A Glycolytic Mechanism Regulating an Angiogenic Switch in Prostate Cancer


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Abstract

The generation of an ‘angiogenic switch’ is essential for tumor growth, yet its regulation is poorly understood. In this investigation, we explored the linkage between metastasis and angiogenesis through CXCL12/CXCR4 signaling. We found that CXCR4 regulates the expression and secretion of the glycolytic enzyme phosphoglycerate kinase 1 (PGK1). Overexpression of PGK1 reduced the secretion of vascular endothelial growth factor and interleukin-8 and increased the generation of angiostatin. At metastatic sites, however, high levels of CXCL12 signaling through CXCR4 reduced PGK1 expression, releasing the angiogenic response for metastatic growth. These data suggest that PGK1 is a critical downstream target of the chemokine axis and an important regulator of an ‘angiogenic switch’ that is essential for tumor and metastatic growth. [Cancer Res 2007;67(1):149–59]

Introduction

Hypoxia is a central feature of many solid tumors, including prostate cancer, making them extremely resistant to chemotherapy. Transcriptional activation of hypoxia-inducible genes are known to play a critical first step in the adaptation of tumors to hypoxia, including positive [vascular endothelial growth factor (VEGF), interleukin (IL)-8, and SDF-1/(CXCL12)] and negative regulation of the angiogenic cascade. Hypoxia-inducible genes also regulate many glycolytic enzymes to facilitate anaerobic metabolism. These molecules are required to provide for the high energetic demands of growing neoplasms and to generate macromolecules critical for growth (1). Consequently, the shift in energy production from oxidative phosphorylation to glycolysis, the so-called “Warburg effect,” is likely to be a fundamental property of cancer cells and is known to correlate with enhanced tumor progression (2–4).

Phosphoglycerate kinase (PGK) 1 is an ATP-generating glycolytic enzyme that forms part of the glycolytic pathway. PGK1 is regulated by hypoxia-inducible factor-1α (HIF-1α; ref. 5) and it is often overexpressed in prostate cancer (6). PGK1 is located in a chromosomal region associated with familial prostate cancers, hypospadias, and androgen insensitivity (X chromosome; Xq11-q13; ref. 7). In the nucleus, PGK1 influences DNA replication and repair. Surprisingly, PGK1 is secreted extracellularly by tumors, where it acts as a disulfide reductase capable of cleaving plasminogen to generate the vascular inhibitor angiostatin (8–13). Overexpression of PGK1 is therefore likely to restrict tumor growth by limiting angiogenesis. Thus, a delicate equilibrium may be established between the hypoxic response needed to generate proangiogenic factors and events essential for anaerobic metabolism (e.g., PGK1) for tumors to grow. The challenge is to regulate PGK1 secretion.

Our previous work has focused on the role that chemokine axis of CXCL12 and its receptor CXCR4 plays in metastatic prostate cancer (14–16). We observed that CXCR4 expression is related to increasing tumor grade (14) and that CXCL12 signaling through CXCR4 triggers the adhesion of prostate cancers to bone marrow endothelial cells, possibly by activating αvβ3 integrins (17) and CD164 (18). Most critically, antibody to CXCR4 significantly reduces the number of prostate cancer bone metastasis in an in vivo model of metastasis (15). Further work has shown that CXCR4 signaling disrupts the delicate equilibrium between proangiogenic and antiangiogenic signals in prostate cancer disease (16).

In the present investigation, we found that CXCR4 signaling regulates PGK1 expression, which regulates angiogenesis by generating angiostatin and by reducing the secretion of the proangiogenic cytokines VEGF and IL-8. At sites of high CXCL12 production, however (bone, lymph node, and liver), PGK1 secretion is inhibited by the CXCL12/CXCR4 axis. Thus, CXCL12 signaling through CXCR4 generates an ‘angiogenic switch’ necessary for metastatic growth. Together, these data further show that CXCL12/CXCR4 chemokine axis and PGK1 represent at least one of the critical events necessary for metastasis of prostate cancer as well as a mechanism for a proangiogenic switch to promote tumor growth. These findings add a new dimension to our understanding of the relationship between chemokines and metabolism. Moreover, they point to potential therapeutic interventions to disrupt these pathways and control prostate cancer growth and its metastasis.

