Role of Chemokine Receptor CXCR7 in Bladder Cancer Progression

Mingang Hao#, Jianghua Zheng#, Kailin Hou c, Jinglong Wang a, Xiaosong Chen b, Xiaojiong Lu d, Junjie Bo c, Chen Xu e, Kunwei Shen b, Jianhua Wang a*

a Department of Biochemistry and Molecular & Cell Biology, Shanghai Jiao Tong University School of Medicine, Shanghai, 200025, China.

b Shanghai Ruijin Hospital, Comprehensive Breast Health Center, Shanghai, 200025, China.

c Department of Urology, Shanghai Renji Hospital, Shanghai, 200001, China.

d Department of Surgery, Division of Trauma/Surgical Critical Care/Burns, Medical Center (Hillcrest), University of California, San Diego, MC8896 UN.

e Department of Histology and Embryology, Shanghai Jiao Tong University School of Medicine, 200025, China

#M Hao, J Zheng, and K Hou contributed equally to this work.

*Corresponding author: Jianhua Wang, PhD, Shanghai Jiao Tong University School of Medicine, Shanghai, 200025, China. Phone: 021-54660871; Fax: 021-63842157; E-mail: jianhuaw2007@gmail.com.
Abstract

Bladder cancer is one of the most common tumors of the genitourinary tract; however, the molecular events underlying growth and invasion of the tumor remain unclear. Here, role of the CXCR7 receptor in bladder cancer was further explored. CXCR7 protein expression was examined using high-density tissue microarrays. Expression of CXCR7 showed strong epithelial staining that correlated with bladder cancer progression. In vitro and in vivo studies in bladder cancer cell lines suggested that alterations in CXCR7 expression were associated with the activities of proliferation, apoptosis, migration, invasion, angiogenesis and tumor growth. Moreover, CXCR7 expression was able to regulate expression of the proangiogenic factors IL-8 or VEGF, which may involve in the regulation of tumor angiogenesis. Finally, we found that signaling by the CXCR7 in bladder cancer cells activates AKT, ERK and STAT3 pathways. The AKT and ERK pathways may reciprocally regulate, which are responsible for in vitro and in vivo epithelial to mesenchymal transition (EMT) process of bladder cancer. Simultaneously targeting the two pathways by using U0126 and LY294002 inhibitors or using CCX733, a selective CXCR7 antagonist drastically reduced CXCR7-induced EMT process.

Taken together, our data show for the first time that CXCR7 plays a role in the development of bladder cancer. Targeting CXCR7 or its downstream-activated AKT and ERK pathways may prove beneficial to prevent metastasis and provide a more effective therapeutic strategy for bladder cancer.
1. Introduction

Bladder cancer is the ninth most common type of cancer worldwide, which affects three times as many men as women. Each year, about 360,000 new cases of bladder cancer are expected, and about 150,000 people will die of this disease in the world [1]. It is the fifth most common type of cancer in China—the fourth most common in men and the ninth in women [2]. The clinical course of bladder cancer carries a broad spectrum of aggressiveness and risk. High-grade non–muscle-invasive cancers frequently progress and muscle-invasive cancers are often lethal and have the highest recurrence rate of any malignancy [1, 3]. Intriguingly, emerging evidence suggests that chemokines are likely to play a crucial role in mediating the continuous series of events leading to tumor growth and metastasis [3-8]. Therefore, it is important to investigate chemokine signaling in bladder cancer to get a better understanding of the underlying mechanisms leading to tumor invasion and metastasis, and to develop prognostic and therapeutic strategies in this disease.

