The IL-8–Regulated Chemokine Receptor CXCR7 Stimulates EGFR Signaling to Promote Prostate Cancer Growth

Rajendra Kumar Singh¹ and Bal L. Lokeshwar¹,²,³

Abstract

The proinflammatory chemokine receptor CXCR7 that binds the ligands CXCL11 and CXCL12 (SDF-1a) is elevated in a variety of human cancers, but its functions are not understood as it does not elicit classical chemokine receptor signaling. Here we report that the procancerous cytokine IL-8 (interleukin-8) upregulates CXCR7 expression along with ligand-independent functions of CXCR7 that promote the growth and proliferation of human prostate cancer cells (CaP cells). In cell culture, ectopic expression or addition of IL-8 selectively increased expression of CXCR7 at the level of mRNA and protein production. Conversely, suppressing IL-8 signaling abolished the ability of IL-8 to upregulate CXCR7. RNAi-mediated knockdown of CXCR7 in CaP cells caused multiple antitumor effects, including decreased cell proliferation, cell-cycle arrest in G1 phase, and decreased expression of proteins involved in G1 to S phase progression. In contrast, addition of the CXCR7 ligand SDF-1a and CXCL11 to CaP cells did not affect cell proliferation. Over expression of CXCR7 in normal prostate cells increased their proliferation in a manner associated with increased levels of phospho-EGFR (epidermal growth factor receptor; pY1110) and phospho-ERK1/2. Notably, coimmunoprecipitation studies established a physical association of CXCR7 with EGFR, linking CXCR7-mediated cell proliferation to EGFR activation. Consistent with these findings, CXCR7-depleted CaP tumors grew more slowly than control tumors, expressing decreased tumor-associated expression of VEGF, cyclin D1, and p-EGFR. Together, these results reveal a novel mechanism of ligand-independent growth promotion by CXCR7 and its coregulation by the proinflammatory factor IL-8 in prostate cancer.

Introduction

Chemokines are proteins that participate in multitudes of normal and abnormal physiological processes, including inflammation, immunity, leukocyte chemotaxis, and tumor metastasis (1–4). The cysteine-X-cysteine chemokines (CXCL) with glutamine-leucine-arginine motif (ELR+ CXCL) regulate physiological functions by binding and activating 1 or more 7-transmembrane CXC chemokine receptors (CXCR; ref. 5). The CXCRs induce cell migration through the activation of a single or multiple members of structurally related GTP binding proteins, the G-proteins (6). The constitutive activation or overexpression of CXCL or CXCRs is linked to tumor growth and metastasis (7–9).

The newest member of the 7 CXCRs, CXCR7 (10), binds to 2 chemokines, CXCL11 and CXCL12 (SDF-1a) and dimerizes with CXCR4, a coreceptor for SDF-1a (11, 12). This dual specificity receptor is upregulated in many tumors. However, CXCR7 does not function like other CXCRs. CXCR7 binding to chemokines does not elicit intracellular calcium mobilization, or motility in normal cells, and thus its function has been intriguing (13). According to some reports, expression of CXCR7 provides "proliferation and survival advantage and increased adhesion properties" (13, 14). These reports did not elaborate, however, the mechanism by which CXCR7 allows growth advantage to tumor cells. Elevated levels of CXCR7 in tumors suggest its expression and function may be regulated by other factors, such as inflammatory chemokines [e.g., interleukin-8 (IL-8)] or tumor promoting transcription factors.

IL-8 is a multifunctional ELR+ CXC chemokine that exerts multitude of cellular signaling in normal or tumor cells (15). We and others have previously reported that IL-8 is an autocrine growth factor that promotes tumor growth by inducing hormone independent growth, motility, invasion, and angiogenesis in prostate and other cancers via the distinct activation of two cognate receptors, CXCR1 and CXCR2 (16–18). Here we show possible transregulation of CXCR4 and CXCR7 by IL-8 that could possibly contribute to increased proliferation and angiogenesis in CaP in a ligand-independent manner. Further, we report on the putative mechanism by which CXCR7 acts independent of its ligand binding and

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stimulation of epidermal growth factor receptor (EGFR) phosphorylation, leading to mitogenic cascade and potentially a multitude of tumor promoting activities.

