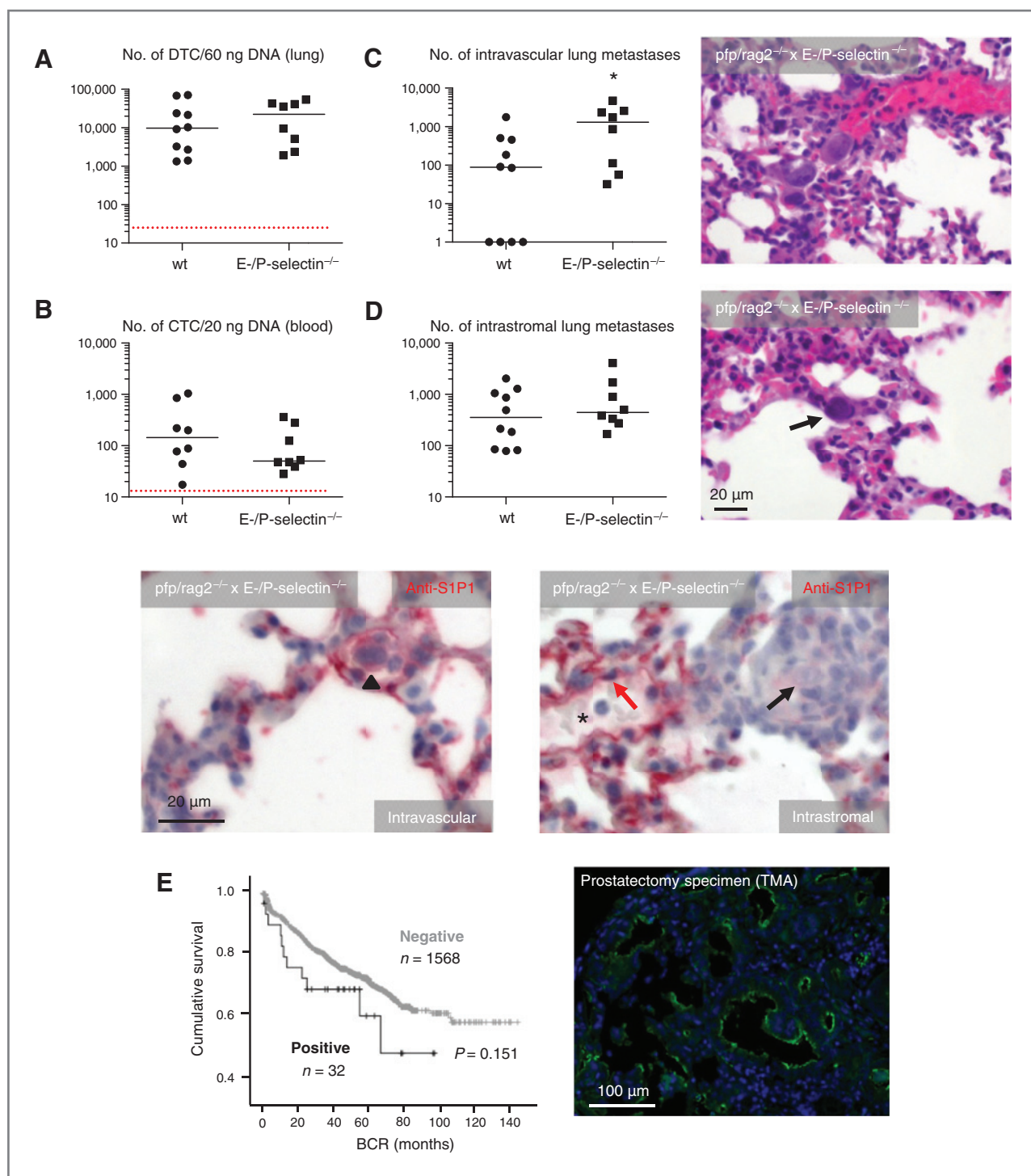
Figure 4. Prostate cancer metastasis formation is largely independent of E- and P-selectin. A-B, The numbers of DTC in the lungs and CTC in the blood remain unchanged (*Alu*-PCR) after s.c. engraftment of PC-3 into E-/P-selectin$^{-/-}$ *Pfp/Rag2*$^{-/-}$ mice. C-D, Morphological analyses reveal an increased number of DTC still present in lung vessels in E-/P-selectin$^{-/-}$ mice. The number of intrastromal metastases, however, is similar in both groups. H.E.-stained photomicrographs show examples of transmigrating PC-3 cells (upper panel) and a single cell metastasis present in the alveolar septum (lower panel, black arrow) in E-/P-selectin$^{-/-}$ mice strongly indicating additional, selectin-independent mechanisms for prostate cancer extravasation. Representative S1P$_1$-immunostainings (middle panel) taken from E-/P-selectin$^{-/-}$ mice illustrate intravascular (left picture) vs. intrastromal (right picture) cancer cells (black arrows) by labeling murine vascular endothelium (red arrows). Intravascular erythrocyte (+)/leukocyte (#). E, Prostatectomy specimens represent E-selectin-binding sites in only 2%of patients without prognostic significance for this small subset. TMA: tissue microarray; * $P < 0.05$.

