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

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Abstract

The pro-inflammatory 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 pro-cancerous cytokine IL-8 up regulates 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 up regulate CXCR7. RNAi-mediated knockdown of CXCR7 in CaP cells caused multiple anti-tumor 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 (pY1110) and phospho-ERK1/2. Notably, co-immunoprecipitation 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 co-regulation by the pro-inflammatory factor IL-8 in prostate cancer.

Keywords

Receptor trans-activation; RNA interference; cell cycle; angiogenesis; EGF-Receptor; apoptosis

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 Glutamineleucine-arginine motif (ELR+ CXCL) regulate physiological functions by binding and activating one or more seven

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CONFLICT OF INTEREST: The authors declare no conflict with any commercial entity.

transmembrane CXC chemokine receptors (CXCRs)(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 over-expression of CXCL or CXCRs is linked to tumor growth and metastasis (7–9).

The newest member of the seven CXCRs, CXCR7 (10), binds to two chemokines, CXCL11 and CXCL-12 (SDF-1a) and dimerizes with CXCR4, a co-receptor for SDF-1a (11,12). This dual specificity receptor is up-regulated 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., interleukine-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 demonstrate 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 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 performed by routine procedures as described (19). In brief, LNCaP, PC3 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 nM 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, Manassas, VA) and used within six months of resuscitation of original cultures. The cell lines used in the study were also authenticated for their origin by Genetica DNA Laboratories Inc. (Cincinnati, OH).

Global Gene expression analysis

The global mRNA expression of IL-8 expressing-LNCaP and LAPC-4 cells (LNCaP-IL8S, LAPC-4IL8S, respectively) was compared to vector-only transfectants (v), generated as reported before (16), using Agilent cDNA expression array (Agilent Technologies, Santa Clara CA). These studies were conducted at University of Miami's DNA Micro-array Core Facility, which also performed the Bioinformatics analysis. To identify differentially expressed genes, mathematical "dye-swap" was performed on arrays and one-class significance analysis of microarrays (SAM) (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 (q-PCR)

Cells cultured for 24h were transfected with gene-specific 21-mer siRNA sets (Smartpool siRNA, Dharmacon/Thermo Scientific Inc, Chicago, IL), using Dharmafect-2 transfection protocol (17). Total RNA isolated from cells 48 h later was subjected to cDNA synthesis and q-PCR using iQ SYBR-Green Supermix (BioRad, Hercules, CA) and the primers described in Table S1 (Supplement). The mRNA levels were normalized to that of Glyceraldehyde 3-phoshate dehydrogenase (GAPDH) based on the threshold cycle (C_t) of each sample in q-PCR. Relative levels of mRNA expression were calculated from ΔC_t, where ΔC_t = (test mRNA C_t – GAPDH C_t). Values are shown as Fold Difference (FD), defined as: FD= $[(2^{\Delta Ct})^{-1} \times 100)]$ (17).

Stable silencing of CXCR7 expression by shRNA

CXCR7 shRNA and scrambled sequence shRNA (Control shRNA) constructs were cloned into pRS plasmid under the control of U6 promoter for stable expression (HuSh-29mer, Origene Technology Inc. Baltimore, MD). CaP cells were transfected with shRNA-pRS using Lipofectamine 2000 (In Vitrogen Inc., Carlsbad, CA). Stable transfectants were selected from transfected cultures following two weeks in puromycin selection medium (2.0 µg/ ml) and evaluating the emergent cell colonies for CXCR7 mRNA knockdown by q-PCR and immunoblotting with rabbit anti-CXCR7 IgG (Cat No. GTX100027, GeneTex Inc, Irvine, CA).

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 q-PCR and immunoblotting. Cell colonies over-expressing 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 performed using 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+ Kit (GE Health Science) and relative protein band intensities were quantified using densitometry (Gel Logic 2200, Care-Stream Instruments, Rochester, NY) (23).