Materials and Methods

Cell lines and culture methods

Acquisition and maintenance of all CaP cell lines (LNCaP, LAPC-4, and PC-3) were carried out by routine procedures as described (19). In brief, LNCaP, PC-3 were cultured in RPMI 1640 medium supplemented with fetal bovine serum (FBS, 10%) and gentamicin. LAPC-4 cells were maintained in Iscove’s medium with 7.5% FBS, 1 nmol/L dihydrotestosterone and gentamicin (16). RWPE-1 cells were cultured in keratinocyte serum-free medium with gentamicin. All cell lines were obtained from an authenticated source (ATCC) and used within 6 months of resuscitation of original cultures. The cell lines used in the study were also authenticated for their origin by Genetica DNA Laboratories Inc.

Global gene expression analysis

The global mRNA expression of IL-8 expressing LNCaP and LAPC-4 cells (LNCaP–IL-8, LAPC-4–IL-8, respectively) was compared with vector-only transfectants (v), generated as reported before (16), using Agilent cDNA expression array (Agilent Technologies). These studies were conducted at University of Miami’s DNA Micro-array Core Facility, which also carried out the Bioinformatics analysis. To identify differentially expressed genes, mathematical “dyo-swap” was carried out on arrays and 1-class significance analysis of microarrays (SAM; ref. 20) was done with the following criteria: false discovery rate was less than 5% and the average fold change was greater than 2. SAM was run on the expressed genes, where at least 2 out of 4 replicates passed quality control.

Gene knockdown with siRNA and quantitative real-time PCR

Cells cultured for 24 hours were transfected with gene-specific 21-mer siRNA sets (Smartpool siRNA, Thermo Scientific Inc.), using Dharmafect-2 transfection protocol (17). Total RNA isolated from cells 48 hours later was subjected to cDNA synthesis and quantitative real-time PCR using iQ SYBR-Green Supermix (BioRad) and the specific 21-mer siRNA sets (Smartpool siRNA, Dharmacon/Thermo Scientific Inc.). Stable transfectants were selected from transfected cultures following 2 weeks in puromycin selection medium (2.0 μg/mL) and evaluating the emergent cell colonies for CXCR7 mRNA knockdown by qPCR and immunoblotting with rabbit anti-CXCR7 IgG (catalogue no. GTX100027; GeneTex Inc.).

Stable expression of CXCR7 in RWPE-1 cells

These were transfected with a human full-length CXCR7 cDNA (True Clones, pCMV6-Neo vector; OriGene) using Lipofectamine 2000. Stable clones were selected with G418 (250 μg/mL) and analyzed for CXCR7 expression by qPCR and immunoblotting. Cell colonies overexpressing CXCR7 (RWCX7) were pooled for further analysis.

Cell proliferation assay and cell-cycle phase analysis

Cell proliferation and cell viability were determined by cell counting and MTT reduction assays, respectively (16, 17). Cell-cycle phase fractionation was carried out using a Beckman-Coulter XCEL flow cytometer (21, 22).

Immunoblotting

Expression of specific proteins in treated cells was analyzed by routine Western blotting. Presence of specific proteins on the Western blot was detected using the ECL ß Kit (GE Health Science) and relative protein band intensities were quantified using densitometry (Gel Logic 2200; Care-Stream Instruments; ref. 23).

Coimmunoprecipitation

Cell lysates were prepared in radioimmunoprecipitation assay buffer (RIPA), centrifuged to obtain nuclei-free lysates, and then immunoprecipitated (24). In brief, lysates were incubated with anti-CXCR7 rabbit IgG (10 μg/mL; MBL International, Cat. No. LS-A1439), with anti-EGFR rabbit monoclonal antibody (Epitomics Inc.) or normal rabbit IgG, overnight at 4°C, followed by incubation with protein A–Sepharose beads for 6 hours at 4°C. The complexes were washed with RIPA buffer and bead bound proteins were eluted in SDS-gel sample buffer, and visualized by immunoblotting.

