EphB4 Expression and Biological Significance in Prostate Cancer

Guangbin Xia,1 S. Ram Kumar,2,3 Rizwan Masood,2 Sutao Zhu,1 Ramchandra Reddy,6 Valery Krasnoperov,4 David I. Quinn,1 Susan M. Henshall,7 Robert L. Sutherland,7 Jacek K. Pinski,1 Siamak Daneshmand,4 Maurizio Buscarini,4 John P. Stein,1 Chen Zhong,2 Daniel Broek,5 Pradip Roy-Burman,2 and Parkash S. Gill2,6

Abstract

Prostate cancer is the most common cancer in men. Advanced prostate cancer spreading beyond the gland is incurable. Identifying factors that regulate the spread of tumor into the regional nodes and distant sites would guide the development of novel diagnostic, prognostic, and therapeutic targets. The aim of our study was to examine the expression and biological role of EphB4 in prostate cancer. EphB4 mRNA is expressed in 64 of 72 (89%) prostate tumor tissues assessed. EphB4 protein expression is found in the majority (41 of 62, 66%) of tumors, and 3 of 20 (15%) normal prostate tissues. Little or no expression was observed in benign prostate epithelial cell lines, but EphB4 was expressed in all prostate cancer cell lines to varying degrees. EphB4 protein levels are high in the PC3 prostate cancer cell line and several folds higher in a metastatic clone of PC3 (PC3M) where overexpression was accompanied by EphB4 gene amplification. EphB4 expression is induced by loss of PTEN, p53, and induced by epidermal growth factor/epidermal growth factor receptor and insulin-like growth factor-I/insulin-like growth factor-IR. Knockdown of the EphB4 protein using EphB4 short interfering RNA or antisense oligodeoxynucleotide significantly inhibits cell growth/viability, migration, and invasion, and induces apoptosis in prostate cancer cell lines. antisense oligodeoxynucleotide targeting EphB4 in vivo showed antitumor activity in murine human tumor xenograft model. These data show a role for EphB4 in prostate cancer spreading and extracapsular invasion. They also suggest targets for treatment of hormone-unresponsive prostate cancers (11).

EphB4 is a member of the largest known family of receptor protein tyrosine kinases, which plays an important role in a variety of processes during embryonic development including pattern formation, cell aggregation and migration, segmentation, neural development, angiogenesis, and vascular network assembly (12–15). Specifically, EphB4, along with its ligand, Ephrin-B2, is essential for vascular remodeling, maturation and directed growth. Both Ephrin-B2 and EphB4 targeted gene knock-outs in mice show lack of remodeling of the primitive vascular plexus to form mature vascular structures (13). EphB4 expression is restricted to venous endothelial cells, whereas Ephrin-B2 is restricted to arterial endothelial cells (16, 17). EphB4 and Ephrin-B2 are also essential for defining the boundaries between arterial and venous domains (17), which persist in the adult (18).

EphB4 selectively binds Ephrin-B2 and not other Ephrin B ligands (19, 20). The B class Ephrins are comprised of transmembrane proteins with an intracellular domain that can elaborate growth factor signaling (21). Ligand-receptor binding leads to protein clustering followed by receptor activation (21). Furthermore, interaction between EphB4 and Ephrin-B2 can provoke bidirectional signaling (23, 24). It has been shown in vitro that EphB4-mediated forward signaling restricts intermingling of cells and supports cellular segregation, whereas reverse signaling from Ephrin-B2 stimulates migration and sprouting angiogenesis (25, 26). Downstream signaling by EphBs regulates cell proliferation, cytoskeletal organization, and migration (27–29).

We focused on EphB4 for its potential role in tumor biology because of its location on chromosome 7q22, a locus frequently amplified in cancers including prostate cancer, and because of its expression in various cancers (30–34). However, little is known about the regulation and role of EphB4 in tumor cell biology. In this report, we show that EphB4 is expressed in prostate tumor cells, and its induction may occur either through gene amplification or the regulatory effect of prosurvival signaling molecules. Furthermore, inhibition of EphB4 expression inhibits tumor cell survival, migration, and invasion. These findings indicate that EphB4 is a novel target for diagnostic and therapeutic application in prostate cancer.

Materials and Methods

Reagents. Neutralizing IGF-IR antibody was obtained from R&D Systems, Inc. (Minneapolis MN), anti-IGF-IR, -EGFR, -EphB4 (C-16), and CD31 (M20) from Santa Cruz Biotech (Santa Cruz, CA), anti-phosphotyrosine (4G10) from Upstate, Inc., (Chicago, IL), anti-Ki-67 from...
DAKO (Carpinteria, CA), anti-human Fc from Jackson Laboratory (Bar Harbor, ME), anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Research Diagnostics, Inc. (Flanders, NJ), and anti-actin monoclonal antibody from Sigma Chemical Co. (St. Louis, MO). Media and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA). AG 1478 [4′-(3′-Chloroanilino)-6′-dimethoxyquinazoline] was synthesized by Calbiochem (San Diego, CA). Ephrin-B2/Fc chimeric protein was from R&D Systems, Inc., and human IgG Fc fragment was from Jackson Laboratory.