refs. 4, 11) are common intermediates for the synthesis of selectin ligands (7, 21), extravasation of CTCs is not crucially dependent on selectin–selectin ligand interactions in prostate cancer. Again, this is a peculiarity of prostate cancer and contrary to different other human malignancies (13–16).

Interestingly, one recent study on the glycosylation potential of human prostate cancer also demonstrates a low mRNA expression of different polypeptide-GalNAc-transferases (pp-GalNAc-T's) in prostate cancer (34), suggesting a minor relevance of *O*-glycosylation initiation in prostate cancer in general. This observation is also reflected by the particularly low incidence of Tn antigen found in prostate cancer (4%–26%; refs. 35), even though it is typically highly presented in several other malignancies (4). In contrast, Gao and colleagues and one of our previous studies rather demonstrated a remarkable increase of N-acetylglucosaminlytransferase V (GnT-V) and GnT-Vb, respectively, indicating a particular relevance of $\beta1,6$-branched complex-type N-glycans in prostate cancer (22, 34). However, approximately 45% of patients with prostate cancer were classified "HPA-positive" in our study. This might be possibly due to the abundant presentation of core 2 *O*-glycans by mucin-1 (36), which is an oncoprotein that has been shown to be overexpressed in up to 60% of patients with prostate cancer (37) and contains numerous carbohydrate chains with terminal GlcNAc-residues. Interestingly, ectopic overexpression of highly core 2-glycosylated mucin-1 in C4-2B prostate cancer cells leads to a decreased prostate cancer xenograft tumor growth *in vivo* (36), which is now supported by the beneficial clinical course of our HPA-positive patient cohort and by the loss of HPA-binding sites in lymph node metastases as well as metastatic xenograft tumors.

The low expression of ppGalNAc-T's, core 1, and 2 synthases especially in metastatic prostate cancer cells is associated with an absence of sLe^A and sLe^X on their surface. Because of the presence of intrastromal lung metastases in E-/P-selectin$^{-/-}$ mice, we concluded that the selectin–selectin ligand axis is not essential for metastasis formation in prostate cancer. We corroborated this conclusion by the low incidence of E-selectin-binding sites in clinical prostate cancer tumors, which, in addition, did not show any significant prognostic importance. These observations strongly suggest selectin-compensating or -independent mechanisms that accomplish transendothelial migration in prostate cancer. Following the steps of the leukocyte adhesion cascade, different integrins and chemokines have actually been proven to be relevant for adhesion and transmigration in prostate cancer. For instance, CXCL13/CXCR5-mediated clustering of $\alpha_v\beta_3$-integrin drives adhesion of prostate cancer cells toward human bone marrow endothelium, with CXCL13 serum levels being positively correlated with prostate cancer progression (38). Furthermore, $\alpha_3\beta_1$-integrin expression in prostatectomy specimens is significantly associated with a poor prognosis (39) and β_4-integrin expression is remarkably upregulated in prostate cancer bone metastases (40). In contrast, another study already pointed out that prostate cancer cells adhere to and traverse bone marrow endothelium via sequential dependence on E-selectin, β_1-, and $\alpha_v\beta_3$-integrin (41). In that and a previous study of the same group (42), however, it was obviously necessary to overexpress α-1,3-fucosyltransferases (FT 3, 6, and 7) in prostate cancer cell lines to observe any adhesive events *in vitro* at all (presumably due to the subsequent elevation of sLe^X on FT-transfected cells). This strongly supports our findings that sLe^A/sLe^X presentation and shear stress-resistant adhesion toward HPMEC and P-selectin are not detectable using native prostate cancer cells *in vitro*. Using the same transfectants for *in vivo* homing studies, the authors showed an increased retention of FT-overexpressing tumor cells within in the bone marrow compared with native prostate cancer cells (41). The genetically engineered overexpression of E-selectin ligands on prostate cancer cells, however, does not represent the clinical situation with respect to the low incidence of E-selectin-binding sites elucidated by our study. Nevertheless, Barthel and colleagues interestingly found that bone retention still occurred in up to 50% of mice after pretreating mice with an E-selectin blocking antibody. In contrast, blockade of prostate cancer cells with a β_1-integrin antibody reduced cell retention by 88% (41).