Tumor generation in athymic mice

Studies on mice were conducted under an NIH approved institutional vertebrate animal-research protocol. Control (PC-3–V) or CXCR7 shRNA transfectants (PC-3–T73 and PC-3–T74) were implanted into the right and left dorsal flanks of 6-week-old athymic mice (1 × 106 cells/site in 50% Matrigel, 5 animals × 2 sites/group; Charles River Labs). Tumor growth was determined by biweekly measurement of tumor volumes for 42 days (25). The expression for VEGF, CXCR7 mRNA, and p-EGFR proteins in tumor tissues were analyzed ex vivo, using tumor tissues collected at necropsy as described before (26).

Results

Malignant prostate tumor cells express high levels of CXCR7

As shown in Table 1 and Fig. 1A, CaP cells express high level of CXCR7 mRNA and protein as compared with RWPE-
Table 1. Expression of CXCR chemokines and their receptors in normal prostate and tumor cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>CXCR4</th>
<th>CXCR7</th>
<th>CXCL11</th>
<th>SDF-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>RWPE-1</td>
<td>ND</td>
<td>0.09 ± 0.13</td>
<td>0.004 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>LAPC-4</td>
<td>0.23 ± 0.04</td>
<td>1.0 ± 0.16</td>
<td>0.091 ± 0.2</td>
<td>0.018 ± 0.1</td>
</tr>
<tr>
<td>LNCaP</td>
<td>0.21 ± 0.12</td>
<td>2.2 ± 0.10</td>
<td>0.023 ± 0.07</td>
<td>0.045 ± 0.04</td>
</tr>
<tr>
<td>PC-3</td>
<td>0.094 ± 0.10</td>
<td>1.3 ± 0.43</td>
<td>0.011 ± 0.04</td>
<td>0.102 ± 0.02</td>
</tr>
</tbody>
</table>

NOTE: Total RNA isolated from various cultures was reverse transcribed and the cDNAs were quantified using qPCR. Values shown are FD as defined in Material and Methods, normalized to GAPDH mRNA. ND: not detected. mean ± SD, n = 3.

1 cells which express very low levels of CXCR7. LNCaP cells expressed highest level of CXCR7 mRNA (FD = 2.2), followed by PC-3 (1.3), LAPC-4 (1.0), and RWPE-1 cells (0.094). LNCaP and LAPC-4 cells, expressed relatively high levels of CXCR4 than that of PC-3. Further, the level of CXCR4 mRNA was 5- to 10-fold lower than that of CXCR7 (Table 1). As many tumor cells coexpress both ligands and receptors, we measured the levels of the target chemokines (SDF-1a and CXCL11). The mRNA or protein expression of CXCL11 and SDF-1a in all CaP cells was insignificant (Table 1).

Global gene expression analysis of IL-8 secreting CaP cells show significant upregulation of CXCR7

We previously reported that constitutive expression of IL-8 in LNCaP and LAPC-4 cells (IL-8S) induce a variety of proinvasive and metastatic function (16). We compared here the
gene expression profile between IL-8S and vector control cultures (V) by cDNA microarray. We found 549 and 377 genes had altered expression in IL-8S cells of LNCaP and LAPC-4, respectively, compared with those of V control pairs. Among them, as shown in Supplementary Table S2, the analysis found the expressions of CXCR7, CXCR4, CD82, caspase-10, MMP-9, VEGF-A, and WISP-2 were consistently altered in both IL-8S transfectants. Importantly, we found 18- and 29-fold increases (Supplementary Table S2) in CXCR7 mRNA expression in LAPC-4-IL-8S and LNCaP IL-8S cells, respectively. We further validated the expression of CXCR7 by qPCR and immunoblotting. The CXCR7 mRNA and protein levels were 6- to 8-fold and more than 2-fold higher in IL-8 transfectants than that of the respective vector-only transfectants (Fig. 1B and C).