**Gene expression microarray studies and analysis.** Fresh-frozen prostate tumor tissues were harvested from 72 patients treated with radical prostatectomy for clinically localized prostate cancer at St. Vincent's Hospital, Sydney between 1996 and 2000 as previously described (35, 36). Total RNA was extracted with Trizol reagent (Life Technologies, Rockville, MD) and was reverse-transcribed using a primer containing oligo (dT) and a T7 promoter sequence. The resulting cDNAs were then in vitro–transcribed in the presence of biotinylated nucleotides (Bio-11-CTP and Bio-16-UTP) using the T7 MEGAscript kit (Ambion, Austin, TX). The biotinylated targets were hybridized to the Eos Hu03, a customized oligonucleotide array comprising 59,000 oligonucleotide probe-sets for the interrogation of a total of 46,000 gene clusters that were based on the first draft of the human genome. Hybridization signals were visualized using phycoerythrin-conjugated streptavidin (Molecular Probes, Eugene, OR). EphB1, EphB2, and EphB4–specific gene expression data was examined in all cases and relative signal intensity was assessed for each of these three receptors.

**EphB4 short interfering RNAs and antisense oligodeoxynucleotides.** Phosphorothioate-modified EphB4-specific antisense and sense oligodeoxynucleotides were used: sense, TCC TGC AAG GAC TTC AC (5′–3′); antisense-1, GTG CAG GGA TAG CAG GGC CAT (5′–3′); antisense-2, 10-AG GAC GCC TCG CTC AGA AA (5′–3′); antisense-9, 9-CAT GCG GAC TTC AGG (5′–3′); and antisense-10, ATG GAC GCC TCG CTC AGA AA (5′–3′), which were synthesized and purified using Qiagen reagents (Valencia, CA). Short interfering RNA (siRNA) sequences were green fluorescent protein (GFP)–siRNA, 5′-GGCUGACCCUGAGGCUAUCUU-3′; EphB4–siRNA (50), 5′-GAGACCUCGUGACACAAUGU-3′; EphB4–siRNA (472), 5′-GGUGAUGUGCUAAGGCGCUUGU-3′. EphB4–siRNA (2303) 5′-CUUUCCGACUCCACCUAU-3′ which were synthesized at the USC Norris Comprehensive Cancer Center Microchemical Core Laboratory.

**Cell lines and culture.** The prostate cancer cell lines, PC3, PC3M, DU145, LNCaP, and BPH-1 were obtained and cultured as described previously (7). Kaposi sarcoma cell line (SK) was obtained from American Type Culture Collection (Rockville, MD).

**Generation of EphB4 monoclonal antibody.** The extracellular domain of EphB4 was cloned into pEEX-F1 to generate glutathione S-transferase–fused extracellular domain. EphB4–extracellular domain expressed as a glutathione S-transferase fusion protein in BL21 E. coli was purified by affinity chromatography and the glutathione S-transferase domain was cleaved by thrombin. A monoclonal antibody was generated and the sensitivity and specificity of the antibody was reconfirmed by Western blot with whole cell lysate of 293 cells stably transfected with EphB4. This monoclonal antibody was used for the Western blot and immunoprecipitation in this study.

**One-Step RT-PCR and quantitative PCR.** Total RNA was extracted using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) from prostate cancer specimens and adjacent normal specimens. For quantitative RT-PCR first-strand cDNA was synthesized from 5 μg of total RNA using SuperScript III (Invitrogen). Quantitative RT-PCR was done on the Stratagene MX3000P system (Stratagene, La Jolla, CA) using SYBR Green 1 Brilliant Mastermix according to the manufacturer’s instructions. Optimized reactions for EphB4 and β-actin (used as the normalizer molecule for comparative gene expression) were 150 nmol/L each of the forward primers (β-actin, 5′-GGA CCT GAC TGA CTA CTA A-3′; EphB4, 5′-AAG GAG ACC TTC ACC GTC TT-3′) and reverse primers (β-actin, 5′-TGG AAG GAT GTT TCG TGG AT-3′; EphB4, 5′-TGG AGT CAG GGT CAC AGT CA-3′) with thermal profile of 95°C for 10 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 1 minute, and 72°C for 1 minute. The specificity of the amplification was confirmed by the presence of a single dissociation peak. All reactions were done in triplicate and with no reverse transcriptase or no template as negative controls.

**Gene amplification was analyzed by quantitative PCR.** The primers were EphB4-forward, 5′-TCC TGC AAG GAC TTC AC-3′; EphB4-reverse, 5′-CAG AGG CCT CGC AAC TAC AT-3′; GAPDH-forward, 5′-GAG GGG TGA TGT GGG GAG TA-3′; and GAPDH-reverse, 5′-GAG GTT CCC GTT CAG CTC AG-3′. DNA was extracted from peripheral blood mononuclear cells of normal donors and the prostate cancer cell lines using the Blood and Cell Culture DNA Midi kit from Qiagen according to the manufacturer’s instructions. Quantitative PCR was done on 50 ng of DNA at the annealing temperature of 64°C for both primers. Amplification signal for EphB4 was normalized to GAPDH and gene copy number was normalized to healthy donor peripheral blood mononuclear cells.