Over the past several years, there is emerging evidence that multiple pairs of chemokines and their receptors play critical roles in cancer progression [4, 5, 7]. Our group and others have shown that CXCL12 and its receptor CXCR4 are critical elements in growth and metastasis of prostate cancer (PCa) to tissues that produce large quantities of CXCL12 [6, 9]. More recently, CXCL12 was shown to bind with high affinity to the orphan receptor CXCR7. CXCR7 was originally cloned on the basis of its homology with conserved domains of G protein-coupled receptors [10, 11]. Combined phylogenetic and chromosomal location studies suggest that CXCR7 represents a chemokine receptor structurally close to CXC receptors [12], pointing to CXC chemokines as potential ligands. CXCR7 shares homology with the viral gene ORF74 encoding a chemokine receptor, which suggests that it may signal constitutively in the absence of ligand [13]. Like CXCR4, CXCR7 serves as a co-
receptor for some human and simian immunodeficiency virus strains [14]. In the vasculature, the expression of CXCR7 is elevated in endothelial cells associated with tumors, and overexpression of CXCR7 in NIH 3T3 cells strongly supports a role for the receptor in tumorigenesis [14]. More recently, CXCR7 expression has been shown to be elevated in endothelial cells associated with tumors [15]. Membrane associated CXCR7 is expressed on many tumor cell lines, on activated endothelial cells, and on fetal liver cells [16]. CXCR7 is expressed by the placenta [17] as well as in the vascular endothelium [18, 19]. Miao et al. [18] further confirmed a critical role for CXCR7 in tumor vascular formation, angiogenesis, and promotion of the growth of breast and lung cancer in vivo. These characteristics suggest that like CXCR4, CXCR7 plays a role in regulating immunity, angiogenesis, stem cell trafficking, and mediating organ-specific metastases of cancer. However, the role of CXCR7 in bladder cancer still remains unsettled.

Importantly, growing evidence has suggested that CXCR7 functions as a decoy receptor, which does not activate Gi pathways of a chemokine receptor that may result in GTP hydrolysis or calcium mobilization [16]. Debate has still arisen whether CXCR7 functions like GPCR mediating the signal transduction process [20]. More recently, it has been demonstrated that CXCR7 interacts with β-arrestin in a ligand-dependent manner [20-23]. CXCR7 can signal through β-arrestin and act as an endogenous β-arrestin-biased receptor, which suggests that other receptors that are currently thought to be orphans or decoys may also signal through non-G-protein-mediated mechanisms [20].
2. Materials and methods

2.1. Cell culture

Human bladder cancer cell lines with different grade T24 (grade 3 bladder cancer), J82 (grade 3 bladder cancer) and RT4 (grade 1 bladder cancer) were purchased from American Type Culture Collection, which have been widely used by other groups as a model for bladder cancer. These cell lines were cultured in DMEM, RPMI 1640, and McCoy's 5a Medium containing fetal bovine serum (FBS) with antibiotics (Invitrogen Corp., Carlsbad, CA), respectively. Additional methodologies are provided in the Supplementary sections.

2.2. Tissue microarrays and immunohistochemical staining

High-density tissue microarrays were constructed by Shanxi Chaoying Biotechnology Co., Ltd. (Catalog No: CC12-11, Xian, China) with clinical samples obtained from a cohort of 78 patients. The clinical samples used to perform in the project have been approved by Shanghai Jiao Tong University School of Medicine Ethical Committee. The method of quantitative analysis was previously described [24]. Additional methodologies are provided in the Supplementary sections.

2.3. Construction of lentiviral vectors

Stable knock-down of CXCR7 in RT4 cells was achieved by expression of shRNA from lentivirus vector pLLU2G-shGremlin under the control of CMV promoter for stable expression (Cyagen Bioscience). Lentivirus vector pLLU2G-shGremlin was purchased from Cyagen Biosciences Inc. (Cyagen Bioscience). Three pairs of annealed DNA oligonucleotides were inserted between HpaI and XhoI restriction sites according to common protocol. Additional methodologies are provided in the Supplementary sections.

