

A Unique Galectin Signature in Human Prostate Cancer Progression Suggests Galectin-1 as a Key Target for Treatment of Advanced Disease

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Abstract

Galectins, a family of glycan-binding proteins, influence tumor progression by modulating interactions between tumor, endothelial, stromal, and immune cells. Despite considerable progress in identifying the roles of individual galectins in tumor biology, an integrated portrait of the galectin network in different tumor microenvironments is still missing. We undertook this study to analyze the "galectin signature" of the human prostate cancer microenvironment with the overarching goal of selecting novel-molecular targets for prognostic and therapeutic purposes. In examining androgen-responsive and castration-resistant prostate cancer cells and primary tumors representing different stages of the disease, we found that galectin-1 (Gal-1) was the most abundantly expressed galectin in prostate cancer tissue and was markedly upregulated during disease progression. In contrast, all other galectins were expressed at lower levels: Gal-3, -4, -9, and -12 were downregulated during disease evolution, whereas expression of Gal-8 was unchanged. Given the prominent regulation of Gal-1 during prostate cancer progression and its predominant localization at the tumor-vascular interface, we analyzed the potential role of this endogenous lectin in prostate cancer angiogenesis. In human prostate cancer tissue arrays, Gal-1 expression correlated with the presence of blood vessels, particularly in advanced stages of the disease. Silencing Gal-1 in prostate cancer cells reduced tumor vascularization without altering expression of other angiogenesis-related genes. Collectively, our findings identify a dynamically regulated "galectin-specific signature" that accompanies disease evolution in prostate cancer, and they highlight a major role for Gal-1 as a tractable target for antiangiogenic therapy in advanced stages of the disease. *Cancer Res*; 73(1); 86–96. ©2012 AACR.

Introduction

Prostate cancer is the second most common cancer in men, and represents a significant cause of mortality worldwide (1). Localized prostate cancer is efficiently treated by association of surgery with radiotherapy and androgen ablation. However, prostate cancer evolves toward stages in which tumor cells acquire properties allowing their distant dissemination (2) and castration-resistant growth (3). No current treatments are

applicable to these situations and the prospect for cure decreases radically. These particular features urge the search of novel prognosis strategies that could delineate the transition from hormone-sensitive toward hormone-resistant tumor growth and innovative therapeutic approaches suitable for castration-refractory stages of the disease.

Effective cancer therapies typically capitalize on molecular differences between healthy and neoplastic tissues. In the

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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-12-1260

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postgenomic era, the study of the glycome has enabled the association of specific glycan structures with the transition from normal to neoplastic tissue (4, 5). The function of deciphering the biologic information encoded by the glycome is assigned to endogenous glycan-binding proteins or lectins, whose expression and function are regulated during tumor progression (5). Galectins, a family of glycan-binding proteins, play pivotal roles as regulators of tumor biology by directly influencing tumor transformation, invasiveness, angiogenesis, and tumor-immune escape (6, 7). These lectins are defined by a common structural fold and a conserved carbohydrate recognition domain (CRD) that recognizes N- and O-glycans expressing the disaccharide N-acetylglucosamine (Gal- β (1-4)-GlcNAc), although differences in glycan-binding preferences of individual members of the family have been reported (7). Galectins that are traditionally classified as "proto-type" (Gal-1, -2, -5, -7, -10, -11, -13, -14, and -15) have 1 CRD that can dimerize, whereas "tandem-repeat" galectins (Gal-4, -6, -8, -9, and -12) contain 2 homologous CRDs in tandem in a single polypeptide chain. Gal-3 is unique in that it contains a CRD connected to a non-lectin N-terminal region that is responsible for oligomerization (7). Extracellularly, galectins interact with cell surface glycoconjugates and trigger cellular signaling to control migration, immunity, and angiogenesis. Intracellularly, galectins can control tumor transformation, proliferation, and survival (7, 8).

Previous studies have identified galectins as key components of the prostate cancer microenvironment (9–11). Expression of galectin-1 (Gal-1) controls the differentiation and survival of prostate cancer cells (9, 12) and inhibits T-cell transmigration (13). On the other hand, Gal-3 controls homotypic and heterotypic aggregation of prostate cancer cells (14–16) and controls their viability (17). Tumor cell expression of Gal-3 has been proposed to delineate the transition from benign stages to castration-resistant malignant disease (18) and its regulated expression is associated with promoter methylation (19). Silencing Gal-3 results in decreased migration, invasion, and proliferation of prostate cancer cells (20). Moreover, Gal-8, which was originally identified as prostate cancer tumor antigen 1 (PCTA1; ref. 21), can modulate integrin-mediated cell–extracellular matrix interactions (22). However, despite considerable progress in dissecting the functions of individual members of the galectin family, there is still no integrated portrait of the "galectin signature" of the human prostate cancer microenvironment.

Our findings identify a unique galectin expression profile, which delineates different stages of prostate cancer progression. From all galectins analyzed, Gal-1 is uniquely expressed at high levels in human prostate cancer and contributes to tumor progression by promoting neovascularization. These results underscore the importance of Gal-1 as an attractive therapeutic target in advanced stages of prostate cancer.

Materials and Methods

Human samples

Radical prostatectomies were obtained from the archival tissue bank of the Department of Pathology, Hospital Alemán (Buenos Aires, Argentina). Samples were classified according to tumor–node–metastasis (TNM) classification [Union for

Table 1. Description of human primary tumors analyzed

Grade	Average age, y	Number of patients
Hyperplasia	65 \pm 10	11
T1	69 \pm 10	10
T2	63 \pm 5	19
T3	60 \pm 5	18
T4	56 \pm 10	3
Gleason \leq 6	64 \pm 6	29
Gleason = 7	57 \pm 9	12
Gleason \geq 8	66 \pm 11	9

NOTE: Radical prostatectomies were classified according to TNM scale. Specimens ($n = 61$) covered all stages of prostate cancer evolution, including T1 (tumor detected in less or 5% of the tissue), T2 (tumor confined to the prostate), T3 (tumor extends beyond the prostatic capsule), and T4 (tumor invades structures other than seminal vesicles), in addition to BHP. Average ages and Gleason indexes from patients are shown (Gleason \leq 6, tumor well-differentiated, low-grade; Gleason = 7, intermediate grade; and Gleason \geq 8, tumor poorly differentiated, high-grade).

International Cancer Control (UICC), 2002] by 2 independent pathologists (Gabriel Casas and O. Mazza). Specimens ($n = 61$) covered all stages of prostate cancer evolution (T1, T2, T3, and T4) in addition to benign hyperplasia (BHP) cases (Table 1). None of these patients received preoperative therapy. Protocols were approved by the Local Ethics Committee (Hospital de Clínicas "José de San Martín", Buenos Aires, Argentina).