**External addition of IL-8 induced immediate increase in CXCR7 expression**

As shown in Figure 1D(i), a rapid increase in CXCR7 mRNA was observed (≤15 minutes) after addition of 3 nmol/L IL-8 to RWPE-1 and LNCaP cells, but not to PC-3 cells (which constitutively produce IL-8). We observed a time-dependent increase in CXCR7 protein following IL-8 stimulation of RWPE-1 and LNCaP cultures [Fig. 1D(ii and iii)]. The IL-8–induced increase of CXCR7 in RWPE-1 cells was more sustained (≥4 hours) when compared with that of LNCaP (≤1 hour), which stabilized after 1 hour with a sustained increase of 55% [Fig. 1D(iii)]. This rapid increase in CXCR7 mRNA following IL-8 addition was due to de novo CXCR7 synthesis as it was completely abolished if incubated with 10 μg/ml Actinomycin D (data not shown). Although external addition of IL-8 did not increase CXCR7 mRNA level in PC-3 cells, which endogenously produce IL-8, depletion of IL-8 in these cells by RNAi caused 73.3% reduction in CXCR7 mRNA as compared with that of c-siRNA transfectants (Fig. 1B). However, CXCR7 depletion did not significantly affect the level of IL-8 mRNA [Supplementary Fig. S1(i)]

**Reciprocal regulation of CXCR4 and CXCR7 by IL-8 in CaP cells**

The cDNA expression analyses showed that IL-8 producing LAPC-4 and LNCaP cells express lower level of CXCR4 mRNA than that of controls (V) [Supplementary Table S2]. We validated by qPCR that LNCaP-V and LAPC-4V cells expressed 2- to 3-fold higher levels of CXCR4 mRNA than their IL-8S counterparts [Fig. 1B(ii)]. Further, depletion of IL-8 in PC-3 cells by siRNA expression of CXCR4 mRNA by 5-fold [Fig. 1B(ii)]. In addition, silencing of CXCR7 by siRNA in both LNCaP-V and PC-3 cells slightly increased CXCR4 mRNA [P > 0.05; Supplementary Fig. S1(iii)]. However, depletion of CXCR4 expression by siRNA in LNCaP cells increased CXCR7 mRNA by 2.5 × (250%) but only modestly (~50%) in PC-3 cells [Supplementary Fig. S1(iii)].

**Both CXCR1 and CXCR2 are required for IL-8–induced CXCR7 expression**

As IL-8–induced intracellular signal transduction occurs via its binding to 2 cell-surface receptors, CXCR1 and CXCR2 (27, 28), we used receptor-specific inhibitors [Repertaxin-Lysine (Sigma) for CXCR1 and SB 225002 (Chemicon) for CXCR2; ref. 29] to abrogate IL-8–induced CXCR7 upregulation. Both inhibitors were effective in abolition of IL-8–induced increase in CXCR7 mRNA [Supplementary Fig S1(iv)].

**CXCR7 is essential for cell proliferation in CaP cells**

Wang and colleagues (14) reported that CXCR7 depletion in C4-2B cells, a derivative of LNCaP cell line, decreased their tumor growth in nude mice (14, 30). We verified the biological significance of CXCR7 in other CaP cells by multiple approaches. Transient depletion of CXCR7 in CaP cells by CXCR7 siRNA caused more than 90% decrease in mRNA and more than 65% decrease in protein expression [Supplementary Fig. S2(i)]. As shown in Figure 2A, depletion of CXCR7 significantly decreased the proliferation of PC-3 (55%), LNCaP-V (67%), and LNCaP-IL-8-S (40%) cells at 72 hours posttransfection. Because siRNA-mediated gene silencing lasts 72 or less hours (31), we generated stable CXCR7-depleted lines of CaP cells (T73 and T74) using shRNA transfection. T73 and T74 clones showed decreased level of CXCR7 mRNA and protein (Fig 2B, inset) and cell counts on day 7 showed a significant (~63%) reduction in cell density, compared with those in vector–only transfected cells (Fig. 2B).