Materials and Methods

Cell cultures. The LNCaP, LNCaP metastatic subline C4-2B and PC3, and MCF-7 breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Invitrogen Corp., Carlsbad, CA). The human prostate epithelial cell line (RWPE-1) was cultured in keratinocyte medium supplemented with 5 ng/ml human recombinant epithelial growth factor and 50 μg/ml bovine pituitary extract (Invitrogen). Human dermal microvascular endothelial cells (HDMEC) were grown as described previously (15, 19, 20).
PGK and CXCR4 expression constructs. A 1.33-kb human PGK (hPGK) cDNA was isolated by reverse transcription-PCR from total RNA extracted from PC3 cells. The reverse and forward primers were 5′-AGTACATATGTCGTTTCTAAACAGCTG-3′ (positions, 80-100) and 5′-AGTAGGATCTCAATGCAAATGATGCA-3′ (positions, 1,409-1,389), respectively. The hPGK cDNA containing the open reading frame of hPGK was cloned to pcDNA3.1D/V5-His-TOPO vector. Cells were transfected with the PGK cDNA in pcDNA3.1D/V5-His-TOPO, pcDNA3.1-CXCR4-Tagged 3′/C2 hemagglutinin (HA), or an empty vector by lipofection (16).

Small interfering RNA knockdown of PGK1. Short hairpin small interfering RNAs (siRNA) under the control of the polymerase IIIH1-RNA promoter were used for these studies (Oligoengine, Seattle, WA) as described previously for CXCR4 (16). For siRNA knockdown of PGK1, two groups of primers corresponding to nucleotide sequences in open reading frame were synthesized [position, 186-203; Si1, 5′-gatccccACAACCAGGATTCGCTTCTGTTTttttta-3′ (forward oligonucleotide) and 5′-agcttaaaaaACAACCAGGATTCGCTTCTGTTTttttta-3′ (reverse oligonucleotide); position, 192-210; Si2, 5′-gatccccACAACCAGGATTCGCTTCTGTTTttttta-3′ (forward oligonucleotide) and 5′-agcttaaaaaACAACCAGGATTCGCTTCTGTTTttttta-3′ (reverse oligonucleotide)]. A group scrambled primers [5′-gatccccAAAACCGACGGCTATCTCTttttta-3′ (forward oligonucleotide) and 5′-agcttaaaaaAAAACCGACGGCTATCTCTttttta-3′ (reverse oligonucleotide)] were used. To generate stable clones, the cells were transfected by empty pSUPER vector, scrambled vector, or PGK1-Si (Si1 and Si2) vector by lipofection (16). The treated cells were selected in ~ 500 μg/mL G418 (Invitrogen), and the resulting clones were screened by Western blot. Further subcloning was done by limiting dilution at a density of 0.2 cells per well after verifying that only 1 cell per well had been plated.

Indirect immunofluorescence. PC3 cells were fixed and permeabilized using Fix and Perm kit according to the manufacturer's protocol (Caltag Laboratories, Burlingame, CA). Nonspecific staining was blocked with 10% FBS in permeabilization buffer and anti-PGK1 sheep IgG (1:200), α-tubulin (1:200; Sigma Chemical Co., St. Louis, MO), and sheep IgG (1:100; Upstate, Lake Placid, NY) antibodies were used followed by anti-mouse fluorescein-conjugated antibody (1:200; Upstate) and anti-mouse rhodamine-conjugated antibody (1:200; Upstate), each used for 1 h in sequence. The samples were then fixed in Slow and Fade light anti-fade kit (Molecular Probes, Eugene, OR). Images were acquired with a Zeiss LSM510 (Thorwood, NY).

Western blot analysis. Prostate cancer cells were cultured to confluence, washed, and then serum starved in RPMI 1640 with 0.1% bovine serum albumin for 24 h (16). PGK1 detection was done using a monoclonal IgM antibody at 1:5,000 (1 μg/mL) dilution (11). Detection of CXCR4 or angiostatin was similarly done using 5% milk with antibody to CXCR4 (1:1,000; Abcam, Inc., Cambridge, MA) or a mouse anti-human monoclonal antibody (1 μg/mL) angiostatin (1:1,000; Sigma Chemical) with anti-species-specific horseradish peroxidase–conjugated antibodies (Sigma Chemical).