Cells and animals

Human prostate cancer cell lines used included: the hormone-responsive LNCaP cell line and the castration-resistant cell lines 22Rv1 and PC-3 with or without androgen receptor (AR) expression, respectively. The LNCaP and 22Rv1 cell lines were provided by A. Chachereau (Institute Gustave Roussy, Villejuif, France). LNCaP cells were also provided by E. Vazquez. These cell lines were originally obtained from the American Type Culture Collection. Cell morphology was evaluated by microscopic examination on a daily basis. Growth properties of LNCaP cells were regularly tested through their responsiveness to androgens using MTT assay. Cells were incubated for 24 hours in phenol red-free RPMI, 10% charcoal-treated serum and medium was supplemented with 10^{-10} mol/L R1881 (AR agonist) for 3 days before analyzing growth and gene expression. Prostate-specific antigen (PSA) induction was evaluated by real-time reverse transcriptase quantitative PCR (RT-qPCR). Routine tests for 22Rv1 cells included examination of androgen-insensitive growth (MTT method) and PSA induction by R1881 (real-time RT-PCR). The PC-3 cell line was provided by E. Vazquez. Growth of these cells was routinely tested for androgen sensitivity and the AR and PSA phenotypes by real-time RT-PCR. Bovine aortic endothelial cells (BAEC)

were provided by M.T. Elola. BAEC were tested for their ability to form tubular structures in the presence of VEGF. Each cell line was routinely tested for *Mycoplasma* contamination by genomic PCR. LNCaP, 22Rv1, and PC-3 cells were cultured in RPMI and BAEC were cultured in Dulbecco's modified Eagle's medium. Medium was supplemented with 10% heat-inactivated FBS (PAA, Cell Culture, Austria), 2 mmol/L L-glutamine, 100 µg/mL streptomycin, and 100 U/mL penicillin. BAEC were used at passage 14 or less. For some experiments, 22Rv1 cells (50,000) were plated into 12-well plates, cultured for 2 days under normal oxygen supply, and then exposed to hypoxic (1% O₂) or normoxic conditions for an additional 15 hours. Nude mice were obtained from The National University of La Plata (La Plata, Argentina) and maintained in accordance with the Institutional Animal Care and Use Committee guidelines (IBYME, Buenos Aires, Argentina).

Reagents

The following anti-galectin antibodies (Santa Cruz Biotechnology, Inc.) were used: rabbit anti-Gal-1 (H-45), anti-Gal-8 (H-80), anti-Gal-3 (H-160), anti-Gal-4 (T-20), anti-Gal-12 (H-166), and goat anti-Gal-9 (C-20) antibodies. A purified anti-Gal-1 polyclonal rabbit immunoglobulin G (IgG) generated in G.A. Rabinovich's laboratory was used (23, 24). Anti-human carbonic anhydrase IX polyclonal antibody (H-120) was obtained from Santa Cruz. Media and trypsin/EDTA were obtained from Gibco-Invitrogen (Life Technologies). Blocking anti-Gal-1 monoclonal antibody (mAb; F8.G7) was generated and validated as described (25, 26). Growth factor-reduced Matrigel was obtained from BD Biosciences.

Immunohistochemistry

Immunohistochemistry was conducted on paraffin-embedded tissue samples. Samples were deparaffinized by 5-minute incubation in xylene, 100%, 95%, and 75% ethanol. Endogenous peroxidase activity was quenched by 10-minute incubation with 1% H₂O₂. Nonspecific binding was blocked using normal horse serum in 0.05% saponin. Samples were incubated with the appropriate antibodies at the optimal dilutions for 1 hour at room temperature. The following antibodies were used: polyclonal anti-Gal antibodies and preimmune sera from Santa Cruz (1:200 dilution) and purified anti-Gal-1 rabbit IgG (1:1,500). Immunoreactions were developed using the avidin-biotin-peroxidase Vectastain ABC Kit (Vector). Galectin expression was graded as follows: 0 (negative); 1+ (poor intensity); 2+ (moderate intensity); 3+ (high intensity), and 4+ (very high intensity). Prostate cancer cell lines were adhered to poly-L-lysine (Sigma)-coated coverslips for 2 hours at 37°C (50,000 cells per coverslip), fixed with 4% paraformaldehyde for 5 minutes, and processed for immunocytochemistry as described for tissues.

Immunohistochemistry of tissue microarrays was conducted using 4-µm thick formalin-fixed, paraffin-embedded sections of tissue microarray slides containing 29 paired cores (2 different areas of each single tumor from 29 tumors analyzed) of invasive prostate cancer (BC19013; US Biomax; Table 2). Slides were soaked in xylene and passed through graded alcohols and distilled water before use. Slides

Table 2. Description of human primary tumors analyzed

Grade	Average age, y	Number of patients
Hyperplasia	69 ± 6	5
Grade 1	74 ± 7	8
Grade 2	73 ± 8	60
Grade 3	73 ± 8	58

NOTE: Tissue arrays of radical prostatectomies obtained from US Biomax (TMA-BC19013) are classified according to: grade 1 or well-differentiated (cells appear normal and do not grow rapidly); grade 2 or moderately differentiated (cells appear slightly different from normal); and grade 3 or poorly differentiated (cells appear abnormal and tend to grow and spread more aggressively).

were then pretreated with citrate buffer pH 6.0 (Invitrogen) in a steam pressure cooker (Decloaking Chamber CD2008US, Biocare Biomedical) according to manufacturers' recommended settings (127°C for 30 seconds, followed by 90°C). Slides were blocked for peroxidase activity using a specific blocker (DAKO) and washed for 5 minutes in buffer. Individual slides were incubated with a mouse anti-human CD34 mAb (clone QBEND-10, RTU, Immunotech) and a rabbit anti-human Gal-1 polyclonal antibody (1:10,000) generated and used as described (23, 24). After 1 hour incubation, slides were washed and processed by the appropriate Envision+ Kit (DAKO) as per manufacturer's instructions, developed using a 3,3'-diaminobenzidine (DAB) chromogen (DAKO) and counterstained with hematoxylin. Stained slides were digitally scanned using Aperio ScanScope XT workstation at the ×20 setting (Aperio Technology, Inc.). Core images were then analyzed using ImageScope software (version 10.0.35.1800, Aperio Technology). Briefly, pathologists (S.J. Rodig and J.L. Kutok) identified areas of tumors as regions of interest (ROI) and excluded areas without significant tumor using standard ImageScope software functions. The ROIs were then analyzed using a standard analysis algorithm (color deconvolution v9.0, Aperio Technology) to quantify the average optical density of Gal-1 staining and the percentage of positive pixels in the annotated tumor area.