**Immunohistochemistry.** Freezing solution (optimum cutting temperature [OCT]) embedded tissues were sectioned at 5 μm and fixed in phosphate-buffered 4% paraformaldehyde. Sections were washed thrice for 5 minutes in PBS and endogenous peroxidase was blocked by incubation in 0.3% H2O2 in PBS for 10 minutes at room temperature. Sections were incubated with EphB4 (C-16) antibody (1:50) for 1 hour at room temperature, followed by three washes in PBS and incubation with donkey anti-goat secondary antibody (Santa Cruz Biotechnology) for 1 hour at room temperature. After three washes in PBS, peroxidase activity was localized by incubation in 3,3-diaminobenzidine substrate solution (Vector Laboratories, Inc., Burlingame, CA) for 10 minutes at room temperature. Sections were counterstained with hematoxylin for 20 seconds, dehydrated and mounted. The negative control for staining was substitution of normal IgG isolate for primary antibody. Immunohistochemical staining on the formalin-fixed prostate tissues microarray (BioMedia, Foster City, CA) and animal tumors was done using avidin–biotin complex method staining system (Santa Cruz Biotechnology) according to the manufacturer’s instructions. The antibodies used were EphB4 (C-16, Santa Cruz Biotechnology; 1:100 dilution), CD3 (M20, Santa Cruz Biotechnology; 1:200 dilution) and Ki-67 (DAKO; 1:100 dilution).

**Western blot.** Cell lysates were prepared using cell lysis buffer (GeneHunter, Basgukke, TN) supplemented with protease inhibitor cocktail (Pierce, Rockford, IL), unless otherwise noted. Total protein was determined using the DC reagent system (Bio-Rad, Hercules, CA). Typically, 20 μg whole cell lysate was run on 4% to 20% Tris-glycine gradient gel. The samples were electrotransferred to polyvinylidene difluoride membrane and nonspecific binding was blocked in TBST buffer (0.5 mmol/L Tris-HCl, 45 mmol/L NaCl, 0.05% Tween 20 [pH 7.4]) containing 5% nonfat milk. Membranes were first probed with primary antibody overnight, washed and probed with the secondary antibody and developed. The membranes were then stripped with Restore Western blot stripping buffer (Pierce) and probed with β-actin to confirm equivalent loading and transfer of protein. Signal was detected using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

**Phosphorylation analysis.** Cells growing in 60 mm dishes were either serum-starved (1% FBS supplemented RPMI 1640 for 24 hours) or cultured in normal conditions (10% FBS) and then treated with or without clustered 1 μg/mL mouse Ephrin-B2/Fc for 10 minutes to activate EphB4 receptor. Cleared cell lysates were incubated with EphB4 monoclonal antibody overnight at 4°C. Antibody–antigen complexes were immunoprecipitated by the addition of 20 μL of protein G-Sepharose in 20 mmol/L sodium phosphate (pH 7.0) with incubation overnight at 4°C. Immunoprecipitates were analyzed by Western blot with phosphotyrosine-specific antibody at 1:1000 dilution. To monitor immunoprecipitation efficiency, a duplicate membrane was probed for EphB4.

**Transient transfection and sorting of transfected cells.** PC3 cells were cotransfected with pMACS 4.1 coding for a truncated CD4 and wild-type p53 (pC53-SN3) or PTEV vector or both using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. The molar ratio of CD4 to p53 or PTEV or vector was 1:3 and total plasmid was 24 μg for a 10 cm2 dish of 90% confluent cells using 60 μL of LipofectAMINE 2000. Twenty-four hours after transfection, a single cell suspension was made and sorted using truncated CD4 as a surface marker according to the manufacturer’s protocol (Miltenyi Biotec, Germany). Equal numbers of sorted cells were lysed in 1× SDS sampling buffer and analyzed by Western blot.
Study of insulin-like growth factor and epidermal growth factor signaling pathway on the expression of EphB4. PC3 cells were seeded onto six-well plates and cultured until 80% confluent and treated with 2 μg/mL neutralizing IGF-IR monoclonal antibody, MAB931 (37), or with 1 μmol/L AG 1478, a strong EGFR-kinase inhibitor (38) for 24 hours. Crude cell lysates were analyzed by Western blot to detect EphB4 and β-actin as described above. Band density was quantified with the Bio-Rad QuantityOne System software.