The endothelial sprout network formation assay in Matrigel. Growth factor-reduced Matrigel was placed into eight-chamber slides (125 μL/chamber; BD Biosciences, San Diego, CA) and 0.8 × 10⁶ endothelial cells were added on top. The chambers were then incubated at 37°C for 24 h.
Figure 1  Continued. C, Western blot analysis of PGK1 expression in PC3-, LNCaP-, and C4-2B–expressing control or siRNA to CXCR4 vector (left). In some cases, the cells were starved (48 h) and stimulated with 200 ng/mL CXCL12 for 0 or 24 h at 37°C (left). A time course of PGK1 expression in response to 200 ng/mL CXCL12 (right). D, Western blot analysis of CXCR4 expression in prostate cancer cells overexpressing PGK1 or where PGK1 was targeted by siRNA. The data show that signaling through or expression of CXCR4 negatively regulates PGK1 expression, whereas PGK1 expression regulates CXCR4 expression. Quantitative densitometry was used for direct comparison. *, P < 0.05, significant difference from the controls. Bars, SD.
After incubation, the slides were fixed with methanol and stained with Diff-Quick solution II (Sigma Chemical). The slides were examined, and the sprouts were counted from five random fields under a microscope (×200). For coculture assays, equal numbers of prostate cancers and endothelial cells were added together, or 500 μl of the prostate cancer conditioned medium or control were added daily.

**Cytokine and PGK1 ELISA analysis.** Antibody sandwich ELISAs were used to evaluate IL-6, IL-8, and VEGF in the prostate cancer conditioned medium (R&D Systems, Minneapolis, MN). PGK1 levels were determined by direct ELISA using 100 μl of 5 μg/ml murine anti-PGK monoclonal IgM against a recombinant hPGK1 (rhPGK1) standard as described (8, 16). PGK1, IL-6, IL-8, and VEGF levels were normalized to total protein in the conditioned medium (Sigma Chemical).

**Intracardiac, s.c., and intratibial injections.** Intracardiac studies, (s.c.) and intratibial (i.t.) injections were done to determine the role that PGK1 plays in metastasis (15). After 4 weeks, bioluminescence was used to follow the prostate cancer-derived bone metastases as the primary outcome. The mice were injected i.p. with luciferin (100 μl at 40 mg/ml in PBS) before Xenogen IVIS (Alameda, CA) (15). Short-term homing capabilities of PC3 \( ^{3} \text{Gki} \text{Luc} \) or PGK \( ^{3} \text{Luc} \) cells were evaluated at 24 h by real-time PCR for luciferase 2CP gene \( ^{3} \text{Luc} \) CGGCTGGCAGAATGTTGAA (forward), TGCGTGCACACCCAGAT (reverse), and 5′-FAM-CTATGGGGCTGATA-CAAACC (TaqMan probe, ABI, Foster City, CA). Data were normalized to mouse \( \beta \)-actin (mm00607939-s1).

Cell implants and tibias were fixed in 10% formalin at 4°C. Tibias were further decalcified in 10% EDTA (pH 7.4) for 10 days and embedded in paraffin. Longitudinal sections of tissues were cut and stained with H&E for histologic evaluation. Where indicated, histomorphometric analyses of the samples were done using a computer-assisted bone histomorphometric analyzing system (Image-Pro Plus version 4.0, Media Cybernetics, Silver Spring, MD). The numbers of stained microvessels were blindly counted in 10 random fields per implant at 100 and 200 magnifications. Four or five implants were analyzed per condition following immunostaining for human von Willebrand factor (28 mg/ml anti-rabbit IgG; DAKO North America, Inc., Carpinteria, CA; ref. 16).

**Statistical analysis.** Numerical data are expressed as mean ± SD. Statistical differences between the means for the different groups were evaluated with Instat 4.0 (GraphPad Software, San Diego, CA) using one-way ANOVA, with the level of significance at \( P < 0.05 \).

**Results**

**Identification of PGK1.** We reported previously the generation of cell lines, which stably express siRNAs to reduce CXCR4 expression (Fig. 1A; ref. 16). To further examine the differential expression of proteins regulated by CXCR4 in prostate cancers, a proteomic approach was taken. High-resolution two-dimensional PAGE analysis of proteins derived from scramble or vector control or cell lines with the reduced CXCR4 expression were examined. More than 500 individual protein signatures with \( \pm 3 \) kDa between 3.5 and 10 and relative molecular masses between 20 and 55 kDa were detected (Supplementary Fig. S1). A computer-assisted comparative analysis of the silver-stained patterns of paired cell line samples identified several proteins that were increased in the CXCR4 down-regulated cells compared with controls. These protein spots were excised from the gels and analyzed by MALDI-TOF-mass spectrometry. Two of the proteins, in which peptides were identified from the tryptic gel digests, had the sequence VLNNMEIGTSLFDEEGAK. A Matrix Science Mascot Search and BLAST analysis identified the sequence belonging to two isoforms of PGK1.