Real-time RT-PCR

Transcriptional profile of galectins was analyzed in human prostate cancer cell lines (log phase of growth) that are representative of different stages of tumor progression. Transcriptional profile of angiogenesis-related genes was analyzed in plugs generated by injection of Gal-1-sufficient or Gal-1-silenced human 22Rv1 cells into nude mice. In all cases, RNA was purified using TRIzol reagent (Invitrogen) coupled to DNase (RQ1, Promega) treatment. Four hundred nanograms of total RNA was used for the reverse transcription reaction by using SuperScript III Reverse Transcriptase, random hexamers (2.5 µg/mL) and deoxynucleotide triphosphates (dNTP; 500 nmol/L) according to manufacturer's instructions (Invitrogen) during 50 minutes at 42°C, following by RNase H treatment for

30 minutes at 37°C. One microliter of a 1:25 dilution of cDNA was used as template in real-time PCR. Relative gene expression was analyzed using SYBR Green PCR Kit (Applied Biosystem, Life Technologies). PCR conditions were as follows: 5 minutes 95°C, 40 cycles 30 seconds at 95°C, 32 seconds at 59°C, and 45 seconds at 72°C. Amplification fragments were analyzed by electrophoresis on a 2% agarose TAE (40mmol/L Tris, 40 mmol/L acetate, 1 mmol/L EDTA, pH 8.2) gel and by thermal dissociation curves (T_m) to characterize the amplicon corresponding to each primer couple. Primers used are listed in Supplementary Tables S1 and S2. Cyclophilin A was used as an internal reference gene (27). Equivalent amounts of RNA were tested to rule out genomic DNA contamination.

Immunoblotting

Specificity of anti-galectin antibodies was evaluated by immunoblotting (Supplementary Fig. S2). Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer [50 mmol/L Tris-HCl pH 8, 150 mmol/L NaCl, 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS, 10 mmol/L EDTA, 1 mmol/L sodium vanadate, and Protease Inhibitor Cocktail Set III (Calbiochem)]. Equal amounts of protein (10–30 µg) were resolved by 15% SDS-PAGE, blotted onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare), blocked with 5% bovine serum albumin (BSA), and probed with anti-galectin or anti-β-tubulin (H-235 1:200, Santa Cruz) antibodies or preimmune rabbit antiserum. The following dilutions of antibodies were used: anti-Gal-1 (1:500), Gal-3 (1:400), Gal-4 (1:100), Gal-8 (1:400), Gal-9 (1:100), Gal-12 (1:100), and rabbit IgG anti-Gal-1 at 1:1,500. Bound antibodies were detected with peroxidase-labeled anti-rabbit total immunoglobulins (1:3,000; Sigma) or by peroxidase-labeled rabbit anti-goat IgG (1:2,000; Sigma). Peroxidase activity was detected using a luminol-based method and chemiluminescence was determined using a Fuji Photo Film Analyzer.

Lentivirus vector production and transduction

pLV-HTM plasmid (provided by Trono Didier, Geneva University, Geneva, Switzerland) is a self-inactivation third generation HIV-1-derived vector (28). Annealed oligonucleotides coding for short hairpin RNA (shRNA) were ligated into *ClaI* and *MluI* double-restricted plasmids by standard cloning. Restriction enzymes and T4 DNA ligase were from New England BioLabs. Production of shRNA was under the control of H1 (human RNA polymerase type III promoter). As reporter gene, GFP was expressed under the control of eukaryotic EF-1α promoter. Plasmids were verified by sequence analysis. Lentiviral particles were produced by transient transfection of 293T cells. Briefly, subconfluent 293T cells were cotransfected with 20 µg plasmid vector, 15 µg pCMVR8.74, and 5 µg pMD.G [pseudotyped vesicular stomatitis virus glycoprotein (VSVG)] using calcium phosphate. Supernatants were harvested at 48 and 72 hours and stored at –80°C until use. Viral titers expressed as TU/mL were determined by assessing transduction of 22Rv1 cells with serial dilutions of virion preparations. 22Rv1 prostate cancer cells were transduced with virus at multiplicity of infection (MOI) = 5 in the presence of 5 µg/mL protamine sulfate (Sigma). After 1 week, transduced cells (GFP⁺) were purified using a FACSAria II cell sorter (BD Bioscience).

In vitro capillary-like tube formation and *in vivo* Matrigel plug assay

Matrigel (150 µL; BD Biosciences) was added to 24-well plates and allowed to polymerize for 2 hours at 37°C. Conditioned media were added to wells and 2.5×10^4 BAEC were seeded on each well. Tube formation was evaluated in 5 different fields of each well and photographed at 18 hours using an inverted microscope. For *in vivo* assays, cold Matrigel was mixed with 5×10^6 22Rv1 cells in the absence or presence of a Gal-1 blocking or isotype control mAb (7.5 mg/kg). The mixture (500 µL) was subcutaneously injected into 6-week-old male nude mice. Five days later, Matrigel plugs were harvested and photographed. Matrigel plugs were homogenized in 500 µL H₂O on ice and cleared by centrifugation at $200 \times g$ for 6 minutes at 4°C. Hemoglobin content was determined using the Drabkin's reagent (Wiener Lab).

Statistical analysis

Data are presented as mean ± SD of at least 3 independent experiments in triplicate. Comparisons between 2 groups were conducted by using paired Student *t* test or Spearman correlation test as indicated. Differences were considered significant when *P* values were less than 0.05.

Results

Identification of "the galectin signature" of human prostate cancer cells

To delineate the galectin expression profile during prostate cancer progression, we first examined the galectin-transcriptional pattern of several human prostate cancer cell lines, which are representative of different stages of the disease. These include the hormone-responsive cell line LNCaP and the castration-resistant cell lines 22Rv1 and PC-3, which are AR-positive (22Rv1) or negative (PC-3) respectively. Total RNA was extracted in the log phase of growth and analyzed by quantitative RT-PCR (Fig. 1A). Gal-1 was found to be the most abundantly expressed galectin in all cells analyzed and its expression was higher in prostate cancer cells exhibiting more aggressive behavior *in vivo* (22Rv1 and PC-3; ref. 27). Transcripts for Gal-8, which has been postulated as prostate cancer marker (21, 29), were expressed at moderate levels in all prostate cancer cell lines tested. Gal-3 mRNA was only detected in castration-resistant, AR-negative PC-3 cells. Transcripts for all other galectin family members (Gal-2, -4, -7, -9, -10, -12, and -13) were expressed at very low levels.

To further delineate the "galectin-specific prostate cancer signature," we assessed the expression of galectin family members at the protein level (focusing on galectins with higher transcript abundance). Immunocytochemical analysis confirmed that Gal-1 was the most abundantly expressed galectin in the prostate cancer cell lines analyzed, showing upregulated expression in the most aggressive cell lines (Fig. 1B). On the other hand, Gal-3 was selectively expressed in the PC-3 cell line, Gal-8 was detected in all the 3 cell lines and Gal-9 and -12 showed a modest expression in all cell lines analyzed. These results indicate a fine regulation of galectin expression in prostate cancer cell lines characterized by differences in phenotype, hormone-dependency, and aggressiveness.