Taken together, these and our own findings indicate selectin-independent, presumably integrin-driven metastasis patterns as a characteristic of prostate cancer. Interestingly, this unusual biologic behavior is obviously associated with unusual metastasis patterns in clinical prostate cancer. As initially shown by Oscar Batson in 1940, metastases to the vertebrae of the lumbar spine are the most frequent ones in prostate cancer and typically occur via a valveless prevertebral vein plexus (Batson's plexus; ref. 43). These metastatic lesions occur independently of systemic dissemination and are predominant in patients with smaller primary tumors, suggesting backward venous spread as an early metastasis route in prostate cancer (44). As the blood flow in prevertebral plexus is normally directed toward the lower vena cava and by this away from the spine (45), vertebrae metastases might rather appear through a kind of growth *per continuitatem*. The patterns of dynamic flow adhesion and thus selectin interactions as recognized to be necessary for systemic dissemination might be less relevant here. However, the precise mechanisms of how integrins or chemokines accomplish extravasation in prostate cancer independent of selectins still remain to be determined.

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

Authors' Contributions
Conception and design: T. Lange, U. Schumacher
Development of methodology: T. Lange, M. Kupfernagel, D. Wicklein, H. Maar, K. Brügge, I. Müller
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Lange, M. Kupfernagel, D. Wicklein, F. Gebauer, H. Maar, I. Müller, R. Simon
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Lange, D. Wicklein, F. Gebauer, T. Schlomm