**Growth-promoting activity of CXCR7 in CaP cells does not require its known ligands**

Because CXCR7 depletion leads to decreased cell growth, we postulated that the activation of CXCR7 by its known ligand(s) is responsible for CXCR7-dependent cell proliferation. Because we could not detect autocrine production of CXCL11 or SDF-1a in CaP cell cultures, we added CXCL11, CXCL12, or both, 50 ng/mL each, to cultures of PC-3, LNCaP, and corresponding CXCR7-shRNA-depleted clones (T74). We found little change (less than 3% increase) in cell growth of PC-3–V or PC-3–T74 cells (that express CXCR4, but CXCR7 was depleted), after 7 days of culture with CXCL11 or SDF-1a, regardless of whether the cells were cultured in the medium supplemented with or without FBS [10%; Fig. 2C and Supplementary Fig. S2(ii and iii)].

As CaP cells express both CXCR4 and CXCR7 (14), and both share SDF-1a as a ligand (13), we investigated whether CXCR4 depletion affects the PC-3 cells’ growth. Depleting CXCR4 mRNA by siRNA reduced CXCR4 mRNA in cells by more than 85% but did not affect cell proliferation when compared with control or CXCR7 siRNA transfectants (Fig. 2D). However, silencing of both CXCR4 and CXCR7 expression together, reduced cell proliferation by 52.33% ± 8.0%, similar to a level of inhibition observed by depleting CXCR7 only (47.5% ± 3.5%; Fig. 2D).

**CXCR7 depletion blocks cell-cycle progression in CaP cells**

As shown in Figure 3A, PC-3 or LNCaP cells transfected with CXCR7 siRNA, showed increased accumulation in G0/G1 phase, by 52%, compared with that of c-siRNA transfectants. Further, this increased accumulation led to a significant decrease in S-phase (54% ± 6.5%) and in G2/M fractions.
25 nmol/L siRNA
50 nmol/L siRNA

(12% ± 2.5%) in PC-3 cultures. CXCR7 depletion in LNCaP cells led to a greater accumulation of G0/G1 (68% ± 2.5%), and decrease in S- and G2/M phase fractions (Fig. 3B).

**CXCR7 depletion decreases G1 to S transition–regulating proteins in CaP cells**

CXCR7 depletion by siRNA significantly decreased cell-cycle regulatory proteins, cyclin D1, cyclin E, and Rb (p-Rb) in both PC-3 and LNCaP cells (Fig. 3C). We found 40-fold decrease in cyclin D1, 1.5- to 2-fold decreases in Cdk-4, and 2- to 6.5-fold decreases in cyclin B1, in both CaP cells (Fig. 3C) and significantly increased P21 (11.5 × in PC-3 and 15 × in LNCaP) protein levels, but none in P27Pkip1 protein levels.

An accumulation at G0/G1 and depletion of S-phase fraction in cultures depleted of CXCR7 could be due to the attenuation of MAP-kinase activity. Therefore, we measured the relative activation of extracellular signal–regulated receptor kinase-1/2 (Erk1/2) ratio of p-Erk1/2 in c-siRNA and CXCR7 siRNA–transfected PC-3 cells, using an ELISA. Levels of p-Erk1/2 in CXCR7 siRNA–transfected cells were 31.70% ± 4% lower than that of c-siRNA–transfected PC-3 (Fig. 3D). To further validate the association of CXCR7 in Erk1/2 phosphorylation, we compared the Erk1/2 activity in PC-3–T74 cells with that of V transfectants. Again, p-Erk1/2 activity was markedly diminished in CXCR7-depleted cells (Fig. 3D).