2.4. RNA extraction and RT-PCR
Total RNA in T24, J82 and RT4 cells were extracted using Trizol (Invitrogen, Carlsbad, CA). RT-PCR was performed using reverse transcriptase cDNA synthesis kit (Fermentas, St Leon-Rot, Germany) according to the manufacturer’s protocol. 1 μg of total RNA were reversely transcribed into cDNA and equal volume of cDNA were used as PCR template using specific primers: CXCR7, Genbank no.: NM_020311, (sense) 5’-AGGAAGTAGAAGACAGCGATAATGG-3’, (anti-sense) 5’-TATGACACGCACTGCTACATCTTG-3’; CXCL11, Genbank no.: NM_005409, (sense) 5’-AGCCTCCATAATGTACCCAA-3’, (anti-sense) 5’-GCACCTTTGTAAACTCCGATG-3’; CXCL12, Genbank no.: NM_199168, (sense) 5’-GAGCCAACGTCAAGCATCT-3’, (anti-sense) 5’-CGGGTCAATGCACTTTGTGTC-3’; GAPDH, Genbank no.: NM_002046, (sense) 5’-TCACCATCTTCCAGGAGCGAGA-3’, (anti-sense) 5’-GCAGGAGGCGATTGCTGATGATC-3’. CXCR7, CXCL11, CXCL12 and GAPDH were amplified by 30 cycles at 95°C for 20 s, 60°C for 60 s and 68°C for 30 s (NEB, Ipswich, MA).

2.5. siRNAs of CXCR4 and transfection

Transient knock-down of CXCR4 by transduction of siRNA duplexes targeting CXCR4 mRNA was achieved as previously described [25]. siRNA duplexes were purchased from RiboBio (RiboBio, Guangzhou, China) and the most effective duplexes are: (sense), 5’-UAAAAUCUUCCGCCCACCdTdT-3’, (anti-sense), 5’-GGUGGGCCAGGAUUUUAdTdT-3’. The scramble siRNA duplexes were used as control. The siRNA duplexes were transduced into RT4 and J82 cells at a final concentration of 120 nM using Lipofectamine2000 (Invitrogen, Carlsbad, CA).

2.6. Flow cytometry analyses

To determine total cell surface CXCR7 and CXCR4 expression, 10^5 cells were incubated at room temperature for 30 min with 10 μL of nonspecific isotype-matched
controls, mouse to human IgG (R&D Systems, Minneapolis, MN) and 10 μL of mouse monoclonal antibodies conjugated PE fluorochrome (anti-human CXCR7, 11G8) (R&D Systems, Minneapolis, MN) and APC fluorochrome (anti-human CXCR4, 12G5) (BD Biosciences, San Diego, CA). Unbound antibodies were removed by washing the cells twice in phosphate-buffered saline buffer. Cells were analyzed by FACScan (BD Biosciences) and the data were analyzed with CellQuest software (BD Biosciences).

2.7. Proliferation assay
After a 24-h serum withdrawal, bladder cancer cells were digested and plated at 1×10^4 cells/well into triplicate 96-well flat-bottomed cell culture plates in 0.1ml complete culture medium. Additional methodologies are provided in the Supplementary sections.

2.8. Cell migration and invasion
Wound healing assay was used to assess cell migration. Cell invasion was examined using a reconstituted extracellular matrix membrane (BD Biosciences, San Jose, CA). Additional methodologies are provided in the Supplementary sections.

2.9. Western blots
Cells were cultured in 3.5 cm diameter plates (80-90% confluence), and washed by PBS, and then serum-starved for overnight. The cells were then stimulated with 200 ng/ml CXCL12 (R&D Systems, Minneapolis, MN) or not for 10 min at 37°C and lysed for 10 min on ice in RIPA buffer (Thermo Scientific, Waltham, MA) containing an anti-protease mix (Roche, Germany), and protein concentration was measured by BCA assay (Thermo Scientific, Waltham, MA). Additional methodologies are provided in the Supplementary sections.

2.10. ELISA
Antibody sandwich ELISAs were used to evaluate IL-8, and VEGF levels in the PCa cell conditioned medium (CM) (R&D Systems, Minneapolis, MN) as previously described.

2.11. Cytokine antibody arrays

The expression of 20 cytokines was evaluated using human angiogenesis antibody arrays (Catalog No: AAH-ANG-1) (Ray-Biotech, Norcross, GA). The membranes were exposed to blocking buffer for 1 h at 25°C, and incubated with CM or control medium up to 2 h at 25°C. After washing three times, the arrays were processed with biotin-conjugated angiogenesis antibody mix and strepavidin-HRP conjugate according to the manufacturer protocol.