Co-immunoprecipitation

Cell lysates were prepared in RIPA buffer, centrifuged to obtain nuclei-free lysates, and immunoprecipitated (24). In brief, lysates were incubated with anti-CXCR7 rabbit IgG (10 µg/ml, GeneTex)), with anti-EGFR Rabbit monoclonal antibody (Epitomics Inc., Santa Clara, CA) or normal rabbit IgG, overnight at 4°C, followed by incubation with protein A-Sepharose beads for 6 h 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-3V) or CXCR7 shRNA transfectants (PC3-T73 and T74) were implanted into the right and left dorsal flanks of 6-week-old athymic mice ($(1 \times 10^6$

cells/site in 50% Matrigel, 5 animals × 2 sites/group, Charles River Labs, MA). 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 to RWPE-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 compared to that of PC-3. Further, the level of CXCR4 mRNA was 5 to 10-fold lower than that of CXCR7 (Table 1). Since many tumor cells co-express 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 up regulation of CXCR7

We previously reported that constitutive expression of IL-8 in LNCaP and LAPC-4 cells (IL-8S) induce a variety of pro-invasive and metastatic function (16). We compared here the gene expression profile between IL-8S and vector control cultures (V) by cDNA micro array. We found 549 and 377 genes had altered expression in IL-8S cells of LNCaP and LAPC-4 respectively, compared to those of V control pairs. Among them, as shown in Supplementary Table 2, 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 2) in CXCR7 mRNA expression in LAPC-4IL-8S and LNCaP IL-8S cells, respectively. We further validated the expression of CXCR7 by q-PCR and immunoblotting. The CXCR7 mRNA and protein were 6–8 folds and >2-fold higher in IL-8 transfectants than that of the respective vector-only transfectants (Fig. 1BC).

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

As shown in Fig. 1D (i), a rapid increase in CXCR7 mRNA was observed (≤ 15 min) after addition of 3 nM 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 & iii). The IL-8 induced increase of CXCR7 in RWPE-1 cells was more sustained (≥ 4 h) when compared to that of LNCaP (≤1 h) which, stabilized after 1 h 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 10ug/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 to that with 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 compared to that of controls (V) cells

(Supplementary Table 2). We validated by Q-PCR that LNCaP-V and LAPC-4V cells expressed 2–3 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 increased the expression of CXCR4 mRNA by 5-fold (Fig. 1Bii). 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 ii). 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 (Fig S1iii).

CXCR1 and CXCR2 both are required for IL-8 induced CXCR7 expression

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

CXCR7 is essential for cell proliferation in CaP cells

Wang et al. **(2008)** 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 >90% decrease in mRNA and >65% decrease in protein expression (Supplementary Fig.S2 (i)] As shown in Fig. 2A, depletion of CXCR7 significantly decreased the proliferation of PC-3 (55%), LNCaP-V (67%) and LNCaP-IL-8-S (40%) cells at 72 h post-transfection. Since siRNA-mediated gene silencing lasts ≤ 72 h (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 to those in vector-only transfected cells (Fig. 2B).

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

Since 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. Since we could not detect autocrine production of CXCL11 or SDF-1a in CaP cell cultures, we added CXCL-11, CXCL-12, 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 PC3-V or PC3-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 medium supplemented with or without FBS (10%) (Fig. 2C and Supplementary Fig. S2-ii & iii).

Since CaP cells express both CXCR4 and CXCR7 (14), and both share SDF1a 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 >85% but did not affect cell proliferation when compared to 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 Fig. 3A, PC-3 or LNCaP cells transfected with CXCR7 siRNA, showed increased accumulation in G0/G1 phase, by 52%, compared to 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 (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 cyclinD1, 1.5 to 2-fold decreases in Cdk-4, and 2 to 6.5- fold decreases in cyclinB1, in both CaP cells (Fig. 3C) and significantly increased P21 (11.5× in PC-3 and 15×-in LNCaP) protein levels, but none in P27^{kip1} 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 Extra cellular 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 CXCR7siRNA 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) compared to the vector-only transfected RWPE-1 cells (RW-V).