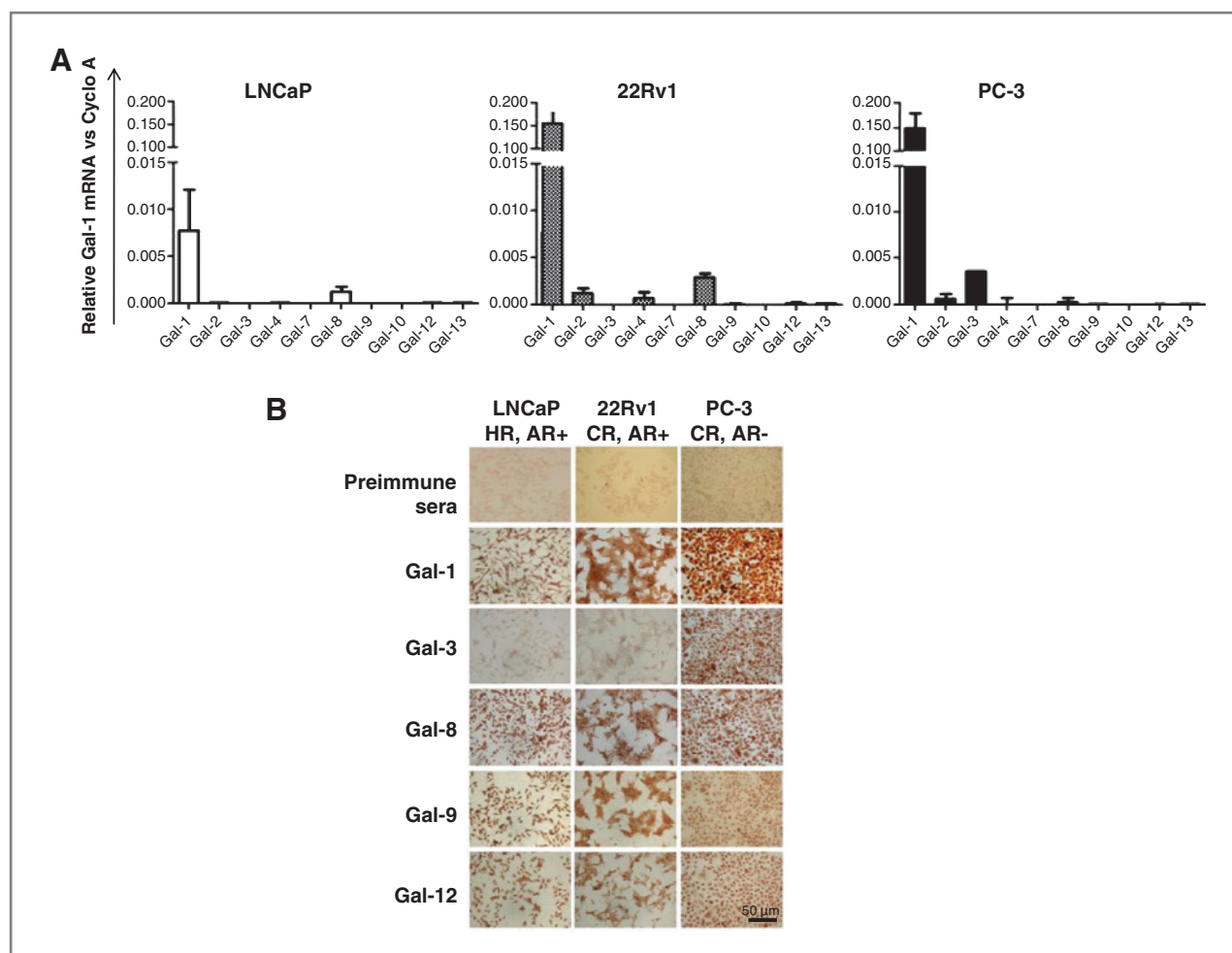


Figure 1. Galectin expression profile in human prostate cancer cell lines. A, transcriptional profile of galectins by real-time RT-PCR. Results are expressed as galectin mRNA relative to cyclophilin A. Cell lines are presented according to androgen sensitivity and AR expression. Left, LNCaP cells [hormone-responsive (HR), AR+]; middle, 22Rv1 cells [castration-resistant (CR), AR+]; right, the more aggressive PC-3 cells (CR and AR-). Data are expressed as mean \pm SD of 4 independent experiments. B, immunocytochemical analysis of galectins in prostate cancer cells adhered onto poly-L-lysine-coated glasses (magnification, $\times 100$).

Analysis of the galectin expression profile of human primary prostate tumors

The differential expression of galectins in prostate cancer cell lines prompted us to investigate the galectin profile in prostatectomies obtained from 61 patients with newly diagnosed untreated disease. Samples included a large spectrum of prostate cancer stages (T1, T2, T3, and T4 according to TNM classification; UICC, 2002) in addition to a benign stage (BHP; Table 1). Similar to prostate cancer cell lines, Gal-1 was highly expressed in primary tumors and its expression was upregulated in more advanced lesions (Fig. 2). On the other hand, although typically expressed at lower levels, Gal-3, -4, -9, and -12 decreased gradually as the disease progressed toward more aggressive stages. Conversely, Gal-8 was expressed at moderate levels in lesions corresponding to all stages. These data delineate a "galectin-specific signature" characterized by selective up- or downregulation of galectins during prostate cancer progression and highlight a potential role for Gal-1 as a sensitive biomarker in advanced stages of the disease.

Galectin-1 is a novel target for antiangiogenic therapies in advanced human prostate cancer

Because Gal-1 expression is associated with prostate cancer aggressiveness and has emerged as a novel proangiogenic factor in other tumor types (30, 31), we asked whether this lectin was differentially expressed in tumor areas associated to blood vessels in human prostate cancer. To address this issue, we investigated whether a correlation exists between Gal-1 and CD34 expression using a human tissue array comprising 29 paired cores of invasive prostate cancer classified according to proliferation rates and cell morphology (Table 2). A positive correlation was found between Gal-1 and CD34 expression in arrays representing advanced stages of human prostate cancer (Fig. 3A). This correlation was not observed in arrays of human breast cancer (Fig. 3A), suggesting tissue-specific proangiogenic effects of this lectin.