Cell viability assay. PC3 cells were seeded on 48-well plates at a density of 1 × 10⁴ cells per well in a total volume of 200 μL. Medium was changed after the cells were attached and triplicate samples of cells were treated with various concentrations (1-10 μmol/L) of EphB4 antisense or sense oligodeoxynucleotides as control. After 3 days, medium was changed and fresh oligodeoxynucleotides were added. Following a further 48 hours incubation, viable cells were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as described previously (39). EphB4 siRNAs (10-100 nmol/L) or control siRNAs were introduced into triplicate samples of 1 × 10⁴ PC3 cells per well of a 48-well plate using 2 μL of LipofectAMINE 2000 according to the manufacturer’s instructions. Four hours post-transfection, the cells were returned to growth medium (RPMI 1640 supplemented with 10% FBS). Viable cells were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide 48 hours following transfection. The SLK cell line, which lacks EphB4 expression, was used as a negative control.

Wound healing migration assay. PC3 cells were seeded onto six-well plates and cultured until confluent. Antisense-10 or sense oligodeoxynucleotides (10 μmol/L) as control were introduced to the wells as described above. After 12 hours before wounding the monolayer by scraping it with a sterile pipette tip. Medium was changed to RPMI 1640 supplemented with 5% FBS and fresh oligodeoxynucleotides. Confluent cultures transfected with 50 nmol/L EphB4 siRNA 472 or GFP siRNA 12 hours prior to wounding were also examined. The healing process was examined dynamically and recorded with a Nikon Coolpix 3000 digital camera with microscope adapter.

Invasion assay. PC3 cells were transfected with EphB4 siRNA 472 or GFP siRNA using LipofectAMINE 2000 and 6 hours later 0.5 × 10⁵ cells were transferred into 8 μm Matrigel precoated inserts (BD Bioscience, Palo Alto, CA). The inserts were placed in companion wells containing RPMI supplemented with 5% FBS and 5 μg/mL fibronectin as a chemoattractant. Following 22 hours of incubation, the inserts were removed and the noninvasive cells on the upper surface were removed with a cotton swab. The cells on the lower surface of the membrane were fixed in 100% methanol for 15 minutes, air-dried, and stained with Giemsa stain for 2 minutes. The cells were counted in five individual high-powered fields for each membrane under a light microscope. Assays were done in triplicate for each treatment group.

Cell cycle analysis. PC3 cells (80% confluence) in six-well plates were transfected with EphB4 siRNA 472 (100 nmol/L) using LipofectAMINE 2000. Twenty-four hours after transfection, cells were trypsinized, washed in PBS and incubated for 1 hour at 4°C in 1 mL of hypotonic solution containing 50 μg/mL propidium iodide, 0.1% sodium citrate, 0.1 Triton X-100, and 20 μg/mL DNase-free RNaseA. Cells were analyzed in linear mode at the USC Flow Cytometry Facility. Results were expressed as percentages of elements detected in the different phases of the cell cycle, namely sub-G₀ peak (apoptosis), G₀/G₁ (no DNA synthesis), S (active DNA synthesis), G₂ (premitosis), and M (mitosis).

Apoptosis ELISA. Apoptosis was studied using the Cell Death Detection ELISA⁴⁺ Kit (Roche, Piscataway, NJ) according to the manufacturer’s instructions. Briefly, PC3 cells in 24-well plates cultured to 80% confluence were transfected using LipofectAMINE 2000 with various concentrations of total cell lysate and Western blot for pTyr (top right) and EphB4 mRNA level was quantitated by real-time RT-PCR (bottom right).

Figure 1. Expression of EphB4 in prostate cell lines. A, Western blot of total cell lysates of various prostate cancer cell lines, benign prostate hyperplasia (BPH) epithelial cell line, and SLK cells probed for EphB4. The membrane was stripped and reprobed for β-actin to show equivalent loading and transfer of proteins. B, gene amplification in prostate cancer cell lines by quantitative PCR as described in Materials and Methods. Columns, mean; bars, ± SD. C, expression of EphB4 copy number in duplicate samples relative to peripheral blood mononuclear cells. D, phosphor-yrosine phosphorylation of EphB4 in PC-3 cells determined by Western blot. PC-3 cells cultured under normal conditions (RPMI 1640 supplemented with 10% FBS) or serum-starved for 24 hours were exposed to 1 μg/mL Ephrin-B2 for 10 minutes. Cell lysates were immunoprecipitated with EphB4 monoclonal antibody and analyzed by Western blot for phosphotyrosine. A duplicate blot was probed for total EphB4. D, PC3 cells were treated with varying concentrations of Ephrin-B2/Fc or Fc alone for the time periods shown. Total EphB4 and β-actin was measured by Western blot (left), EphB4 phosphorylation status was assessed by immunoprecipitation of EphB4 from 50 μg of total cell lysate and Western blot for pTyr (top right) and EphB4 mRNA level was quantitated by real-time RT-PCR (bottom right).
(0-100 nmol/L) of EphB4 siRNA or GFP siRNA. Sixteen hours later, 
cells were detached and 1 × 10⁷ cells were incubated in 200 μL lysis 
buffer. Nuclei were pelleted by centrifugation and 20 μL of supernatant 
containing the mono- or oligonucleosomes was taken for ELISA analysis. 
Briefly, the supernatant was incubated with anti-histone-biotin and anti-
DNA-POD in streptavidin-coated 96-well plates for 2 hours at room 
temperature. The color was developed with ABST and absorbance at 405 
nm was read in a microplate reader (Molecular Devices, Sunnyvale, CA). Apoptosis was detected in deparaffinized sections of tumor tissue from 
murine xenograft studies using the in situ cell death detection kit (Roche) 
according to manufacturer's instructions.