2.12. Endothelial sprout formation assays

Growth factor reduced basement membranes were placed into 4-chamber slides (Matrigel™ 125 µl/chamber; BD Biosciences, San Diego, CA) and 0.8 × 10^4 endothelial cells were added on top. Additional methodologies are provided in the Supplementary sections.

2.13. cDNA microarray analysis

Illumina Human HT12 v 3 Expression BeadChips (Illumina) were used in this study. This BeadChip targets > 25,000 genes with > 48,000 probes derived from the RefSeq (Build 36.2, rel22) and UniGene (Build 99) databases. Additional methodologies are provided in the Supplementary sections.

2.14. Subcutaneous tumor growth

All experimental animal procedures were performed in compliance of the institutional ethical requirements and were approved by the Shanghai Jiao-Tong University School of Medicine Committee for the Use and Care of Animals. Additional methodologies are provided in the Supplementary sections.

2.15. Immunohistochemistry
For immunostaining with CD31, tissue sections were blocked with Sniper for 5 min and incubated overnight at 4 °C with 28 mg/ml rabbit anti-human CD31 antibody (Dako North America Inc., Carpinteria, CA) diluted in PBS. Additional methodologies are provided in the Supplementary sections.

2.16. Statistics

Numerical data are expressed as means ± standard deviation. Statistical differences between the means for the different groups were evaluated with Instat 4.0 (GraphPAD software) using one-way analysis of variance (ANOVA) with the level of significance at $p < 0.05$. Where indicated, a Krusal-Wallis test and Dunn’s multiple comparisons tests were utilized with the level of significance set at $p < 0.05$. 
3. Results

3.1. Expression of CXCR7 in bladder cancer

To explore the role of CXCR7 in bladder cancer, high density tissue microarrays were stained with an anti-human CXCR7 from clinical samples obtained from a cohort of 78 patients. Representative images are shown in Fig. 1A, indicating that expression of CXCR7 in bladder cancer tissues (grade I, II, III) is markedly higher than the normal tissues. High grade II and III also indicated stronger staining than low grade I (Fig. 1A). By quantitative analysis, these findings suggested that CXCR7 expression increases with increasing tumor grade (Fig. 1B). Moreover, we recently showed by Kaplan-Meier analysis that in 148 patients followed up for 2-95 months, overexpression of CXCR7 is significantly associated with shorter recurrence-free survival (RFS) rate (log -rank, 3.256; \( p = 0.002 \)) [26]. Therefore, these results demonstrate that CXCR7 expression is correlated with bladder cancer progression.

3.2. CXCR7 modulates phenotype of bladder cancer cells.

RT-PCR and FACS analyses of CXCR7 showed higher expression in the RT4 compared with J82, T24 cell lines (Fig. 2A, up and bottom). Next CXCR7 expression was modulated by overexpressing the receptor in J82, T24 cells (noted as J82\textsubscript{CXCR7}, T24\textsubscript{CXCR7}) or by reducing its expression by shRNA (noted as RT4\textsubscript{shCXCR7}). After clone selection, individual clones were pooled and evaluated by FACS for CXCR7 expression in these cell lines (Fig. 2B). As shown in Fig. 2C, overexpression of CXCR7 increased the basal proliferation rates of the J82 and T24 cells in 4 day, whereas reducing the receptor expression in RT4 cells decreased the effects.

One possible explanation of the increased proliferation observed after transfection with CXCR7 is that the receptor may protect the cell lines from apoptosis. The loss of cellular membrane integrity as a reflection of cells undergoing apoptosis was therefore determined by staining the cells for annexin-V. As shown in supplemental
Fig. 1, CXCR7 overexpression decreased the apoptotic fraction of cells in culture for both J82 and T24 cells compared with the respective controls (8.9 % versus 4.5 % and 13.8 % versus 12.5 %). The total number of dead or dying cells (double-positive for PI and annexin-V) (10.7 % J82_{CXCR7} versus 6.1 % J82_{control}; 20.0 % T24_{CXCR7} versus 16.0 % T24_{control}) was less as well.