Given the promising value of antiangiogenic therapies in castration-resistant advanced prostate cancer (32), we examined the role of Gal-1 in prostate cancer angiogenesis. We

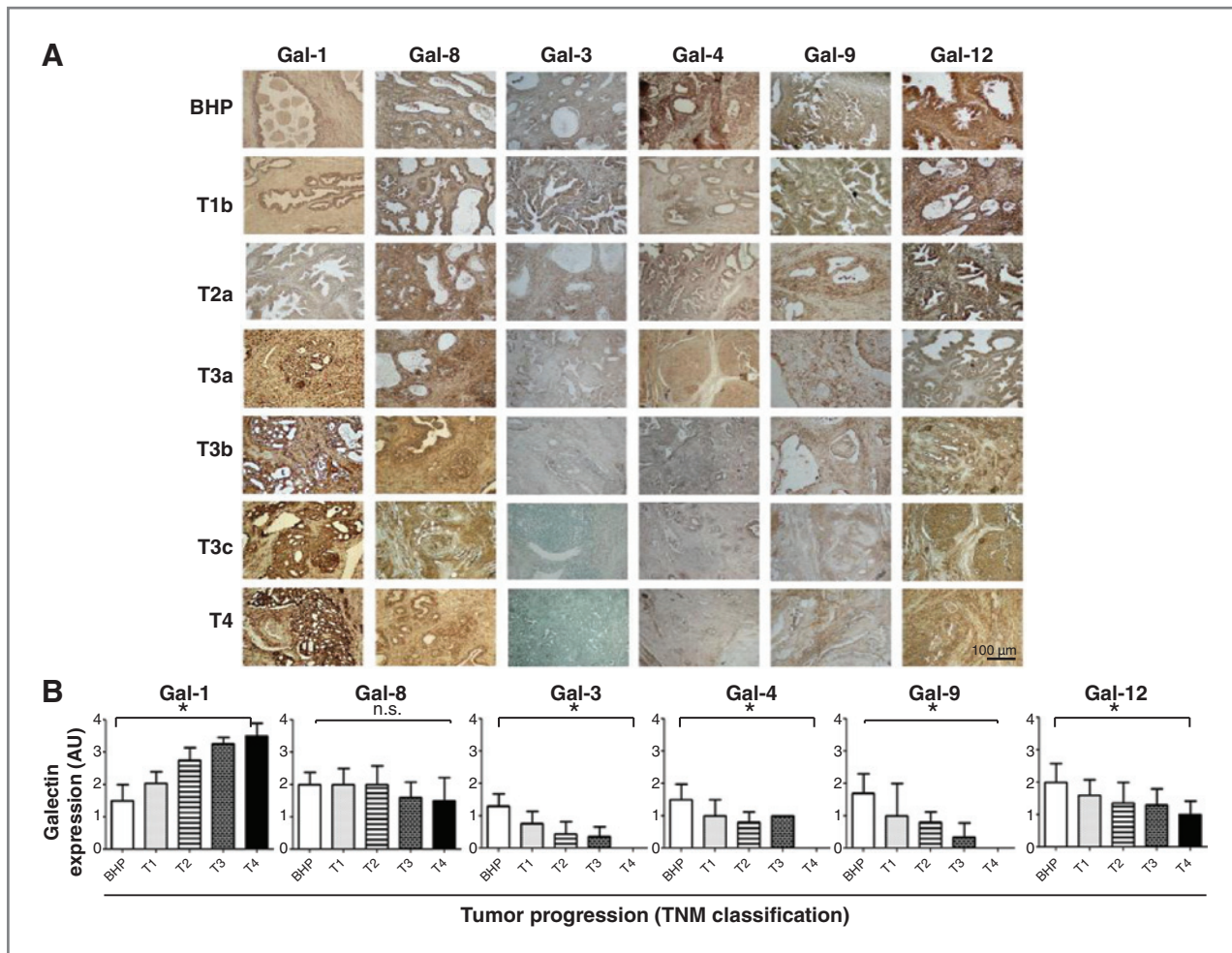


Figure 2. Galectin expression profile of human primary prostate tumors. A, radical prostatectomies from naïve patients ($n = 50$ carcinomas and $n = 11$ BHP) were arranged according to T grade (TNM classification; UICC). Galectin expression was analyzed by immunohistochemistry in paraffin-embedded tissue sections from patients (Table 1). Magnification, $\times 400$. B, galectin expression in patient samples was graded as follows: 0, negative; 1, low intensity; 2, moderate intensity; 3, high intensity; and 4, very high intensity. Results are representative (A) or are the mean \pm SD (B) of individual patient samples. *, $P < 0.05$ (Student t test); n.s., not statistically different.

evaluated the effect of conditioned medium obtained from a Gal-1–positive prostate cancer cell line (22Rv1 conditioned medium) on *in vitro* BAEC tubulogenesis. As shown in Fig. 4, conditioned medium from 22Rv1 cells (Gal-1 concentration, 10.7 ng/mL) induced the formation of tubular structures reflective of endothelial cell morphogenesis. To evaluate the involvement of Gal-1, we exposed BAEC to conditioned medium from 22Rv1 prostate cancer cells in the presence of an anti-Gal-1 neutralizing mAb (25, 26). Neutralization of soluble Gal-1 considerably reduced tube formation (3.76 ± 1.50 fold; $n = 10$) compared with BAEC exposed to prostate cancer conditioned medium in the presence of a control isotype Ab (Fig. 4A and B).

The *in vitro* effects of Gal-1–expressing prostate cancer cells on endothelial cell morphogenesis prompted us to investigate the role of this endogenous lectin in angiogenesis *in vivo* using 2 different approaches to differentiate the source of Gal-1 (tumor and microenvironment vs. tumor alone). First, we injected a Matrigel mixture containing 22Rv1 prostate cancer cells and a

blocking anti-Gal-1 mAb (or its isotype control) into nude mice. Second, we used 22Rv1 tumor cells that were transduced with a human-specific Gal-1 shRNA-coding lentivirus (Gal-1-shRNA-LV) purified to homogeneity by cell sorting. Nonsorted bulk transduced 22Rv1 cells, with partial downregulation of Gal-1, were also tested (Fig. 5A). A marked reduction of microvessel density was observed using both experimental approaches (anti-Gal-1 mAb and Gal-1 shRNA-LV), indicating that tumor cells were the main source of Gal-1, at least at early time points of tumor implantation (Fig. 5B and C). Intermediate effects were observed when Gal-1 was partially downregulated in prostate cancer cells (Fig. 5B and C). Altogether, our results reveal a key role for Gal-1 as a mediator of prostate cancer–induced angiogenesis.