CXCR7 stimulates EGFR phosphorylation

Strong change in ERK1/2 activity upon 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 RWCX7, LNCaP and PC-3 but not in RWPE-1 cells (Fig. 4B; also see Supplement Fig S3 (i & ii) for quantitation). CXCR7 depleted-cells showed lower levels of both p-EGFR and p-Erk1/2, as compared to those in c-siRNA transfectants (Fig 4C, see also Fig. 3D).

Since 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-1a, or CXCL11. As shown in Fig. 4D p-EGFR levels remained unchanged in cultures exposed to recombinant SDF-1a, or CXCL11 in both RWPE-1 and RWCXC7 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 pEGFR levels in Lane 1 of RWPE-1 and lane 5 of RWCX7 in Fig 4D or when stimulated with EGF (Lane 4 and 8, respectively, in Fig 4D, also see Suppl. Fig. S3 (iii).

CXCR7 co-localizes with EGFR

Since expression of CXCR7 constitutively activates EGFR, we examined whether CXCR7 and EGFR co-localize or aggregate on plasma membrane (33). We attempted to co-immunoprecipitate CXCR7 and EGFR from RWPE-1 or CaP cells, using anti-EGFR or anti-CXCR7 antibodies and non-ionic detergent solubilized cell lysates. As shown in Fig. 5A, and 5B, both molecules are immunoprecipitated by either anti- p-EGFR or anti-CXCR7 antibodies. We demonstrated further more p-EGFR in RWCX7 cells than that in RWPE-1V

cells and CXCR7 over-expression increased phosphorylation of EGFR only at tyrosine$_{1110}$ but, not serine-$_{1070-71}$ (Fig. 5C). In addition, we performed confocal microscopy of LNCaP cells labeled with anti-EGFR (mouse anti-human IgG) and anti-CXCR7 (Rabbit anti-human IgG, GeneTex) antibodies followed by, fluorescent labeling with secondary antibodies labeled with Alexafluor 555 and Alexfluor488, 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 two labeled antibodies were observed (see Supplementary Fig. S4 A–D).

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

We characterized *in vivo* tumor growth of two CXCR7-depleted PC-3 sub-lines, T73 and T74 and compared their tumor growth with that of vector-only transfectants (v). 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 to 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 vs 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 to total EGFR and cyclinD1 levels (Fig. 6C), and VEGF mRNA (Fig. 6D) in T73 and T74 tissues as compared to 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 four major observations: first, transient exposure or constitutive expression of a pro-inflammatory chemokine (IL-8) modulates non-target chemokine receptors (CXCR4 and CXCR7); second, although CXCR7 is a "decoy" receptor, it is highly modulated by IL-8, it is constitutively up regulated 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 (CXCL-11 and SDF-1a), and four, CXCR7 is capable of stimulating cell proliferation, cell cycle progression and angiogenesis by coupling/co-localizing 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 up regulation of CXCR7 and down regulation 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 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, down regulation 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 et al (2010) were unable to detect ligand binding or cell surface expression of CXCR7 in PC-3 cells and they disputed earlier work by Wang et al (2008) that observed PC-3 expression of CXCR7 in PC-3 may be attributed to non-specific 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 Gene Tex) and with qRT-PCR. 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 q-PCR (see Figs. 1,2, 4, 5 and 6 plus Supplementary Fig. S5).

We showed that inhibition of IL-8 specific receptors, CXCR1 or CXCR2 can abrogate IL-8 induced CXCR7 up-regulation (Supplementary Fig S1-iv). This could be due to the co-regulation of CXCR2 by CXCR1, as we previously reported (26, 35). Since 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 up regulation 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. 1Bii). Although CXCR4 is implicated in bone metastasis of CaP, its relative abundance on CaP cells is significantly lower than CXCR7, and therefore, these two 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). Since 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). Since survival following androgen deprivation is a critical step in the emergence of castration-resistant tumors, IL-8 induced up-regulation of CXCR7 may enhance the survival and proliferation of those tumor cells. These finding suggest that elevated CXCR7 on CaP cells may 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 over-expression 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 two independent techniques (Fig.5A–C, and supplementary Fig S4 A–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.