**PGK1 expression is regulated by CXCL12.** To confirm that the CXCL12/CXCR4 axis regulates PGK1 expression, indirect immunofluorescence and Western blot analysis were done. Strong membrane and cytoplasmic staining of PGK1 was observed in cells expressing siRNA targeting CXCR4 (Fig. 1B). Western blots of prostate cancer cell lysates also show significant elevations of PGK1 expression in the siRNA cells compared with the respective controls (Fig. 1C, left). Stimulation of wild-type PC3 cell lines with CXCL12 decreased PGK1 expression by as early as 30 min for PC3 cells (Fig. 1C, right). The C4-2B cells also decreased their expression of PGK1 in response to CXCL12, but the response was not as robust or rapid as seen for PC3 cells (Fig. 1C, right).

**PGK1 and CXCR4 reciprocally regulate each other expression.** As shown, down-regulation of CXCR4 expression or CXCL12 stimulation of prostate cancer cells alters PGK1 expression. To determine if overexpression of CXCR4 directly regulates PGK1 expression, a HA-tagged vector containing the full-length cDNA of CXCR4 was used for transfection of the prostate cancer cell lines. Overexpression of CXCR4 resulted in down-regulation of PGK1 in the prostate cancer cell lines (Supplementary Fig. S2A). To determine whether CXCR4 and PGK1 reciprocally regulate each other, stable transfection of the prostate cancer cells with PGK1 was done. Overexpression of PGK1 increased the expression of CXCR4 in all of the prostate cancer cell lines (Fig. 1D, left). To determine if inhibition of PGK1 regulates CXCR4 expression, siRNA was used to generate stable clones with decreased PGK1 expression. Following selection, PGK1 levels were evaluated and individual clones were selected and pooled. Inhibition of PGK1 reduced the expression of CXCR4 at both level of transcription (data not shown) and protein level (Fig. 1D, right).

Because elevated PGK1 levels are a feature of the neoplastic transformation, the effects of overexpression of PGK1 on CXCR4 expression were also evaluated in the normal human prostate epithelial cell line, RWPE-1. Overexpression of PGK1 enhanced the expression of CXCR4 in the RWPE-1 cell line (Supplementary Fig. S2B). Similar alterations in CXCR4 expression were observed by overexpressing PGK1 in the breast cancer cell line MCF-7 (Supplementary Fig. S2B). Likewise, siRNA targeting of CXCR4 increased PGK1 expression in both cell lines (Supplementary Fig. S2C). Together, these findings suggest that signaling through or expression of CXCR4 inversely correlates with PGK1 expression, whereas PGK1 expression directly correlates with CXCR4 expression.

Downstream targets of PGK1 were next evaluated using gene expression arrays. Overexpression of PGK1 in prostate cancer cell lines induced several molecules involved in adhesion (E-cadherin and \( \beta \)-catenin), angiogenesis (thrombospondin-1, KILLER/death receptor 5/tumor necrosis factor–related apoptosis-inducing ligand receptor 2, epidermal growth factor receptor, and VEGF), apoptosis (APAF1, Bax, BIRC5, and telomerase), and transcriptional regulation [RASAI, IκB kinase-α (IKK-α), and c-Src; Supplementary Table S1]. Western blots confirmed that CXCR4 and \( \beta \)-catenin were down-regulated in LNCap-PGK1 and C4-2B-PGK1 cell lines compared with their respective controls. These data suggest that overexpression of PGK1 reduces tumor cell-cell adhesiveness, suggesting that invasion and metastasis regulated by PGK1 may be a possibility (Supplementary Fig. S3A).

Most germane to the present investigations were the findings that regulation of CXCR4 expression by PGK1 links with extracellular signal-regulated kinase (ERK) 1/2 signaling (Supplementary Fig. S3B) and activation of IκK-α (data not shown). These data suggest that regulation of CXCR4 expression by PGK1 may involve both mitogen-activated protein/ERK kinase/ERK signaling cascade and nuclear factor-κB activation.
Overexpression of PGK1 increases the metastatic rate in vivo. Previously, we showed that antibody to CXCR4 blocked prostate cancer cell adhesion to endothelial cells and invasion into extracellular matrices. Most importantly, antibody to CXCR4 blocked prostate cancer cell metastasis in an animal model (14, 21). Therefore, overexpression of PGK1 should increase the metastatic rate due to its effect on enhanced CXCR4 expression. To test this hypothesis, luciferase-labeled control (PC3\textsuperscript{Control}) and PGK1-overexpressing cells (PC3\textsuperscript{PGK1}) were injected into the left cardiac ventricle of nude mice (Fig. 2A). The short-term homing capabilities of the prostate cancer cells were assessed in a variety of tissues at 24 h by real-time PCR. The ability to form metastatic lesions was also determined at 1 month by Xenogen imaging (Fig. 2C).