As angiogenesis relies on the expression of hypoxia-regulated genes and Gal-1 is regulated by hypoxia in different tumor types (26, 33, 34), we then examined the effects of hypoxia on the galectin expression profile of human prostate cancer. For

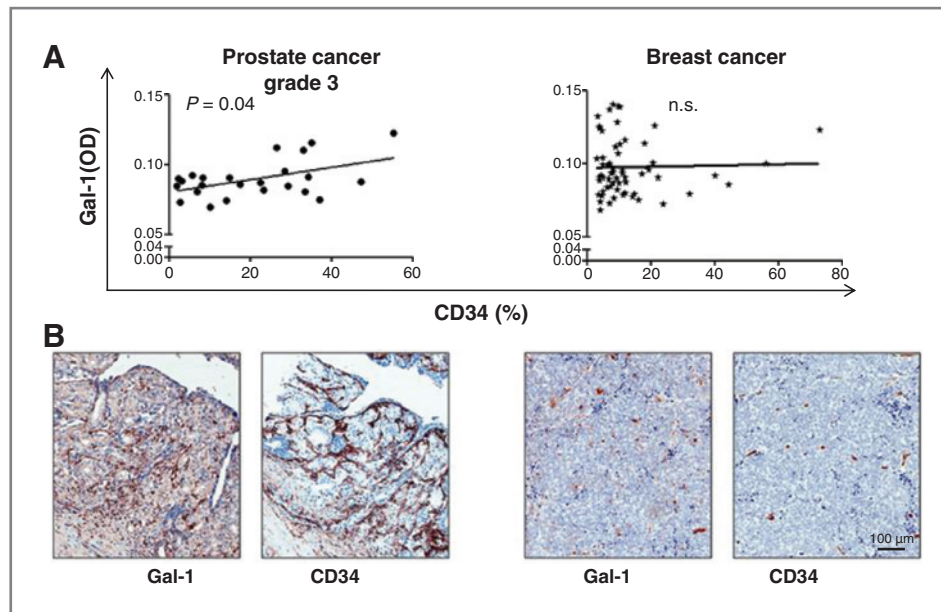


Figure 3. Gal-1 expression positively correlates with the number of CD34+ blood vessels in advanced human prostate cancer. Expression of Gal-1 and CD34 was evaluated in an invasive prostate cancer tissue microarray (slides containing 29 paired cores: BC19013, US Biomax) by immunohistochemistry. Stained slides were digitally scanned using Aperio ScanScope XT workstation and evaluated by ImageScope software. **A**, correlation between Gal-1 and CD34 expression in tumor areas of grade 3 prostate cancer (classification based on grade of proliferation and cell morphology as described in Materials and Methods). Breast cancer tissue was analyzed for comparison purposes. **B**, examples of prostate cancer samples with intense or low Gal-1 and CD34 expression. Results are representative of 29 paired core samples. P = Spearman correlation test.

this purpose, 22Rv1 prostate cancer cells were cultured under hypoxic or normoxic conditions and the galectin transcriptional profile was evaluated. We could observe no significant modification of the galectin expression profile except for Gal-1 that was modestly upregulated in response to hypoxia (1.3-fold, $P = 0.015$; data not shown). To further understand the molec-

ular mechanisms underlying Gal-1–mediating angiogenesis, we screened different molecules classically associated with angiogenesis (bFGF, VEGF-A, CD142, uPA, CXCR4, PDGF-AA, and MMP-9 as activators of angiogenesis; TSP-1, TIMP-1, CXCL10, and SPP1 as inhibitors of angiogenesis; and CA-IX as a marker of hypoxia) in Gal-1–silenced human prostate

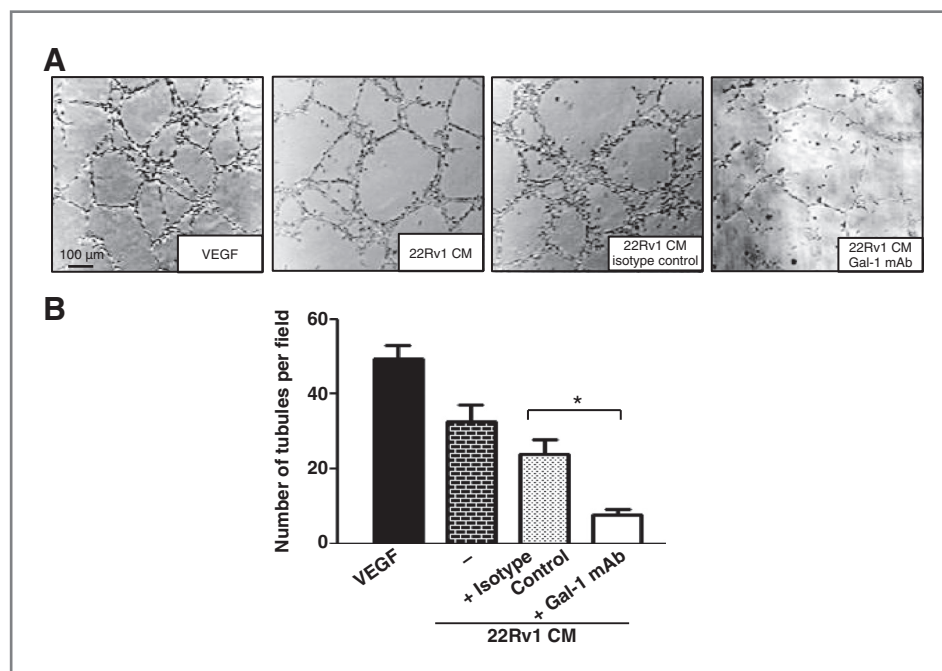
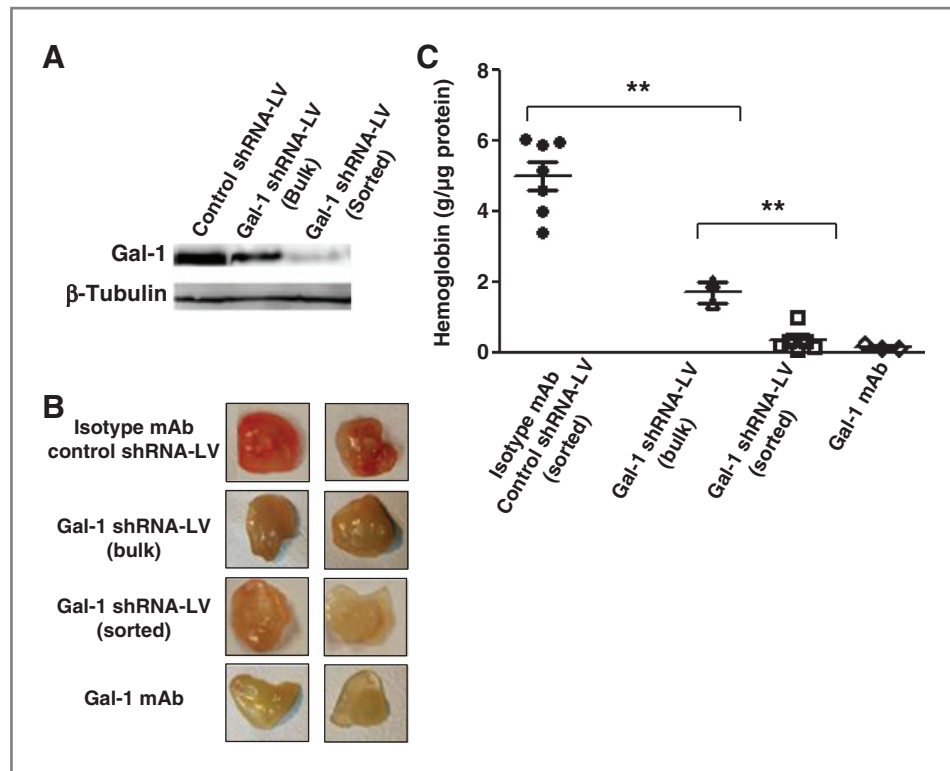