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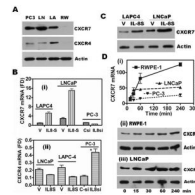
Fig. 1. IL-8 up-regulates CXCR7 and down regulates CXCR4 expression in prostate cells
A. Detection of CXCR4 and CXCR7 proteins in CaP and normal cells by Western blotting
(LN: LNCaP; LA: LAPC-4; RW: RWPE-1). B. CXCR7 (i) and CXCR4 (ii) mRNA. **C**.
CXCR7 protein levels in LNCaP and LAPC-4 cells continuously-expressing IL-8 (IL8-S)
and their vector-only transfectants (e.g., IL-8V). **D**. Increased expression of CXCR7 mRNA
(**i**) and protein levels (**ii & iii**) following IL-8 addition to growth factor-starved (24 h)
RWPE-1 and LNCaP cells, respectively.
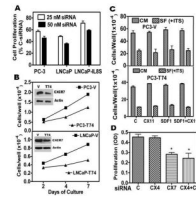
Fig. 2. CXCR7 depletion causes inhibition of proliferation in CaP cells
A, Decrease in cell density (cells/well, mean ± SEM) of CaP cell cultures following depletion of CXCR7 by siRNA. **B (i)–ii)**, Decreased cell growth in PC-3 or LNCaP cells, stably-expressing either control-shRNA (V), or CXCR7 shRNA (T-74), determined by cell counting. Western blot analysis of CXCR7 in the cells transfected with V and T74 shRNA (**Inset, i–ii**). **C**, Cell density of PC-3 cultures expressing either control-shRNA (PC-3V), or CXCR7 shRNA (PC3T-74) in the presence or absence of CXCL11 (CX11) or SDF1 (10 ng / ml), cultured with 10% FBS or without FBS (ITS) medium for 5 days. **D**, Cell viability determined by MTT reduction assay in PC-3 cells transiently transfected with indicated siRNA. Data presented are mean ± S.E.M. n=3. *P < 0.05 versus control, Student's t test.
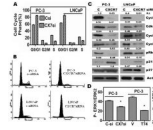
Fig. 3. CXCR7 depletion causes cell cycle arrest in CaP cells
A. Percent of cells in each cell-cycle phase was estimated from flow cytometry analysis of DNA contents in CaP cells 48 hours after transfection with CXCR7 siRNA or c-siRNA. **B**. DNA content histograms of CaP cells transfected with c-siRNA or CXCR7 siRNA. **C**, Comparison of expression of cell cycle regulatory proteins by Western blotting as in **A**, and relative expression (R.I.) shown are normalized to protein band intensities of β-actin. **D**, Levels of P- Erk1/2 measured with an ELISA in C-siRNA (c-si), CXCR7 siRNA (CXC7si), CXCR7shRNA (T74) or V transfectants following EGF stimulation (5 nM, 10 min). The p-Erk1/2 levels were lower in CXCR7 depleted PC-3 cells (27% in siRNA and 63% in shRNA depleted PC-3-cells) (* = P ≤0.05, n=3).