In vivo tumor growth studies. Balb/C nu/nu mice (male, 9 weeks old) 
were implanted with tumor cells propagated in vitro. Orthotopic xenografts 
were done under ketamine anesthesia. A lower abdominal midline incision 
was made. The posterior face of prostatic lobe was exposed and PC3 cell 
(1 × 10⁷) in 20 μL PBS was injected under the capsule of prostate. The 
abdominal wound was closed in two layers with 4-0 surgical sutures. Mice 
bearing the xenografts were treated with EphB4 antisense-10 or sense oligodeoxynucleotide dissolved in sterile physiologic saline (0.9% NaCl) 
given i.p. at a dose of 20 mg/kg, daily for 3 weeks (n = 6).

Statistical analysis. The significance of EphB4 expression in normal and 
prostate cancer tissues was analyzed by χ² test. Tumor volumes in vivo and 
number of CD31-positive vessels and Ki-67 or terminal nucleotidyl 
transferase–mediated nick end labeling positive cells were compared with 
Students' t test and P < 0.05 was accepted for significance.

Results

Expression of EphB4 in prostate cancer. We first examined 
the expression of EphB4 protein in a variety of prostate cancer cell 
lines by Western blot. Prostate cancer cell lines uniformly 
expressed EphB4 but showed a marked variation in expression 
protein level. The levels were relatively high in PC3 and even higher 
in PC3M, a metastatic clone of PC3, whereas benign prostate 
epithelial cells and SLK cells showed no expression (Fig. 1 A). Using 
quantitative PCR, we found that the gene copy number of EphB4 
was amplified in PC3M when compared with peripheral blood 
mononuclear cells or PC3 cells (Fig. 1B). We next examined if 
EphB4 on tumor cells is functional by testing whether EphB4 is 
phosphorylated in response to ligand (Ephrin-B2). A low-level basal 
signal was detected in cells grown in serum-containing conditions, 
and this signal markedly increased following optimal time exposure 
to 10 μg/mL ligand, Ephrin-B2/Fc (Fig. 1C). Serum-deprived PC3 
cells showed no basal phosphorylation of EphB4 (Fig. 1C). Thus,

![Image of prostate tumor specimens](image-url)

Figure 2. Expression of EphB4 in prostate tumor specimens. A, immunostaining on clinical prostate cancer samples: a, b, e, and g, prostate cancer showing EphB4 in tumor cells; c, isotype control IgG was used in place of primary antibody to probe a serial section of (a); d, lack of EphB4 staining in benign prostate hyperplasia; f, high-power view of (e) showing membrane staining of EphB4; h, EphB4 signal is blocked when a serial section of (g) is probed with primary antibody premixed with antigen. B, summary of EphB4 staining in formalin-fixed prostate tissue microarray consisting of 62 prostate cancers and 20 normal prostate 
tissues. C, gene expression for EphB1, EphB2, and EphB4 by DNA microarray in 72 prostate cancer tissues. EphB4 was expressed in 64 (89%) cases.
EphB4 in PC3 cells is functional. We next determined the fate of EphB4 following treatment with Ephrin-B2/Fc for prolonged periods. EphB4 levels began to decline following 4 hours of stimulation with 3 or 10 μg/mL Ephrin-B2/Fc, whereas 1 μg/mL of Ephrin-B2/Fc or Fc (10 μg/mL) had no effect (Fig. 1, left). These results are expected due to the endocytosis of receptor-ligand complex and degradation (50, 51). Concomitant to the decrease in EphB4 level, the extent of receptor phosphorylation also declined. Whereas robust phosphorylation was observed following stimulation with 10 μg/mL Ephrin-B2/Fc for 15 minutes, there was no detectable phosphorylation after 8 or 24 hours of stimulation (Fig. 1D, top right). EphB4 mRNA levels were quantitated by real-time RT-PCR in PC3 cells treated simultaneously with Ephrin-B2/Fc (10 μg/mL) for 15 minutes, 8 hours, and 24 hours. No significant change was observed in mRNA levels (Fig. 1D, bottom right). In parallel experiments, cell viability was studied in PC3 cells treated with Ephrin-B2/Fc at 10 μg/mL. A 45% decline in cell viability was observed after 24 hours, whereas Fc alone at the same dose had no effect (data not shown). Decline in EphB4 was thus associated with reduction in the measure of viable cells.