Figure 4. Prostate cancer–derived Gal-1 promotes endothelial cell morphogenesis. **A**, *in vitro* formation of tubular structures by BAEC cultured in Matrigel with 22Rv1-conditioned media (22Rv1 CM) in the absence or presence of an anti-Gal-1 mAb (F8.G7) or isotype control mAb. Recombinant VEGF was used as a positive control. **B**, quantification of the number of tubular structures per field. Results are representative (**A**) or are the mean \pm SD (**B**) of 3 independent experiments. *, $P < 0.001$ (Student t test).

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Figure 5. Prostate cancer-derived Gal-1 promotes angiogenesis *in vivo*. Nude mice were injected with 5×10^6 22Rv1 prostate cancer cells incorporated in Matrigel plugs. The role of Gal-1 was assessed by 2 different strategies: (i) using 22Rv1 cells transduced with Gal-1 shRNA-coding lentivirus (bulk; $n = 3$ or sorted cells; $n = 8$ with ~40% and 80% Gal-1 downregulation, respectively) compared with control shRNA-LV cells ($n = 8$); or (ii) by adding an anti-Gal-1 mAb ($n = 3$) or isotype control ($n = 3$). A, immunoblot of Gal-1 in 22Rv1 cells. First lane, cells transduced with a control shRNA-LV and sorted according to GFP expression; second lane, Gal-1 shRNA-LV (bulk); third lane, cells transduced with a Gal-1 shRNA-LV and sorted. B, representative photographs of *in vivo* plugs at day 5. C, hemoglobin content in plugs at day 5, normalized to microgram of protein. Results are representative (A and B) or are the mean \pm SD (C). Data show individual samples analyzed and the mean of individual plugs. **, $P < 0.005$ (Student *t* test).



cancer tumors. RNA was purified from *in vivo* plugs generated by injection of Gal-1-sufficient and Gal-1-silenced human 22Rv1 cells into nude mice, and angiogenesis-related genes were determined by real-time RT-PCR. Silencing Gal-1 in

prostate cancer cells growing in Matrigel plugs *in vivo* did not alter expression of angiogenesis-related genes neither in the tumor itself (human) nor in the tumor microenvironment (mouse; Fig. 6). These results suggest that Gal-1-induced

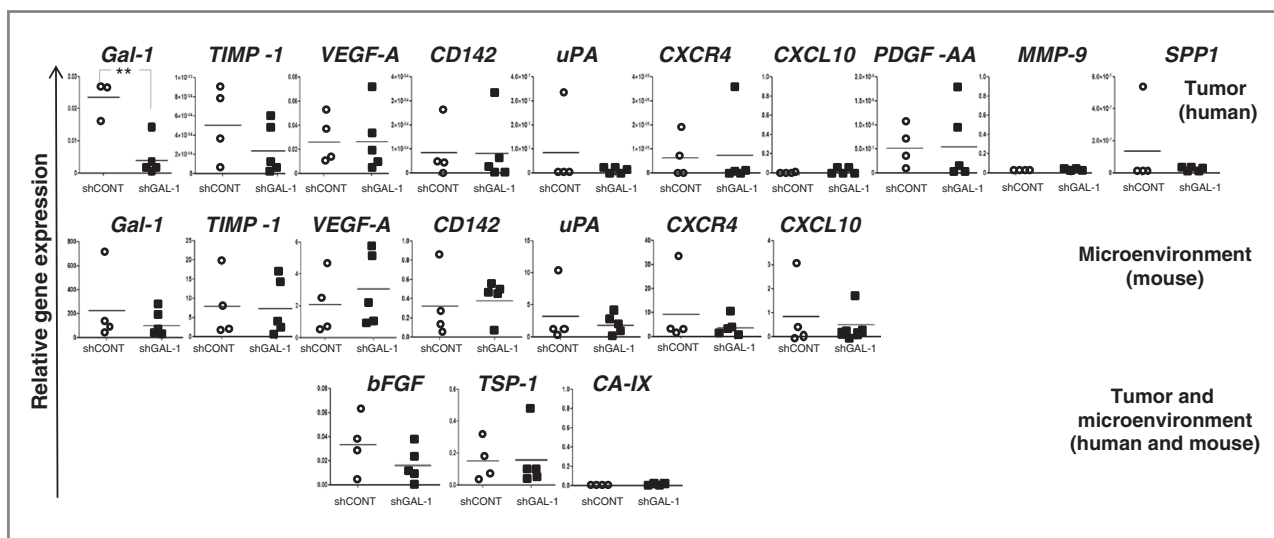


Figure 6. Gal-1 silencing does not alter expression of angiogenesis-related genes in prostate cancer. Total RNA was extracted from plugs generated *in vivo* by injection of Gal-1-sufficient ($n = 5$) and Gal-1-silenced ($n = 5$) human 22Rv1 cells into nude mice. Angiogenesis-related genes, including activators of angiogenesis (bFGF, VEGF-A, CD142, uPA, CXCR4, PDGF-AA, and MMP-9), inhibitors of angiogenesis (TSP-1, TIMP-1, CXCL10, and SPP1), and a marker of hypoxia (CA-IX) were screened by real-time RT-PCR relative to *cyclophilin A*. This screening allows the distinction of human genes (derived from the tumor) and mouse genes (reflecting the mouse microenvironment). Bottom, includes genes derived from both tumor and microenvironment, as these primers react with both human and mouse genes. Data show individual samples analyzed and the mean of individual plugs. **, $P < 0.01$ (Student *t* test).

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prostate cancer angiogenesis is independent of the upregulation or downregulation of classic proangiogenic or antiangiogenic factors and places Gal-1 as a critical mediator of tumor angiogenesis.