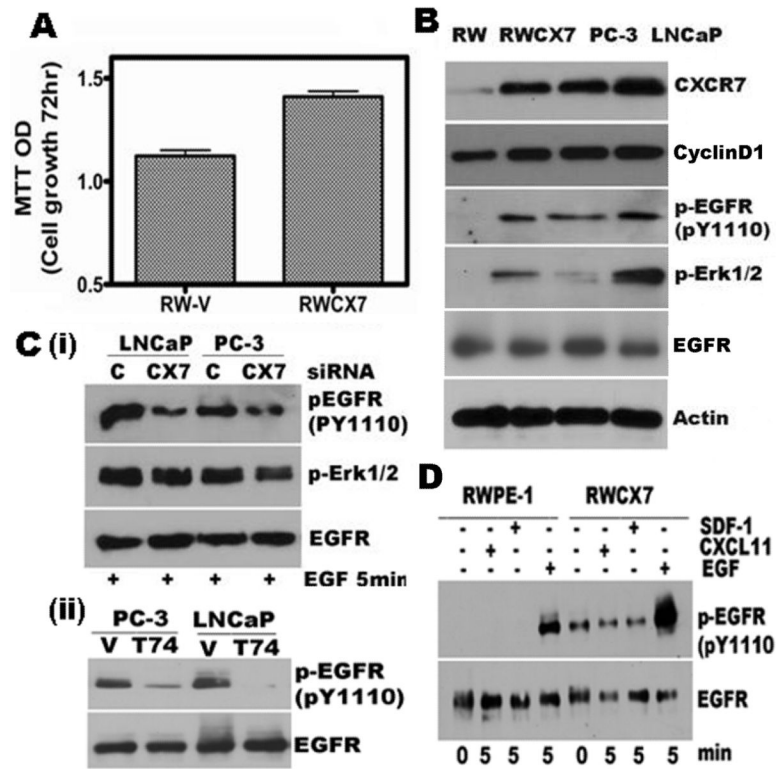**Fig. 4. Forced Expression of CXCR7 increases cell proliferation and p-EGFR levels**
A, Increase in cell density of RWPE-1 cultures stably expressing CXCR7 (RWCX7) as compared to RW-V cells, **B**, A comparison of levels of p-EGFR, p-Erk1/2 and cyclinD1 in cells described in **A** and CaP cells. **C**, Levels of p-EGFR and p-Erk1/2 in CaP cells following transient transfection with either c-siRNA (c-si) or CXCR7 siRNA (CX7) and stimulated with EGF (5 nM) for 5 min. CXCR7siRNA transfectants had lower level of p-EGFR and p-Erk1/2 compared to that of c-siRNA transfected cells (**C** Lanes 2 and 4). **D**, Western blots of pEGFR$_{y1110}$ from cells described in **A** that were growth factor starved for 24 h and then stimulated with 5 nM EGF, CXCL11 or SDF-1 (both 100 ng/ml). Total EGFR and ß-actin levels are shown as loading controls.
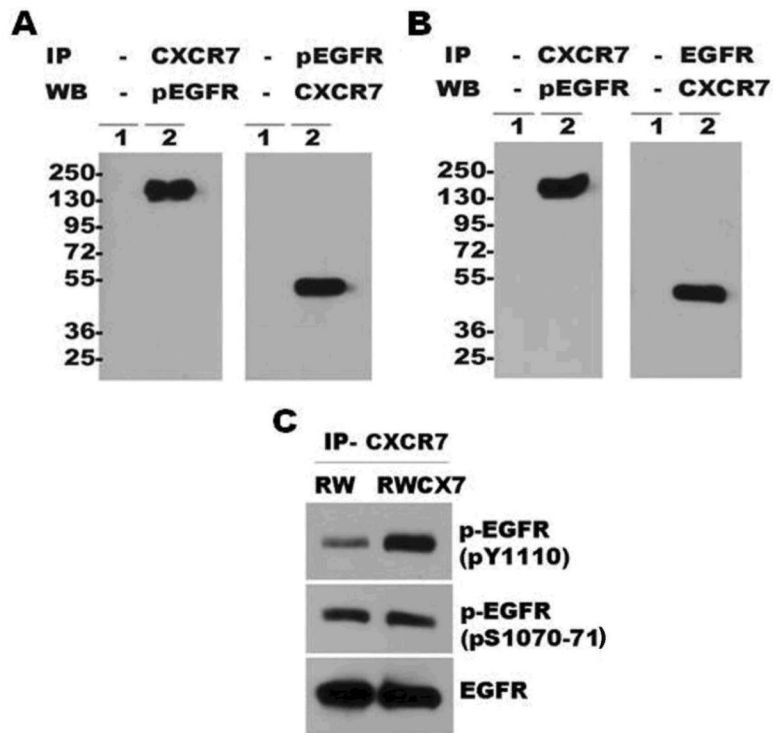
Fig. 5. CXCR7 associates with EGFR in CaP cells