**Constitutive expression of CXCR7 in normal prostate epithelial cells increases cell proliferation**

The CXCR7-expressing RWPE-1 cells (RWCX7) showed 23% ± 3.5% increase in cell growth (Fig. 4A) than the vector-only-transfected RWPE-1 cells (RW-V).

**CXCR7 stimulates EGFR phosphorylation**

Strong change in ERK1/2 activity on CXCR7 depletion led us to test potential association of CXCR7 with growth factor receptors such as EGFR (32). Indeed, we observed constitutively increased phosphorylation of EGFR and Erk1/2 in cells that express high level of CXCR7, such as RWCE7, LNCaP, and PC-3 but not in RWPE-1 cells [Fig. 4B; also see Supplementary Fig. S3(i and ii) for quantitation]. CXCR7-depleted cells showed lower levels of both p-EGFR and p-Erk1/2, as compared with those in c-siRNA transfectants (Fig. 4C, see also Fig. 3D).

As CXCR7 expression stimulates cell proliferation without its ligand, we hypothesized that activation of EGFR and Erk1/2 phosphorylation may also be independent of CXCR7 ligands.
We noticed increase in p-EGFR following stimulation with EGF, but not by SDF-1α, or CXCL11. As shown in Figure 4D, p-EGFR levels remained unchanged in cultures exposed to recombinant SDF-1α, or CXCL11 in both RWPE-1 and RWCX7 cells, indicating ligand-independent role of CXCR7 in EGFR activation. In contrast, there was a time-dependent increase in p-EGFR in RWCX7 cells following stimulation with EGF, suggesting expression of CXCR7 increases the activation of EGFR either constitutively (compare p-EGFR levels in lane 1 of RWPE-1 with lane 5 of RWCX7 in Fig. 4D) or when stimulated with EGF [lanes 4 and 8, respectively, in Fig. 4D, also see Supplementary Fig. S4A–D].

CXCR7 colocalizes with EGFR

As expression of CXCR7 constitutively activates EGFR, we examined whether CXCR7 and EGFR colocalize or aggregate on plasma membrane (33). We attempted to communoprecipitate CXCR7 and EGFR from RWPE-1 or CaP cells, using anti-EGFR or anti-CXCR7 antibodies and nonionic, detergent-solubilized cell lysates. As shown in Figure 5A and B, both molecules are immunoprecipitated by either anti-p-EGFR or anti-CXCR7 antibodies. We showed further more p-EGFR in RWCX7 cells than that in RWPE-1 cells and CXCR7 overexpression increased phosphorylation of EGFR only at tyrosine1110 but, not serine1070–71 (Fig. 5C). In addition, we carried out confocal microscopy of LNCaP cells labeled with anti-EGFR (mouse anti-human IgG) and anti-CXCR7 (rabbit anti-human IgG; GeneTex Inc.) antibodies followed by fluorescent labeling with secondary antibodies labeled with Alexafluor 555 and Alex fluor488, respectively. The confocal micrographs, although could not completely reveal whether the colocalization of CXCR7 and EGFR is inside or outside of the plasma membrane, showed several focal points at which the colocalization of 2-labeled antibodies were observed (see Supplementary Fig. S4A–D).

Stable silencing of CXCR7 in CaP cells decreases tumor growth in vivo

We characterized in vivo tumor growth of 2 CXCR7-depleted PC-3 sublines, T73 and T74, and compared their tumor growth with that of vector-only transfectants. The T73 and T74 cells express reduced levels of CXCR7 mRNA (by 61.23%± 1.2% and 70.57% ± 2.2%), and proteins (50% and 75%), compared with PC-3V cells (Fig. 2). We found a significant growth delay (>35%) in both T73 and T74 tumors at the time of terminal measurement on day 42 (Fig. 6A). A significant difference in the slopes of the tumor growth from day 22 to 42 in the control versus T73 or T74 tumors further verified the slow growth rate of CXCR7-depleted tumor cells. Furthermore, ex vivo analysis of tumor tissues revealed a significant decrease in CXCR7 expression in T73 and T74 tumors (Fig. 6B). In addition, there was a significant decrease in p-EGFR as compared with total EGFR and cyclin D1 levels (Fig. 6C), and VEGF mRNA (Fig. 6D) in T73 and T74 tissues as compared with PC-3V tumors, suggesting a link between tumor growth and decrease in angiogenesis, cell proliferation, and cell-cycle arrest resulting from CXCR7 depletion.
Discussion