The ability of CXCR7 to regulate migration was assessed by scratch healing assay. The results showed that the CXCR7-transfected cells nearly closed the wound at 36 h for J82 cells or 12h for T24 cells after scratch, whereas the respective control cells were unable to heal the wound at the respective time. The mean wound distances of the CXCR7-transfected cells and the control cells at 36 h for J82 cells or 12h for T24 cells were significantly different (165.28 ± 6.18 µm vs 435.231 ± 53.06 µm; 8.17 ± 6.41 µm vs 189.31 ± 24.67 µm p < 0.001) (Fig. 2D). The results indicate that CXCR7 expression is capable of inducing migration of bladder cancer cells.

Once individual tumor cells are bound to the endothelium, they must invade through the extracellular matrix to establish a metastasis. The ability of CXCR7 to regulate invasion was next studied using reconstituted extracellular matrices in porous culture chambers (Matrigel; Beckman Coulter Labware). J82 cells with overexpression of CXCR7 resulted in markedly higher invasive ability compared with the control (Fig. 2E) in the presence of CXCL12, but not in CXCR7-overexpressing T24 cells. The possibility is that CXCR4 expression is absent in T24 cells, which is responsible for insignificant invasion (Supplemental Fig. 2). In contrast, reducing the expression of CXCR7 decreased invasive abilities of J82 cells in the presence of CXCL12 (Fig. 2E).

To determine whether CXCR7 and CXCR4 reciprocally regulate each other in bladder cancer, similar studies were performed in cells with altered CXCR7 levels. As shown in supplemental Fig. 2, alterations of CXCR7 expression did not significantly regulate CXCR4 levels in these bladder cell lines as previously reported in PCa [19].
In contrast, knockdown of CXCR4 expression in J82 and RT4 cells resulted in enhanced levels of CXCR7 as compared with the respective controls (Supplemental Fig. 2).

3.3. Effect of altered CXCR7 expression on cytokine secretion.

The growth of new blood vessels (angiogenesis) within tumors by altered cytokine secretion is essential for tumor growth, maintenance, and metastasis [7-9]. Based on these findings, we hypothesized that modulated CXCR7 expression is potentially linked to regulating expression of angiogenic growth factors in bladder cancer cells. To test this possibility, antibody arrays targeting angiogenic cytokines were used. As shown in Fig. 3A, high levels of VEGF and IL-8 were commonly presented in the CM derived from T24 and J82 cells with overexpression of CXCR7 compared with the respective controls. To further explore the proangiogenic signals regulated by CXCR7, CM derived from the various CXCR7 transfectants were analyzed by ELISA. Fig. 3B indicated that IL-8 and VEGF levels in T24 and J82 cells overexpressing CXCR7 were higher than the control cells, consistent with our antibody array data. In contrast, shRNA targeting of CXCR7 expression in RT4 cells resulted in significant reductions in the levels of IL-8 and VEGF compared with the control cells.

To explore whether CXCR7 expression is biologically relevant to vascular recruitment by bladder cancer cells, human endothelial cell sprout formation was used as in vitro assay measuring proangiogenic activity. Shown in Fig. 3C, little or no sprout formation occurred in the absence of external stimuli applied to endothelial cells alone in vitro. Co-culture of the endothelial cells with either the CM derived from J82 or T24 control cells stimulated robust vascular sprout formation (Fig. 3C). However, overexpression of CXCR7 in both cells dramatically increased blood vessel sprout formation (Fig. 3C) compared with their respective controls. In contrast, reduced expression of CXCR7 in RT4 cells decreased vessel formation relative to the
respective control (Fig. 3C).

3.4. CXCR7 promotes EMT of bladder cancer cells

As shown in Supplementary Fig. 3, altering expression of CXCR7 induced the morphological appearance similar to EMT or MET phenotype, suggesting that CXCR7 may be associated with EMT or MET in bladder cancer progression. To test the possibility, we assessed the expression of EMT marker proteins as previously reported [27]. The results showed that although E-cadherin, a universal epithelial marker, is absent in J82 and T24 cells, overexpression of CXCR7 in both cells induced up-regulation of N-cadherin and β-catenin, two mesenchymal markers. In contrast, CXCR7 shRNA-targeted RT4 cells increased E-cadherin expression coincided with the reduction of N-cadherin and β-catenin expression (Fig. 4A).