To determine whether EphB4 is expressed in clinical prostate samples, tumor tissues and adjacent normal tissue from prostate cancer surgical specimens were examined. The histologic distribution of EphB4 in the prostate specimens was determined by immunohistochemistry. EphB4 protein expression is confined to the neoplastic epithelium. Both membrane and cytoplasmic staining were seen (Fig. 2A, a, b, e, f, and g), whereas the expression is absent in normal glands (Fig. 2A, d). Antibody specificity was shown by loss of signal when the antibody was premixed with antigen/peptide (Fig. 2A, g and h). In a prostate tissue microarray generated from archival tissue, 41 of 62 (66%) prostate cancers examined were positive, whereas 3 of 20 (15%) normal prostate tissues showed expression at low intensity (p < 0.01; Fig. 2B).

We also examined gene expression in 72 prostate cancer patients using DNA microarrays. EphB4 was expressed in the majority of the cases (64 of 72 or 89% with normalized expression above 50 units).
with signal intensity higher than that seen for EphB1 and EphB2 (Fig. 2C).

**Regulation of EphB4 expression by tumor suppressor genes and growth factors.** PC3 cells are known to lack wild-type p53 function (8) and PTEN expression (9). We investigated whether the relatively high expression of EphB4 is related to p53 and/or PTEN by reintroducing wild-type p53 and/or PTEN into PC3 cells. Cells were cotransfected with a truncated-CD4 expression plasmid to allow selection of transfected cells. Transfected cells were then sorted for the cotransfected truncated CD4 marker. We found that the expression of EphB4 in PC3 cells was reduced >75% by the reintroduction of either wild-type p53 or PTEN (Fig. 3A). The cotransfection of both p53 and PTEN did not further inhibit the expression of EphB4. EGFR and IGF-IR have both been shown to promote PC3 cell growth and survival. We postulated that EphB4 expression might correlate with these prosurvival growth factors. We tested the relationship by independently blocking EGFR and IGF-IR signaling. In the PC3 cell line, EphB4 was downregulated by >50% after blocking EGFR signaling using the EGFR kinase inhibitor AG 1478 (Fig. 3B, left) or upon blockade of the IGF-IR signaling pathway using IGF-IR neutralizing antibody (Fig. 3B, right).

**EphB4 short interfering RNA and antisense oligodeoxynucleotides inhibit PC3 cell viability.** To define the significance of EphB4 overexpression in the prostate cancer model, we concentrated our study on PC3 cells, which have a relatively high expression of EphB4. The two approaches used for decreasing EphB4 expression were siRNAs and antisense oligodeoxynucleotides. A number of different phosphorothioate-modified antisense oligodeoxynucleotide complementary to different segments of the EphB4 coding region were tested for specificity and efficacy of EphB4 inhibition. Antisense-10 was found to be a specific and potent inhibitor of EphB4 expression (Fig. 3C). A similar approach was applied to the selection of specific siRNA. EphB4 siRNA 472 effectively knocks down EphB4 protein expression, whereas EphB4 siRNA 2,303 was inactive (Fig. 3C). Another EphB4-specific siRNA 50 was also highly effective in knocking down protein expression (data not shown). Both EphB4 siRNAs (472 and 50) and antisense-10 oligodeoxynucleotide reduced the number of viable PC3 cells (by as much as 80%) in a dose-dependent manner (Fig. 3D, top and bottom left). Unrelated (GFP-siRNA) or inactive (2,303) siRNAs or sense oligodeoxynucleotide had no effect on the number of viable cells. We found that neither siRNA 472 nor antisense-10 had an inhibitory effect on viable cell number in the EphB4-negative cell line, SLK (Fig. 3D, top right and bottom).

**EphB4 short interfering RNA and antisense oligodeoxynucleotides inhibit prostate tumor cell migration and invasion.** PC3 cells can grow locally and form lymph node metastases when injected orthotopically into mice. In an effort to study the role of EphB4 on migration of PC3 cells in vitro, we did a wound-healing assay.
assay. When a wound was introduced into a monolayer of PC3 cells, over the course of the next 20 hours, cells progressively migrated into the cleared area. However, when cells were transfected with 50 nmol/L EphB4 siRNA 472 and the wound was introduced, this migration was significantly inhibited when compared with control (GFP-siRNA; Fig. 4A). Pretreatment of PC3 cells with 10 μmol/L EphB4 antisense-10 for 12 hours also generated inhibition of cell migration when compared with a sense oligodeoxynucleotide (Fig. 4B). In addition, knockdown of EphB4 expression in PC3 cells with 50 nmol/L EphB4 siRNA 472 severely reduced the ability of these cells to invade Matrigel as assessed by a double-chamber invasion assay (Fig. 4C), whereas control GFP-siRNA showed little or no effect.