At 24 h, fewer PC3\textsuperscript{PGK1 Luc} cells were recovered from the peripheral blood than the PC3\textsuperscript{Control Luc} cells (Fig. 2B). At the same time, more cells overexpressing PGK1 were found in the heart, spinal, and tibial marrow than control cells (Fig. 2B). These findings roughly correlate to the levels of CXCL12 found in murine tissues (14). At 1 month, bioluminescence imaging showed that most of the animals treated with either of the PC3 cells had developed metastatic tumors. Quite unexpectedly, however, the total metastatic burden of the animals receiving PC3\textsuperscript{PGK1 Luc} cells was considerably less than those animals injected with PC3\textsuperscript{Control Luc} cells (Fig. 2C). Together, these data suggest that overexpression of PGK1 enhanced the metastatic homing but interfered with the ability of the metastatic cells to grow. Accordingly, proliferation assays were done, which showed that PGK1 levels in culture did not correlate directly with proliferative activity in the various cell lines (data not shown).

PGK1 regulates an angiogenic phenotype in vitro. The effects of PGK1 on metastatic rates suggest that overexpression of PGK1 may lead to a diminished ability to recruit a vasculature necessary to support metastasis while increasing the expression of CXCR4 necessary for metastatic homing (14). Previously, we and others have reported that changes in CXCR4 levels alter the secretion of IL-6, IL-8, tissue inhibitor of metalloproteinase-2, and VEGF by prostate cancer cells (16, 22–24). To further determine if PGK1 regulates an angiogenic phenotype, conditioned medium derived
Figure 3. The effect of PGK1 on an angiogenic phenotype in vitro. The levels of IL-6, IL-8, VEGF, and PGK1 were determined at 48 h by ELISA in the conditioned medium derived from (A) PC3 cells that overexpressed PGK1 or control vectors or for cells expressing a siRNA targeting PGK1 or a scramble vector control. Columns, mean of triplicate determinations and normalized against total protein; bars, SD. *, P < 0.05, significant difference from controls. The data show that overexpression of PGK1 reduces the secretion of proangiogenic factors by prostate cancer cell lines, whereas reducing PGK1 expression increases their secretion.

B. secreted PGK1 levels were evaluated by ELISA for prostate cancer cells overexpressing CXCR4 or a siRNA targeting CXCR4 or a scramble vector control at 48 h. The data show that signaling through or the expression of CXCR4 reduces the secretion of PGK1.

C. prostate cancer cells were cultured for 12 h in the presence or absence of CXCL12 (100 ng/mL), with or without Co2+ (400 μmol/L; Sigma Chemical) as a mimic for hypoxic conditions. The data show that CXCL12 signaling decreases PGK1 secretion even under hypoxic conditions.

D. angiostatin levels were determined by Western blot for prostate cancer cells overexpressing PGK1, in prostate cancer cell lines with reduced PGK1 expression by siRNA targeting compared with a scrambled vector control (scramble; top left) and in prostate cancer cell lines overexpressing CXCR4 or control vectors (bottom left). Angiostatin levels in normal prostate epithelium (RWPE) or MCF-7 breast cancer cells transiently overexpressing PGK1 levels (bottom right). Quantitative densitometry of independent investigations was used for direct comparison of the treatment (top right and bottom right). *, P < 0.05, significant difference from the controls. The data show that PGK1 levels correspond with increased angiostatin levels.
from cells overexpressing PGK1 was evaluated for alterations in IL-6, IL-8, and VEGF levels. PC3 and C4-2B cells that overexpressed PGK1 secreted less VEGF into their conditioned medium than did the parental or control cells (Fig. 3A; data not shown in C4-2B cells). At the same time, it was observed that elevated PGK1 levels correlated with reductions of IL-6 and IL-8 levels by PC3 cells (Fig. 3A). Conversely, PC3 cells expressing a siRNA that targeted PGK1 secreted more IL-6, IL-8, and VEGF into their conditioned medium compared with controls (Fig. 3A).