To explore potential cellular pathways of EMT induced by CXCR7, we analyzed the genome-wide transcriptome profile of CXCR7 shRNA-targeted RT4 and the control cells by Illumina Human HT12 v 3 Expression BeadChips (Illumina). According to fold-change (X2.0) screening between RT4 control and RT4CXCR7 cells, we found 310 up-regulated genes and 267 down-regulated genes (Supplementary Table 1). As shown in Fig. 4B, 47 cancer-associated genes for cluster mapping on the MeV microarray analysis platform (www.tm4.org/mev.html). We also identified genes related to molecular function of EMT and picked up the top 9 gene sets that overlapped with different function-clusters for exhibition (Fig. 4C).

3.5. EMT induced by CXCR7 is associated with the activated AKT, ERK and STAT3 pathways.

The above microarray data imply that CXCR7 signaling may be able to activate some pathways, which acts as a player in EMT process of bladder cancer. As shown in Fig. 5A, overexpression of CXCR7 in J82 and T24 cells induced marked phosphorylation of Akt at Thr-308 site, but not at Ser-473 site (Fig. 5B); In contrast, knockdown of
CXCR7 in RT4 cells reduced intense phosphorylation of Akt (Thr-308). Similar induced trend were observed in these cells that altering CXCR7 expression is responsible for activating ERK and STAT3 (Tyr-705) pathways (Fig. 5A). Interestingly, we noted that targeting the ERK pathway by using U0126 in CXCR7-expressing J82 cells can activate AKT pathway. Yet, targeting the AKT pathway by using LY294002 can also activate ERK pathway (Fig. 5B). The results imply that AKT and ERK signaling by CXCR7 may reciprocally regulate in bladder cancer cells.

EMT has been noted as a critical process that will contribute to tumor invasive phenotypes, which is associated with multiple activated pathways, including AKT and ERK signaling et al (25). Fig. 5B showed that activating AKT and ERK pathways by CXCR7 can both significantly up-regulated expression of β-catenin and N-cadherin. In this case, simultaneously targeting the two pathways by using U0126 and LY294002 can greatly reduce expression of β-catenin and N-cadherin compared with inhibiting the one pathway alone, however. Similarly, using CCX733, a selective CXCR7 antagonist, drastically reduced CXCR7-induced expression of β-catenin and N-cadherin compared with another antagonist CCX771 and negative control CCX704, followed by inhibiting phosphorylation of Akt (Thr-308) and ERK signaling. Taken together, the results imply that activating AKT and ERK pathways by CXCR7 are responsible for in vitro EMT process of bladder cancer cells. However, whether the activated STAT3 pathway by CXCR7 also involved in EMT process is warranty needed to further investigate.

3.6. Effects of CXCR7 expression on tumor growth, angiogenesis and EMT in vivo.

To confirm that CXCR7 plays a role in tumor growth, SCID mice were implanted s.c. with cells engineered to overexpress CXCR7 level. As shown in Fig. 6A, B, tumor burdens generated from CXCR7-overexpressing J82 cells were much larger than the
tumors generated by the transfected control cells (535 mm$^3$ versus 196 mm$^3$).

To address directly whether CXCR7 was able to play a role in tumor angiogenesis, the tumors were stained with an antibody to CD31, a specific marker for vascular endothelial cells. Overexpression of CXCR7 in J82 cells resulted in more large and abundant blood vessel formation than the tumors generated by the control transfected cells (Fig. 6C) (26.2 ± 1.6 versus 9.8 ± 2.6 per high powered field, respectively).