Discussion

Prostate cancer is no longer viewed as a disease of abnormally proliferating epithelial cells, but rather as a disease involving complex interactions between prostate cancer epithelial cells and the tumor microenvironment. Multiple signaling pathways and biologic events mediate tumor growth, including AR signaling, tyrosine kinase receptor signaling, angiogenesis, and tumor-immune escape (35). Interactions between multivalent lectins and glycans participate in this complex network by modulating stromal, endothelial, and immune cell compartments (6, 7). Although original assumptions based on conserved carbohydrate specificity and structural homology suggested that galectins may have redundant functions, recent information challenged this view showing specific roles for each member of the galectin family in the regulation of tumor cell invasiveness, inflammation, and angiogenesis (7). In search for novel biomarkers and therapeutic targets, here we identified a "galectin-specific signature" associated with prostate cancer progression. Galectin expression was profiled in prostate cancer cell lines with diverse androgen-dependence properties and AR expression and in human primary tumors obtained from treatment-free patients at different stages of the disease.

Originally described as PCTA-1, Gal-8 has been reported to be ubiquitously expressed in several tissues, but is upregulated in prostate cancer (29). Our results confirm that this "tandem-repeat" galectin is expressed by prostate cancer cell lines and primary tumors, but indicate that the degree of Gal-8 expression is comparable in all tumor stages. Given the complexity of Gal-8 isoforms, the variability of its intracellular localization and its ubiquitous expression pattern, this galectin is likely to be an important component of prostate cancer biology (29), including modulation of cell proliferation, adhesion, and angiogenesis (22, 36).

Interestingly, our results reveal that Gal-3, -4, -9, and -12 are downmodulated in advanced stage primary tumors. These data are consistent with earlier reports showing that Gal-3 expression decreases during tumor growth (10, 18) through mechanisms including promoter methylation (19) and metalloproteinase-mediated protein cleavage (20). Depending on its selective intracellular or extracellular localization, different biologic properties have been assigned to Gal-3, resulting in a dual pro- or antitumorigenic effect (10, 37). Our results suggest that downregulation of Gal-3, combined with the expression of other galectin members, is a hallmark of prostate cancer progression. More importantly, our findings show that Gal-1 is the most abundantly expressed galectin in prostate cancer and its expression correlates with disease severity, underscoring the relevance of this endogenous lectin as a possible biomarker and therapeutic target in high-grade castration-refractory prostate cancer.

Previous studies aimed at delineating the galectin transcriptional profile of a panel of human tumor cell lines revealed that all prostate cancer cell lines analyzed (DU145, PC-3, and LNCaP) were negative for Gal-2, -4, -7, and -9 but expressed considerable amounts of Gal-8 (38). Moreover, Gal-1 and -3 were expressed in DU.145 and PC-3 but not in the LNCaP cell line (38). In contrast to these findings, we detected significant expression of Gal-1 in LNCaP cells both at the mRNA and protein levels, although at 20-fold lower levels than the androgen unresponsive 22Rv1 and PC-3 tumor cells. Moreover, Gal-1 expression augmented when LNCaP cells were cultured for several weeks in the absence of hormones (LNCaP-CR; Supplementary Fig. S1A). As castration-sensitive or resistant LNCaP cells were both PSA-positive and responsive to an AR agonist (R1881; Supplementary Fig. S2B), the discrepancies in Gal-1 expression among different studies might reflect different culture conditions, cell line sources, or selection protocols. In this regard, our studies were conducted on prostate cancer cells isolated during the log phase of growth and the results were substantiated using primary tumors isolated at different stages of the disease.

Given the pleiotropic functions of Gal-1 in the tumor microenvironment, including its role in angiogenesis (26, 31, 39, 40), cell adhesion and invasiveness (16, 30), and immunosuppression (23–25, 41), upregulation of Gal-1 may dramatically influence prostate cancer progression. In this regard, Gal-1 is expressed in endothelial cells (42–44) and is upregulated in various cancer types (6). Here, we show that Gal-1 is the most highly expressed and regulated galectin in the prostate cancer microenvironment and plays essential roles in prostate cancer angiogenesis. The role of Gal-1 in angiogenesis seems to be tissue-specific as Gal-1 expression correlates with endothelial cell markers in advanced prostate cancer but not in human breast cancer. These findings are consistent with the ability of Gal-1 to induce angiogenesis in oligodendroglioma (30), B16 melanoma (31), and Kaposi's sarcoma (26) but not in other tumor types such as Lewis lung carcinoma (41).

Selective silencing strategies in tumors clearly showed that the main cellular source of Gal-1 is represented by tumor cells. However, mechanisms by which endothelial cells capture Gal-1 from the tumor microenvironment or tumor-induced endothelial cell activation upregulates Gal-1 expression have also been described (31, 39). Moreover, as Gal-3 and -8 also contribute to angiogenesis in other tumor types, the spatiotemporal regulation of distinct members of the galectin family might ultimately dictate the vascularization phenotype (36, 45, 46, 47). Finally, Gal-1 silencing in prostate cancer cells did not alter the expression of classic proangiogenic or antiangiogenic mediators neither in tumor cells nor in the tumor microenvironment, highlighting a direct and critical role of this lectin in prostate cancer angiogenesis.

In summary, our findings identify a distinctive "galectin signature," which delineates tumor progression in human prostate cancer and highlight a major role of Gal-1 as a novel target of antiangiogenic therapies in advanced castration-resistant stages of prostate cancer, where effective treatments are still lacking.

Disclosure of Potential Conflicts of Interest

D.O. Croci, M.A. Shipp, J.L. Kutok, S.J. Rodig, and G.A. Rabinovich have ownership interest in a patent application regarding composition, kits and methods for the diagnosis, prognosis, and monitoring of immune disorders using galectin-1; and a patent application regarding application composition, kits and methods for the modulation of immune responses using galectin-1. No potential conflicts of interest were disclosed by the other authors.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Casas
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Acknowledgments

The authors thank Drs. Karim Fizazi and Catherine Gaudin (INSERM U981; IGR-France), Geraldine Gueron (University of Buenos Aires), Mr. Juan Stupirski (IBYME), and Carla Saleh (Pasteur Institute, France) for help and advice.

Grant Support

This study was supported by grants from Prostate Action (UK) to G.A. Rabinovich, D.J. Laderach, and D. Compagno, Agencia Nacional de Promoción Científica y Técnica Argentina (ANPCyT; PICT 2008-134 to D.J. Laderach; PICT 2010-870 to G.A. Rabinovich), Programa de Cooperación Franco-Argentino ECOS-Sud (A10S03 to G.A. Rabinovich, A. Chauchereau, D.J. Laderach, and D. Compagno), Fundación Sales to G.A. Rabinovich, University of Buenos Aires to G. A. Rabinovich, and Association pour la Recherche sur les Tumeurs de la Prostate (ARTP), France, to D. Compagno.

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Received April 3, 2012; revised September 10, 2012; accepted October 8, 2012; published OnlineFirst October 29, 2012.

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