**Secreted PGK1 results in enhanced angiostatin generation.** Our previous work linked CXCR4 signaling with the generation of angiostatin *in vitro* (16), and secreted PGK1 is known to function extracellularly as a disulfide reductase–cleaving plasminogen to generate angiostatin (8–11). To explore the possibility that the CXCL12/CXCR4 axis alters angiostatin levels through secreted PGK1, the levels of secreted PGK1 were evaluated in the conditioned medium of the prostate cancer cell lines. As shown in Fig. 3B, reducing CXCR4 expression resulted in enhanced secretion of PGK1 into the conditioned medium. Overexpression of CXCR4, however, resulted in decreased PGK1 levels detected in the conditioned medium (Fig. 3B).

As PGK1 is regulated by both HIF-1α and CXCL12, we next evaluated whether the secretion of PGK1 is regulated by either of these factors. For these studies, prostate cancer cell lines were

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**Figure 4.** PGK1 levels alter angiogenesis *in vitro*. HDMECs were plated in growth factor–reduced Matrigel as a functional, *in vitro* assay of blood vessel formation. After 24 h, the cultures were fixed and stained. **A**, left, PC3 cells that overexpressed PGK1 or control vectors, or cancer cells expressing a siRNA targeting PGK1 expression (or a scrambled control) were co-plated with either the HDMECs or their conditioned medium; right, vessel sprout formation was quantified by direct microscopic counting. **B**, the vascular response to PC3 cells overexpressing CXCR4 or stimulated with 200 ng/mL CXCL12 was reversed by rhPGK1 (2,000 ng/mL; top) and quantified (bottom). Columns, mean of triplicate determinations; bars, SD. *, P < 0.05, significant difference from control. Original magnification, ×10. Bar, 200 μm.
cultured in the presence or absence of CXCL12 with or without Co2⁻ (400 \mu M) at 12 h to mimic hypoxic conditions. In each case, hypoxic conditions enhanced the secretion of PGK1 into the conditioned medium (Fig. 3C). Under these conditions, CXCL12 reduced the secretion of PGK1 (Fig. 3C).

Next, it was determined whether the expression of PGK1 corresponds with the generation of the vascular inhibitor angiostatin. As predicted, the overexpression of PGK1 resulted in elevated angiostatin levels (Fig. 3D, top left). Decreasing PGK1 expression by siRNA resulted in reduced levels of angiostatin (Fig. 3D, top right). Similarly, overexpression of CXCR4 (Supplementary Fig. S2A) resulted in less angiostatin generated (Fig. 3D, bottom left). The effect of overexpression of PGK1 on angiostatin levels was also evaluated in RWPE-1 under normoxic conditions. As shown in Fig. 3D (bottom right), overexpression of PGK1 enhanced the expression of angiostatin by the RWPE-1 cell line and the MCF-7 cell line.

**PGK1 levels alter angiogenesis in vitro.** To examine whether the effect of secreted PGK1 has a biologically relevant effect on endothelial cells, human microvascular endothelial cell migration through Matrigel membranes was examined as a functional, *in vitro* assay measuring proangiogenic capacity (25). As shown in Fig. 4A, little or no endothelial sprout formation took place in the absence of external stimuli. Coculture of the endothelial cells with either conditioned medium of the PC3-Control cells or the cells themselves stimulated robust sprout formation. Overexpression of PGK1 in PC3 cells (PC3-PGK1) dramatically reversed the activity on sprout formation of the parental cells. Downregulation of PGK1 by siRNA had a stimulatory effect on blood vessel tube formation (Fig. 4A). In parallel, overexpression of CXCR4 or stimulation of PC3 cells with CXCL12 enhanced endothelial vessel formation that was reversed by the addition of rhPGK1 (Fig. 4B).

**CXCL12/CXCR4 signaling regulates an angiogenic switch through PGK1.** The previous results suggest that overexpression of PGK1 may promote metastasis by virtue of its effect on CXCR4 expression. However, the effect of PGK1 secretion may be to limit
the size of the metastatic lesions based on its effects on angiogenesis. To directly test the role of PGK1 on angiogenesis in vivo, mice were implanted s.c. with cells engineered to express altered levels of PGK1 in an environment that expresses low levels of CXC12. At 1 month, the tissues were recovered, weighed, and stained for factor VIII to identify blood vessels. Overexpression of PGK1 in PC3 cells significantly reduced the number of blood vessels formed in the tissue from 14.3 ± 2.1 to 6.8 ± 0.8 per high-power field relative to controls (Fig. 5A and B). Cancer cells with reduced expression of PGK1 or overexpression of CXCR4 generated significantly larger and more abundant blood vessels than controls (Fig. 5A and B). To further test the role of PGK1 on angiogenesis, mice were implanted s.c. with scaffolds containing a mixture of human dermal endothelial cells with PC3Control or PC3PGK1 cells. Overexpression of PGK1 resulted the generation of smaller tumor-derived tissues and significantly reduced the number of human blood vessels formed in the tissue from 11.0 ± 2 to 1.8 ± 0.8 per high-power field (Supplementary Fig. S4).