Given that CXCR7 functions as a player in EMT process of bladder cancer in vitro by activating AKT and ERK pathways, we next assay whether the effects may contribute to tumor growth in vivo. Fig. 6C indicated that, CXCR7-overexpressing J82 in tumor tissues expressed higher phosphorylated AKT (Thr-308) and ERK levels relative to the respective controls. In this case, expressions of N-cadherin and β-catenin are significantly increased in CXCR7-overexpressing J82 tumor compared with the controls. Taken together, these data suggest that activation of AKT and ERK signaling by CXCR7 in J82 cells indeed play a role in EMT process of bladder cancer.
4. Discussion

CXCR7, formerly an orphan receptor, has recently been identified as a chemokine receptor for CXCL12, where it is expressed by many human cell lines, vascular endothelial cells, and in rodent brain, kidney, lung, heart, spleen, pancreas, small intestine, blood, colon, and blood vessels [11-13]. In addition, recent findings suggest that expression of CXCR7 is elevated in endothelial cells associated with tumors [28]. Shimizu et al, recently found that intracellular CXCR7 expression is mainly located into cytoplasm of human adult brain and differentiated neurons under normal and pathological conditions [29]. We recently demonstrated that CXCR7 expression is correlated with PCa metastasis and progression and suggest potential targets for therapeutic intervention [19, 30]. However, to our knowledge, there is no report on the expression and functional contribution of CXCR7 in bladder cancer development. Here, we found that CXCR7 expression in clinical patients is correlated with bladder cancer progression. Notably, Fig2.A indicated that CXCR7 expression in RT4, a grade 1 bladder cancer cell line, is higher than T24 and J82, grade3 bladder cancer cell lines, suggesting that these cell lines as the bladder cancer model may not be perfect representative of phenotype of clinical patients. The potential reason is that these cell populations established from bladder cancer patients are heterogeneous, comprising different genomic characteristics and different abilities to metastasize to distant secondary sites. By using in vivo selection in SCID mice, it may prove effective in isolating highly representative of phenotype from the original bladder cancer [31]. However, In vitro and in vivo studies in bladder cancer cell lines showed that alterations in CXCR7 expression were associated with the activities of proliferation, apoptosis, migration, invasion and tumor growth. This may correlate with the signaling of CXCR7 through activation of the AKT and ERK pathways. We also observed that CXCR7 appears to regulate blood vessel formation in bladder
cancer progression as it was noted that both IL-8 and VEGF levels were altered in response to changes in CXCR7 expression. These findings were in keeping with the number of blood vessels formed by the tumors *in vivo*. IL-8 and VEGF have been shown to be important factors involved in the development of tumor blood supply in the progression of solid tumors upon activating AKT and ERK pathways [32, 33]. Together, our results suggest that CXCR7 may act as a player in the disease.

CXCR7 was originally cloned on the basis of its homology with conserved domains of G protein-coupled receptors [10, 11] as an orphan receptor. Currently, it is accepted that CXCR7 has two ligands, the chemokines CXCL12 and CXCL11, which also bind to the chemokine receptor CXCR3 [10, 11]. CXCL12 mRNA is highly expressed in lymph nodes, lung, liver and bone marrow, and multiple tumor cells [10, 11], whereas CXCL11 is primarily expressed in peripheral blood leukocytes, pancreas, liver, thymus, spleen and lung and less expressed in small intestine, placenta and prostate [10, 11]. Recently, Singh RK, et al. showed that addition of CXCL12 and CXCL11 to PCa cells did not affect cell proliferation. Overexpression of CXCR7 in normal prostate cells increased their proliferation in a manner associated with increased levels of p-EGFR and p-ERK1/2, which suggest that CXCL12 or and CXCL11 may be nonessential to induce mitogenic response of CXCR7-overexpressing cells [34]. Indeed, we found that overexpression of CXCR7 in J82 and T24 cells only enhanced CXCL11 gene expression, but not altered CXCL12. Administration of different concentration of CXCL11 to these cells didn’t induce a significant proliferating response (Supplementary Fig 4).