We report a novel mechanism of transregulation of chemokine/receptor-induced alteration in prostate tumor cells. We report 4 major observations: first, transient exposure or constitutive expression of a proinflammatory chemokine (IL-8) modulates nontarget chemokine receptors (CXCR4 and CXCR7); second, although CXCR7 is a "decoy" receptor, it is highly modulated by IL-8, it is constitutively upregulated in tumor cells, extensively involved in cell proliferation; thereby participating in multiple protumorigenic activities. Third, CXCR7 is capable of modulating a variety of cellular functions without being activated by its identified ligands (CXCL11 and SDF-1a). Finally, CXCR7 is capable of stimulating cell proliferation, cell-cycle progression, and angiogenesis by coupling/colocalizing with EGFR. The last attribute may be responsible for increased tumor growth due to CXCR7 elevation.

The observation that extrinsic addition of IL-8 also results in a rapid rise in CXCR7 expression shows a dynamic interaction between IL-8–induced intracellular signaling that results in transcriptional upregulation of CXCR7 and downregulation of CXCR4. Further, an induction of CXCR7 by external IL-8 was more pronounced in cells that do not constitutively express IL-8, such as RWPE-1 and LNCaP, but not the cells (PC-3) that constitutively synthesize it (Fig. 1D). This subdued response in PC-3 cells may be due to desensitization of CXCR1 and CXCR2 in the presence of constitutively produced IL-8. The regulation of CXCR7 by IL-8 in PC-3 cells was further supported by RNAi studies, where as expected, downregulation of IL-8 decreased CXCR7 and increased CXCR4 levels (Fig. 1B). However, IL-8 may be one of the several factors modulating CXCR7 and reciprocally regulate both receptors.

Berahovich and colleagues (34) were unable to detect ligand binding or cell surface expression of CXCR7 in PC-3 cells and they disputed earlier work by Wang and colleagues (14) that observed PC-3 expression of CXCR7 in PC-3 may be attributed to nonspecific nature of some commercial antibodies (34). We have revisited this claim by testing CXCR7 expression on PC-3 cell surface with immunofluorescence, and flow cytometry using antibodies from several commercial sources (R&D Systems and GeneTex Inc.) and with qPCR. In all these tests, we found positive expression of CXCR7 in PC-3 cells and the fluorescence intensity corresponded with the level of CXCR7 expression as verified by qPCR (see Figs. 1, 2 and 4–6 and Supplementary Fig. S5).

We showed that inhibition of IL-8–specific receptors, CXCR1 or CXCR2 can abrogate IL-8–induced CXCR7 upregulation [Supplementary Fig. S1(iv)]. This could be due to the
coregulation of CXCR2 by CXCR1, as we previously reported (26, 35). As receptor cross-linking between CXCR1 and CXCR2 is required in IL-8–mediated intracellular signaling (36), it is likely that both receptors are essential for IL-8–mediated upregulation of CXCR7.

In contrast to CXCR7, IL-8 negatively regulates CXCR4 in CaP cells. When autocrine production of IL-8 was halted by RNAi, CXCR4 mRNA levels rose 4-fold in PC-3 cells, indicating IL-8 down modulates the transcription of CXCR4 (Fig. 1B(ii)). Although CXCR4 is implicated in bone metastasis of CaP, its relative abundance on CaP cells is significantly lower than CXCR7, and therefore, these 2 receptors may play more complex roles in bone metastasis of CaP than previously thought (37).