EphB4 short interfering RNA induces cell cycle arrest and apoptosis in PC3 cells. Because knockdown of EphB4 resulted in a reduced measure of viable cells, we sought to determine whether this was due to effects on the cell cycle. In comparison with GFP-siRNA–transfected cells, 50 nmol/L EphB4 siRNA 472 resulted in an accumulation of cells in the sub-G0 and S phase fractions. The sub-G0 fraction increased from 1% to 7.9% in EphB4 siRNA 472–treated cells compared with GFP-siRNA–treated cells (Fig. 5A). Apoptosis as a result of EphB4 knockdown was confirmed by measuring levels of cytoplasmic nucleosomes (a marker of DNA fragmentation) and the effect was dose-dependent when PC3 cells were transfected with EphB4 siRNA 472, but not GFP siRNA. DNA fragmentation in EphB4 siRNA 472-transfected PC3 cells increased 15-fold at a dose of 100 nmol/L when compared with GFP-siRNA-treated cells (Fig. 5B).

In vivo activity of EphB4 antisense oligodeoxynucleotide in a prostate cancer xenograft model. Following implantation of PC3 cells in their prostate glands, male athymic mice were either treated with EphB4 antisense-10, sense oligodeoxynucleotide, or diluent (PBS). Sense oligodeoxynucleotide- or diluent-treated mice showed large tumors in each of the implanted prostate glands, whereas those treated with antisense-10 oligodeoxynucleotides had small tumors in only two of the six prostate glands (Fig. 6A). Furthermore, antisense-10 reduced cell proliferative index as measured by Ki-67 staining, increased apoptosis and reduced microvascular density in the tumor (Fig. 6B). The in vivo study was also conducted with a s.c. xenograft model using PC3 cells. Tumor volumes in antisense-10-treated mice were significantly smaller than those in control groups (sense oligodeoxynucleotide- or PBS-treated; P < 0.01). Western blot of extracts of tumor tissue showed that the EphB4 expression is inhibited in the antisense-10-treated group (Fig. 6C). There was no evidence of systemic toxicity to the mice as evidenced by normal food intake and body weight. Furthermore, histologic evaluation of heart, lung, liver, and kidney did not reveal any evidence of toxicity (data not shown).

Discussion

EphB4 protein is expressed in a majority of prostate carcinomas with expression limited predominantly to the neoplastic epithelium. This is in contrast with little or no expression in normal prostate gland. To our knowledge, this is the first documentation of EphB4 expression in prostate cancer. Previous studies have reported overexpression of EphB4 in colon cancer and in a variety of human lung and breast cancer cell lines (30, 31, 40). However, these studies provided limited functional significance of EphB4 expression in cancer. Our survey of prostate cell lines first indicates an association between the level of EphB4 expression and aggressive growth. This is suggested by the fact that cells derived from benign prostatic hyperplasia has no expression of EphB4 compared with the PC3 prostate cancer metastasis-derived cell line, and a clone of PC3 with higher metastatic potential (PC3M), which expressed the highest amounts of EphB4. This trend corroborates the observations in endometrial carcinoma and breast cancer where overexpression of EphB4 has a suggested potential role in cancer promotion (31–33, 41), although a contradictory report exists (42). We hypothesize that there is a direct correlation of EphB4 expression with higher grade and metastatic potential that needs to be further explored in subsequent studies.

One possible mechanism of increased EphB4 expression may be gene amplification as we have observed in PC3M cells. Amplification of chromosome 7q is a common occurrence in androgen-independent prostate carcinomas (43), and the EphB4 locus is at 7q22. We next sought to investigate the functional role and
mechanism of EphB4 expression in prostate cancer using the PC3 cell line as a model. The EphB4 pathway in PC3 cells is functional as shown by markedly increased phosphorylation of EphB4 following stimulation by Ephrin B2 above the basal level when cells were grown in serum. Loss-of-function mutations in tumor suppressor genes or induction of growth factor pathways participate in prostate cancer development or progression (4–10). It is thus not surprising that introduction of the native forms of PTEN and p53 reduced EphB4 levels in PC3 cells, which lack PTEN expression and wild-type p53 function (8, 9, 44). Notably, concurrent introduction of both PTEN and p53 did not result in additive down-regulation of EphB4, suggesting overlapping pathways. In light of this regulation by tumor-suppressor genes, it is interesting to note that EphB4 promotes oncogenesis and metastasis initiated by the neuT oncogene. Mammary tumor latency is reduced by 50% in EphB4/neuT double transgenic mice compared with neuT mice, which develop spontaneous mammary carcinomas (45). In addition, these tumors produce metastasis, whereas tumors in neuT mice are localized. EphB4 expression was also reduced by inhibition of both the EGFR and IGF-IR pathways, which are implicated in prostate cancer and may contribute to escape from androgen dependence (4–6). Together, these results suggest that EphB4 can be up-regulated by a variety of mechanisms. EphB4 induction in prostate cancer may be complex, as we have shown that EphB4 is regulated by oncogenes (EGFR and IGF-IR) and tumor suppressor genes (PTEN and p53). Other possible mechanisms of regulation include disruption of Notch and activation of hoxA9 signaling (46–48).