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin 2012;62:10–29.
2. Schumacher U, Adam E. Lectin histochemical HPA-binding pattern of human breast and colon cancers is associated with metastases formation in severe combined immunodeficient mice. Histochem J 1997;29:677–84.
3. Reis CA, Osorio H, Silva L, Gomes C, David L. Alterations in glycosylation as biomarkers for cancer detection. J Clin Pathol 2010;63:322–9.
4. Varki A, Kannagi R, Toole BP. Glycosylation Changes in Cancer. In: Varki A, Cummings RD, Esko JD, et al. editors. Essentials of Glycobiology. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2009.
5. Schultz MJ, Swindall AF, Bellis SL. Regulation of the metastatic cell phenotype by sialylated glycans. Cancer Metastasis Rev 2012;31:501–18.
6. Mitchell BS, Schumacher U. The use of the lectin Helix pomatia agglutinin (HPA) as a prognostic indicator and as a tool in cancer research. Histol Histopathol 1999;14:217–26.
7. Brooks SA. The involvement of Helix pomatia lectin (HPA) binding N-acetylgalactosamine glycans in cancer progression. Histol Histopathol 2000;15:143–58.
8. Laack E, Nikbakht H, Peters A, Kugler C, Jasiewicz Y, Edler L, et al. Lectin histochemistry of resected adenocarcinoma of the lung: helix pomatia agglutinin binding is an independent prognostic factor. Am J Pathol 2002;160:1001–8.
9. Kakeji Y, Tsujitani S, Mori M, Maehara Y, Sugimachi K. Helix pomatia agglutinin binding activity is a predictor of survival time for patients with gastric carcinoma. Cancer 1991;68:2438–42.
10. Thies A, Moll I, Berger J, Schumacher U. Lectin binding to cutaneous malignant melanoma: HPA is associated with metastasis formation. Br J Cancer 2001;84:819–23.
11. Rambaruth ND, Greenwell P, Dwek MV. The lectin Helix pomatia agglutinin recognizes O-GlcNAc containing glycoproteins in human breast cancer. Glycobiology 2012;22:839–48.
12. Shimodaira K, Nakayama J, Nakamura N, Hasebe O, Katsuyama T, Fukuda M. Carcinoma-associated expression of core 2 beta-1,6-N-acetylglucosaminyltransferase gene in human colorectal cancer: role of O-glycans in tumor progression. Cancer Res 1997;57:5201–6.
13. Stübke K, Wicklein D, Herich L, Schumacher U, Nehmann N. Selectin-deficiency reduces the number of spontaneous metastases in a xenograft model of human breast cancer. Cancer Lett 2012;321:89–99.
14. Köhler S, Ullrich S, Richter U, Schumacher U. E-/P-selectins and colon carcinoma metastasis: first in vivo evidence for their crucial role in a clinically relevant model of spontaneous metastasis formation in the lung. Br J Cancer 2010;102:602–9.
15. Gebauer F, Wicklein D, Stübke K, Nehmann N, Schmidt A, Salamon J, et al. Selectin binding is essential for peritoneal carcinomatosis in a xenograft model of human pancreatic adenocarcinoma in pfp-/rag2-mice. Gut 2013;62:741–50.
16. Wicklein D, Schmidt A, Labitzky V, Ullrich S, Valent P, Schumacher U. E- and p-selectins are essential for repopulation of chronic myelogenous and chronic eosinophilic leukemias in a scid mouse xenograft model. PLoS One 2013;8:e70139.
17. St Hill CA. Interactions between endothelial selectins and cancer cells regulate metastasis. Front Biosci 2011;16:3233–51.
18. Labelle M, Hynes RO. The initial hours of metastasis: the importance of cooperative host-tumor cell interactions during hematogenous dissemination. Cancer Discov 2012;2:1091–9.
19. Julien S, Ivetic A, Grigoriadis A, QiZe D, Burford B, Sproviero D, et al. Selectin ligand sialyl-Lewis x antigen drives metastasis of hormone-dependent breast cancers. Cancer Res 2011;71:7683–93.
20. Martin-Satue M, Marrugat R, Cancelas JA, Blanco J. Enhanced expression of alpha(1,3)-fucosyltransferase genes correlates with E-selectin-mediated adhesion and metastatic potential of human lung adenocarcinoma cells. Cancer Res 1998;58:1544–50.
21. Valentiner U, Hall DM, Brooks SA, Schumacher U. HPA binding and metastasis formation of human breast cancer cell lines transplanted into severe combined immunodeficient (scid) mice. Cancer Lett 2005;219:233–42.
22. Lange T, Ullrich S, Muller I, Nentwich MF, Stubke K, Feldhaus S, et al. Human prostate cancer in a clinically relevant xenograft mouse model: identification of beta(1,6)-branched oligosaccharides as a marker of tumor progression. Clin Cancer Res 2012;18:1364–73.
23. Kalinina T, Gungor C, Thieltges S, Moller-Krull M, Penas EM, Wicklein D, et al. Establishment and characterization of a new human pancreatic adenocarcinoma cell line with high metastatic potential to the lung. BMC Cancer 2010;10:295.
24. Korenchuk S, Lehr JE, L MC, Lee YG, Whitney S, Vessella R, et al. VCaP, a cell-based model system of human prostate cancer. In Vivo 2001;15:163–8.
25. Richter U, Schroder C, Wicklein D, Lange T, Geleff S, Dippel V, et al. Adhesion of small cell lung cancer cells to E- and P-selectin under physiological flow conditions: implications for metastasis formation. Histochem Cell Biol 2011;135:499–512.
26. Mierke CT. Cancer cells regulate biomechanical properties of human microvascular endothelial cells. J Biol Chem 2011;286:40025–37.
27. Ahmed MN, Zhang Y, Codipilly C, Zaghloul N, Patel D, Wolin M, et al. Extracellular superoxide dismutase overexpression can reverse the course of hypoxia-induced pulmonary hypertension. Mol Med 2012;18:38–46.
28. Jojovic M, Schumacher U. Quantitative assessment of spontaneous lung metastases of human HT29 colon cancer cells transplanted into SCID mice. Cancer Lett 2000;152:151–6.
29. Nehmann N, Wicklein D, Schumacher U, Muller R. Comparison of two techniques for the screening of human tumor cells in mouse blood: quantitative real-time polymerase chain reaction (qRT-PCR) versus laser scanning cytometry (LSC). Acta Histochem 2010;112:489–96.
30. El Gammal AT, Bruchmann M, Zustin J, Isbarn H, Hellwinkel OJ, Kollermann J, et al. Chromosome 8p deletions and 8q gains are associated with tumor progression and poor prognosis in prostate cancer. Clin Cancer Res 2010;16:56–64.
31. Fleischmann A, Schlomm T, Huland H, Kollermann J, Simon P, Mirlacher M, et al. Distinct subcellular expression patterns of neutral endopeptidase (CD10) in prostate cancer predict diverging clinical courses in surgically treated patients. Clin Cancer Res 2008;14:7838–42.
32. Minner S, Enodien M, Sirma H, Luebke AM, Krohn A, Mayer PS, et al. ERG status is unrelated to PSA recurrence in radically operated prostate cancer in the absence of antihormonal therapy. Clin Cancer Res 2011;17:5878–88.
33. Bubendorf L, Kononen J, Koivisto P, Schraml P, Moch H, Gasser TC, et al. Survey of gene amplifications during prostate cancer progression