A–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 co-precipitated with CXCR7 in RWCX7 cells. RWCX7 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 residue 1070-71 in EGFR (pS1070-71) (Epitomics).
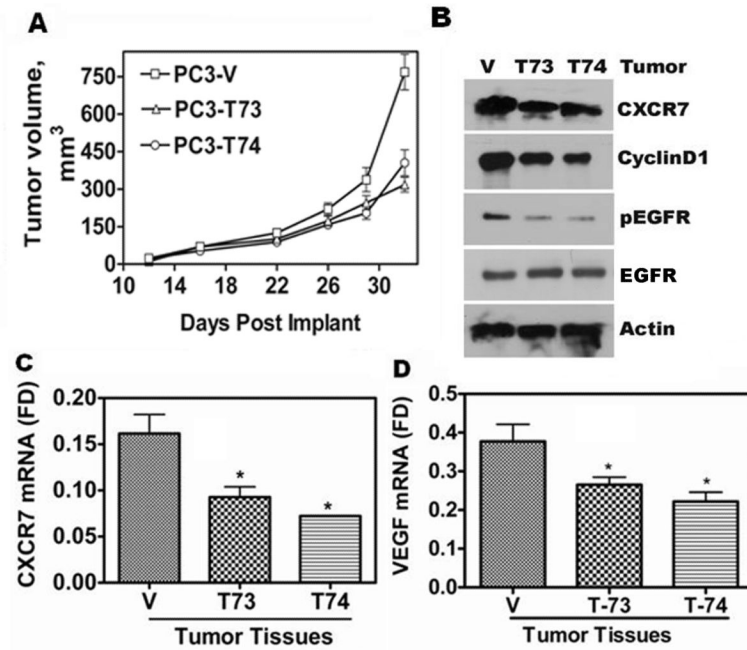
Fig.6. CXCR7 depletion slows PC-3 tumor growth in mice
A, Tumor growth over time in athymic mice was recorded for mice injected with PC-3 cells
stably expressing CXCR7 shRNA (T73 and T74) or scrambled sequence-shRNA (V) **B**,
Blots of CXCR7, cyclin D1 p-EGFR CXCR7 tumor tissue of PC-3V, T73 and T74 **C–D**,
CXCR7 and VEGF mRNA level were decreased in T73 and T74 tumors as analyzed by q-
PCR. There was a significant delay (p<0.03) in tumor growth and in the terminal tumor
volume of T73 and T74 tumors.

Table 1

Expression of CXCR Chemokines and their receptors in normal prostate and tumor cells

Total RNA isolated from various cultures was reverse transcribed and the cDNAs were quantified using q-PCR. Values shown are Fold differences (FD) as defined in the methods, normalized to GAPDH mRNA. ND: Not detected. Mean ± SD, n= 3

Cells	mRNA level relative to GAPDH mRNA (FD)			
	CXCR4	CXCR7	CXCL11	SDF-1
RWPE-1	ND	0.09 ± 0.13	0.004 ± 0.2	ND
LAPC4	0.23 ± 0.04	1.0 ± 0.16	0.091 ± 0.2	0.018 ± 0.1
LNCaP	0.21 ± 0.12	2.2 ± 0.10	0.023 ± 0.07	0.045 ± 0.04
PC-3	0.094 ± 0.10	1.3 ± 0.43	0.011 ± 0.04	0.102 ± 0.02