Targeted disruption of EphB4 expression in prostate cancer cells proved to be highly informative. We showed that EphB4 is required for tumor cell viability, migration, invasion, and cell cycle progression. In endothelial cells, where EphB4 is normally expressed, it regulates cell migration, proliferation, and invasion (29). However, in contrast with endothelial cells, EphB4 expression in prostate cancer cell lines provides a critical survival signal. Here, we showed that EphB4 knockdown increased the fraction of cells in sub-G0 fraction by cell cycle analysis. In addition, a dose-dependent increase in cytoplasmic nucleosomes (a hallmark of apoptosis) was observed after ablation of EphB4 expression in PC3 cells. Thus, reduction in EphB4 levels leads to apoptosis. In a recent report, Noren et al. (49) found that activation of EphB4 with clustered Ephrin-B2/Fc inhibited tumor proliferation and concluded that EphB4 activation inhibits tumor growth. This seemingly contradicts our results. However, we found that prolonged incubation with Ephrin-B2/Fc reduced levels of total and phosphorylated EphB4 without any change in mRNA levels. This may occur by ligand-induced receptor endocytosis and degradation (50, 51). Thus, Noren et al., over a period of 5 days, see a decrease in total cell number, likely due to the loss of EphB4.

In vitro studies were confirmed in vivo using both orthotopic and s.c. xenograft prostate tumor models. Inhibition of EphB4 gene expression with antisense-10 oligodeoxynucleotides but not sense oligodeoxynucleotides, or EphB4 antisense-10 oligodeoxynucleotides. Treatment was begun 10 days after implantation and continued daily throughout the study. Prostate glands were harvested and sectioned for H&E staining at the conclusion of the study. Tumor volume was measured as described in Materials and Methods. Pictures were taken under 10× magnification. Black arrow, tumor; yellow arrow, normal prostate glands; bar, mean volume. B, immunohistochemical staining on tumor-bearing glands for microvascular density (CD31), cell proliferation index (Ki-67), and apoptosis (TUNEL) in tumor (black arrow) and normal mouse prostate (yellow arrow). Median values of positive signal counted in five random high power fields, and P values comparing control and antisense-10 treated tumors are shown. C, athymic mice bearing s.c. PC3 xenografts were treated with PBS (control), sense or EphB4 antisense-10 oligodeoxynucleotide for 21 days. Tumors were harvested and lysates analyzed by Western blotting for EphB4, EGFR, and β-actin.

Figure 6. In vivo effect of EphB4 antisense-10 on prostate tumors. A, mice implanted with PC3 cells in the prostate gland were treated with PBS, sense oligodeoxynucleotide, or EphB4 antisense-10 oligodeoxynucleotides. Treatment was begun 10 days after implantation and continued daily throughout the study. Prostate glands were harvested and sectioned for H&E staining at the conclusion of the study. Tumor volume was measured as described in Materials and Methods. Pictures were taken under 10× magnification. Black arrow, tumor; yellow arrow, normal prostate glands; bar, mean volume. B, immunohistochemical staining on tumor-bearing glands for microvascular density (CD31), cell proliferation index (Ki-67), and apoptosis (TUNEL) in tumor (black arrow) and normal mouse prostate (yellow arrow). Median values of positive signal counted in five random high power fields, and P values comparing control and antisense-10 treated tumors are shown. C, athymic mice bearing s.c. PC3 xenografts were treated with PBS (control), sense or EphB4 antisense-10 oligodeoxynucleotide for 21 days. Tumors were harvested and lysates analyzed by Western blotting for EphB4, EGFR, and β-actin.
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15. Oligodeoxynucleotides showed marked reduction in tumor growth. This reduction corresponds to a down-regulation of EphB4. We found that intratumoral microvesSEL density and postulate that down-regulation of EphB4 may also deter reverse signaling of Ephrin-B2, which is involved in angiogenesis. An antiangiogenic effect in tumor by blocking EphB4/Ephrin-B2 signaling has been reported recently (49, 52). To our knowledge, this is the first study that has related the expression of EphB4 to cancer cell growth, survival, migration, and invasion. Inhibition of EphB4 expression did not cause any evidence of toxicity in adult mice in contrast with embryonic lethality in EphB4 knockout mice. These results are not unexpected as angiogenesis in adult life is limited to sites of remodeling.

In summary, EphB4 is a novel marker for prostate cancer that seems to positively correlate with an invasive and metastatic phenotype. Its expression is actively regulated under the control of growth factors and tumor suppressor genes. EphB4 endows tumor cells with increased migratory and invasive properties. Loss of EphB4 retards cell cycle progression, reduces cell viability, and induces apoptosis. EphB4 is highly expressed in a majority of clinical prostate cancer specimens. Future studies will address the relationship of EphB4 expression with other known genetic lesions in prostate cancer for diagnostic and prognostic implications. Furthermore, targeting EphB4 has the potential to provide novel therapeutic opportunities. Blocking EphB4 function could block angiogenesis as well as directly inhibit survival and invasion of EphB4-positive prostate cancers.

Acknowledgments

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