To directly determine if CXCL12 regulates an angiogenic switch through PGK1 activity, PC3Control, PC3CXCR4, PC3PGK1, or PC3PGK1 siRNA cells were injected i.t. into an environment rich in CXCL12 (15). After 4 weeks, the skeletal lesions were identified. Tumors generated in high CXCL12 environment produced larger and more abundant blood vessels. Overexpression of PGK1 still reduced the number of blood vessels, although these effects were reversed by the high levels of CXCL12 (Fig. 5A and B). Cancer cells with enhanced expression of CXCR4 or reduced expression of PGK1 generated larger and more abundant blood vessels than those produced by tumors expressing scrambled siRNA controls (Fig. 5A and B). A radiographic and histologic analyses of the resulting tumors displayed extensive bone destruction in the control groups (Fig. 5C and D). In comparison, animals receiving PC3PGK1 cells had smaller lesions with less osteolytic damage (Fig. 5C and D). Quantitative histomorphometry confirmed that the overall tumor area and bone area associated with tumor were more extensive in the PC3Control animals versus the PC3PGK1-injected animals (21.51 ± 1.3 versus 7.64 ± 1.6 mm; 12.08 ± 1.1 versus 2.21 ± 0.8 mm; Fig. 5D). A striking feature of the resulting tissues was the formation of extracortical woven bone in the animals injected with the PC3PGK1 cells (Fig. 5C, dashed line). Here, the number of osteoblasts per millimeter of bone surface was higher in tissues resulting from the PC3PGK1 versus PC3Control cells (4.86 ± 0.8/mm versus 3.01 ± 0.4/mm). However, there were no differences in the number of osteoclasts (2.95 ± 0.3/mm versus 2.37 ± 0.6/mm; Fig. 5C).

Figure 5 Continued. C, radiographic (top) and histologic [middle (Mason’s tetracrome stain) and bottom (TRAP stain)] analysis of lesions resulting from i.t. injection of PC3CXCR4 versus PC3Control cells. Black arrows, osteoclast; dashed arrows, osteoblast; dashed line, cortical bone border. D, quantitative histomorphometry for tumor area, bone area associated with tumor, and osteoblast and osteoclast number per millimeter of bone surface using a computerized image analysis system (VisioLab 2000, Biocom, Paris, France). Columns, mean of triplicate determinations; bars, SD. *, P < 0.05, significant difference from control. Original magnification, ×20. Bar, 100 μm (A).
Together, these in vivo and in vitro data suggest that PGK1 is a critical downstream target of CXCL12/CXCR4 chemokine axis and likely to play a critical role in the regulation of an ‘angiogenic switch’ essential for tumor growth and metastasis.

**Discussion**

In this investigation, the connection between angiogenesis, anaerobic metabolism, and chemokine signaling was examined. Using siRNA targeting of CXCR4 in prostate cancer cell lines, we identified that the expression of PGK1 was enhanced following down-regulation of the chemokine receptor. Western blot analysis showed that CXCL12 signaling through CXCR4, or overexpression of CXCR4, inhibited the expression of PGK1. Similarly, when PGK1 was overexpressed, CXCR4 expression was enhanced. In vivo studies corroborated these findings to show that more tumor cells exited the blood and found their way to the spinal and tibial marrow of recipient animals when the cells overexpressed PGK1. However, the size of the resulting tumors generated by PC3PGK1 cells were smaller than those made by PC3Control cells. Similarly, tumors generated by the PC3PGK1 cells following s.c. or i.t. injections were also smaller. These results are most likely due to the fact that cells secreting PGK1 have a diminished ability to recruit a vasculature necessary to support metastasis. This may occur for two reasons; elevated PGK1 levels in prostate cancer cell lines reduced the secretion of proangiogenic factors, including VEGF, IL-6, and IL-8. Elevated intracellular levels of PGK1 also resulted in enhanced secretion of PGK1 that generated the angiogenesis inhibitor angiostatin (8–13). Alternatively, the reduced metastatic burden at 1 month might suggest a decrease in survival of the early metastatic colonies due to the apoptotic deregulation (Supplementary Table S1). Thus, our data suggest that the role of PGK1 in cancer is a double-edged sword. PGK1 regulates CXCR4 expression resulting in changes in the metastatic rate. Yet, the enhanced expression of PGK1 also leads to decreased secretion of proangiogenic factors and secretion of PGK1 itself that inhibits angiogenesis through the generation of angiostatin. These results lead us to conclude that there is a reciprocal relationship between CXCL12/CXCR4 signaling and PGK1 expression (model; Fig. 6).