by high-throughout fluorescence in situ hybridization on tissue micro-arrays. Cancer Res 1999;59:803–6.

34. Gao Y, Chachadi VB, Cheng PW, Brockhausen I. Glycosylation potential of human prostate cancer cell lines. Glycoconj J 2012;29: 525–37.

35. Li Q, Anver MR, Butcher DO, Gildersleeve JC. Resolving conflicting data on expression of the Tn antigen and implications for clinical trials with cancer vaccines. Mol Cancer Ther 2009;8:971–9.

36. Premaratne P, Welen K, Damber JE, Hansson GC, Backstrom M. O-glycosylation of MUC1 mucin in prostate cancer and the effects of its expression on tumor growth in a prostate cancer xenograft model. Tumour Biol 2011;32:203–13.

37. Kirschenbaum A, Itzkowitz SH, Wang JP, Yao S, Eliashvili M, Levine AC. MUC1 expression in prostate carcinoma: correlation with grade and stage. Mol Urol 1999;3:163–8.

38. Singh S, Singh R, Sharma PK, Singh UP, Rai SN, Chung LW, et al. Serum CXCL13 positively correlates with prostatic disease, pros-tate-specific antigen and mediates prostate cancer cell invasion, integrin clustering and cell adhesion. Cancer Lett 2009;283:29–35.

39. Pontes-Junior J, Reis ST, de Oliveira LC, Sant'anna AC, Dall'oglio MF, Antunes AA, et al. Association between integrin expression and prognosis in localized prostate cancer. Prostate 2010;70: 1189–95.

40. Yoshioka T, Otero J, Chen Y, Kim YM, Koutcher JA, Satagopan J, et al. beta4 Integrin signaling induces expansion of prostate tumor progeni-tors. J Clin Invest 2013;123:682–99.

41. Barthel SR, Hays DL, Yazawa EM, Opperman M, Walley KC, Nimrichter L, et al. Definition of molecular determinants of prostate cancer cell bone extravasation. Cancer Res 2013;73:942–52.

42. Barthel SR, Wiese GK, Cho J, Opperman MJ, Hays DL, Siddiqui J, et al. Alpha 1,3 fucosyltransferases are master regulators of prostate cancer cell trafficking. Proc Natl Acad Sci U S A 2009;106:19491–6.

43. Batson OV. The function of the vertebral veins and their role in the spread of metastases. Ann Surg 1940;112:138–49.

44. Bubendorf L, Schopfer A, Wagner U, Sauter G, Moch H, Willi N, et al. Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients. Hum Pathol 2000;31:578–83.

45. Geldof AA. Models for cancer skeletal metastasis: a reappraisal of Batson's plexus. Anticancer Res 1997;17:1535–9.

46. Springer GF. Tn epitope (N-acetyl-D-galactosamine alpha-O-serine/ threonine) density in primary breast carcinoma: a functional predictor of aggressiveness. Mol Immunol 1989;26:1–5.

47. Sheikh S, Rainger GE, Gale Z, Rahman M, Nash GB. Exposure to fluid shear stress modulates the ability of endothelial cells to recruit neutrophils in response to tumor necrosis factor-alpha: a basis for local variations in vascular sensitivity to inflammation. Blood 2003;102:2828–34.