Our findings have relevance to prostate cancer in men, where inflammation is considered a significant cause of disease progression (38–42). As IL-8 is a ubiquitous chemokine induced by acute or chronic inflammation, hypoxia, or necrosis, IL-8 is widely available in tumor microenvironment (43, 44). Furthermore, we reported previously that IL-8 expression in primary CaP tissues strongly correlates with biochemical (PSA) recurrence (45). Increased serum IL-8 levels have been reported in patients with advanced, metastatic CaP, indicating systemic increase in IL-8 (46). As survival following androgen deprivation is a critical step in the emergence of castration-resistant tumors, IL-8–induced upregulation of CXCR7 may enhance the survival and proliferation of those tumor cells. These finding suggest that elevated CXCR7 on CaP cells may

**Figure 5.** CXCR7 associates with EGFR in CaP cells. A and B, demonstration of coimmunoprecipitation of CXCR7 and EGFR. Cell lysates of PC-3 and LNCaP were immunoprecipitated with CXCR7 antibody followed by Western blotting against p-EGFR and vice versa. C, EGFR coprecipitated with CXCR7 in RWCX7 cells. RW-CX7 cells or control vector (RW-V) were immunoprecipitated with CXCR7 antibody followed by Western blotting with an anti-p-EGFR (pY1110) or an antibody recognizing phospho-Ser at residues 1070–1071 in EGFR (pS1070–71; Epitomics).

**Figure 6.** CXCR7 depletion slows PC-3 tumor growth in mice. A, tumor growth over time in athymic mice was recorded for mice injected with PC-3 cells stably expressing CXCR7 shRNA (T73 and T74) or scrambled sequence shRNA (V). B, blots of CXCR7, cyclin D1 p-EGFR CXCR7 tumor tissue of PC-3–V, PC-3–T73, and PC-3–T74. C and D, CXCR7 and VEGF mRNA level were decreased in T73 and T74 tumors as analyzed by qPCR. There was a significant delay (p < 0.03) in tumor growth and in the terminal tumor volume of T73 and T74 tumors.
be associated with autocrine IL-8 synthesis plausibly induced by inflammatory factors in the tumor microenvironment.

At present, little is known about ligand-independent, chemokine receptor–mediated, mitogenic signaling [47, 48]. Our results show that overexpression of CXCR7 alone is sufficient to increase cell proliferation in both normal and transformed cells (Figs. 2A and 4A). Furthermore, increased CXCR7 expression also led to, constitutively and rapidly, EGF-induced increase of p-EGFR (Fig. 4B). We found a stable complex formation between EGFR and CXCR7 in CaP cells that over express CXCR7, by 2 independent techniques (Fig. 5A–C, and Supplementary Fig. S4A–D).

We found CXCR7-depleted PC-3 cells grow significantly slower in vitro and their tumors grew much slower in vivo, indicating CXCR7 may indeed control CaP cell proliferation in vivo. Further, CXCR7-depleted tumor tissues show significantly reduced, cyclin D1, VEGF mRNA, and p-EGFR, further suggesting multiple roles of CXCR7 in tumorigenesis. These and other reports (13, 14) show CXCR7 may facilitate adhesion between tumor cells and host (mouse) endothelial cells that might induce rapid angiogenesis and tumor growth. In conclusion, present discoveries highlight the novel mechanism of chemokine and chemokine receptor interactions to facilitate proliferation and cell survival. The relative contribution of CXCR7 over CXCR4 in several CaP cells examined in this report, further corroborate the possibility of prominent role of CXCR7 in prostate, and potentially in other epithelial cancers.

Disclosure of Potential Conflicts of Interest

The authors declare no conflict with any commercial entity.

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References

CXCR7 Promotes Cell Proliferation via EGFR Activation


The IL-8–Regulated Chemokine Receptor CXCR7 Stimulates EGFR Signaling to Promote Prostate Cancer Growth

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