Prostate tumors as well as those arising in many other tissues display a remarkable propensity to invade and survive in bone. Although the reasons for this are not entirely clear, several mechanisms seem to facilitate this complex phenomenon. Our previous work has shown that prostate cancers use CXCR4 as a key element in their migration to tissues rich in CXCL12, including the lymph nodes, liver, adrenal glands, and bone (15, 16, 21). One mechanism to account for these observations is that CXCL12 transiently regulates adhesion molecules, including CD164 and the number and affinity of the αvβ3 integrin expressed by prostate cancer cells to enhance their binding to marrow endothelial cells and components of the bone marrow extracellular matrix (17, 18). Furthermore, recent data indicate that CD44 expression may regulate the adhesion of prostate and breast cancer cells to haluronic acid expressed by human bone marrow endothelial cells (26). The participation of CD44 in PGK1-mediated events is also possible as PGK1-overexpressing prostate cancer cells significantly overexpressed the protein (Supplementary Table S1).

Recent studies have also shown that E-cadherin/β-catenin/Wnt signaling regulates cell-cell adhesion, migration, and tumorigenesis (27). Likewise, PGK1 was shown to regulate E-cadherin/β-catenin, suggesting that overexpression of PGK1 promotes decreased tumor cell-cell adhesion that potentially linked to Wnt signaling and up-regulation of cell migration (27). A second

**Figure 6.** Model of PGK1 action in prostate cancer. CXCL12 signaling through the CXCR4 receptor leads to an ‘angiogenic switch’ that is mediated by extracellular PGK1 levels in CaP cells.
mechanism is that CXCL12 may be critical for tumor growth. Such that antibody to CXCL12 decreased PC3 proliferation in vitro (14) and anti-CXCR4 antibody or a anti-CXCR4 peptide decreased the size of the skeletal lesions generated in mice following i.t. injection (15). A third and novel mechanism is supported by the PGK1 data; when prostate cancers metastasize to bone, they enter an environment that is high in CXCL12. PGK1 in this new environment, which may also be achieved if metastatic cells were to secrete CXCL12 themselves, undergo an ‘angiogenic switch,’ in which vascular inhibition by PGK1 is reduced and angiogenic growth of the tumor is promoted (Fig. 6). Critically, these data also suggest a mechanism to explain metastatic localization patterns first observed by Paget (28). Only tissues rich in CXCL12 are able to overcome the vascular inhibition produced by PGK1 to facilitate tumor growth.

One of the most striking findings was the effect of PGK1 expression by prostate cancers on bone. Overexpression of PGK1 lead to the generation of extraosseous bone with enhanced levels of osteoblasts and decreased numbers of osteoclasts present in the bone. Previous work has shown that PGK1 can be secreted by tumors, where it cleaves plasminogen generating the vascular inhibitor angiostatin (8–11). Angiostatin limits tumor size by limiting angiogenesis and by directly suppressing osteoclast development (3, 4). This is thought to be critical as active osteoclast-mediated bone resorption may be required for both intraosseous growth and metastatic localization (14, 29). Other work by our colleagues has shown that VEGF produced by prostate cancers induce initial differentiation of osteoblasts but require other factors, present in C4-2B but not LNCaP cells, to induce mineralization. Therefore, how the PGK1 effects on secretion of VEGF and other proangiogenic factors by prostate cancers result in extracortical bone formation is unclear.

In summary, the results presented herein show that high levels of PGK1 are essential for tumor growth but limit angiogenesis when secreted extracellularly. However, at sites of high CXCL12 production, such as bone, lymph node, and liver; however, PGK1 secretion is likely to be inhibited. Thus, CXCL12 signaling through CXCR4 generates an ‘angiogenic switch’ that may be necessary for metastatic growth (Fig. 6). Together, these data further show that CXCL12/CXCR4 chemokine axis and PGK1 represent at least one of the critical determinants for metastasis of prostate cancer as well as a mechanism for a proangiogenic switch that promotes tumor growth. These findings add a new dimension to our understanding of relationship between chemokine and glucose metabolism enzyme and point to potential therapeutic interventions.

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References

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