Notably, since CXCR7 has been confirmed as a second receptor for CXCL12, debates are still arisen whether CXCR7 functions like GPCR mediating the signal transduction process. We and other groups indicated that CXCL12 engagement to CXCR7 can indeed transmit a range of cellular responses, such as, activation of ERK
and AKT pathways, cell survival, proliferation, and invasion et al, and finally chemotaxis of CXCR4-negative cells [35-37]. However, growing evidence has suggested that CXCR7 function as a decoy receptor, which also constitutively interacts with inactive G proteins but dramatically fails to activate them and mobilize intracellular calcium mobilization once engaged by CXCL12 [10, 16, 18, 19]. Recently, Levoye et al proposed that chemotaxis mediated by CXCR7 may involve the ability of CXCR7 to modulate CXCR4 signaling by scavenging CXCL12, thereby modifying the chemokine concentration in the extracellular environment [38]. These findings raise one potential mechanism that CXCR7 can regulate CXCR4 activity through heterodimer formation [38, 39]. Moreover, Decaillot et al [40] further demonstrate that CXCR7, which cannot signal directly through G protein-linked pathways, can nevertheless affect cellular signaling networks by forming a heterogenic complex with CXCR4. The CXCR4-CXCR7 heterodimer complex recruits β-arrestin, which results in preferential activation of β-arrestin-linked signaling pathways over canonical G protein pathways. It can potentiate cell proliferative kinase pathways, including p38MAPK, SAPK and ERK1/2 activation leading to increased cell migration of CXCR4-expressing breast cancer cells [40].

Here, we found that signaling by the CXCR7 in bladder cancer cells simultaneously activates AKT and ERK signaling. Interestingly, in CXCR7-expressing J82 cells, targeting the ERK pathway by using U0126 can activate AKT pathway; Yet, targeting the AKT pathway by using LY294002 can also activate ERK pathway. The results imply that AKT and ERK signaling by CXCR7 may reciprocally regulate each other in bladder cancer cells, for which it is unclear whether the induced cellular signaling networks involve in CXCR4-CXCR7 heterodimer complex; however, further studies are under investigation.

EMT has been noted as a critical process that will contribute to tumor invasive
phenotypes, associated with multiple activated pathways, including AKT and ERK signaling et al.[41-43]. Our results show that activating AKT and ERK pathways by CXCR7 are responsible for *in vitro* and *in vivo* EMT process of bladder cancer. Simultaneously targeting the two pathways by using U0126 and LY294002 inhibitors or using CCX733, a selective CXCR7 antagonist drastically reduced CXCR7-induced EMT process. These findings are consistent with the results reported by Burns *et al.*[16], in which small molecular antagonists to CXCR7 impeded tumor growth *in vivo*. Moreover, Boudot *et al.* indicated that overexpression of CXCR7 in breast cancer cell lines increases cell basal proliferation and leads to an estrogen-independent growth of cells whereby it may play a role in triggering initial step of EMT [44]. Thus, CXCR7 signaling may be an attractive new therapeutic target for treatment of multiple tumors, including bladder cancer.

In summary, these data demonstrate a role for CXCR7 in bladder cancer progression. Elucidation of newly identified receptor roles in tumorigenesis and progression may indicate new avenues of potentially therapeutic intervention in bladder cancer.

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Figure Legends

Figure. 1. CXCR7 expression is correlated with bladder cancer development.

(A) Expression of CXCR7 in human bladder normal (a) and cancer tissues (b, c d). Formalin-fixed paraffin-embedded tissues were incubated overnight at room temperature with anti-human CXCR7 antibody. MBA171 (IgG2a) was used as a negative control. Representative micrographs were taken at an original magnification 20×, where the black bars represent 100 µM.

(B) Quantitative histological evaluation of CXCR7 expression. CXCR7 expression intensity was scored by a genitourinary pathologist on a four point scale as negative (1), weak (2), moderate (3), or strong (4). Mean expression scores multiplied by percent positive cells in the field (Quick’s combined score system) are presented for normal, grade I, grade II and grade III cases in a graphic format using error bars with 95% confidence intervals (CI). * presents statistically significant differences between normal and cancer, p < 0.001, and # indicates marked differences between grade I and grade II or grade III, p < 0.001.

Figure. 2. CXCR7 modulates phenotype of bladder cancer cells.

(A) Top: mRNA level of CXCR7 in human bladder cancer cells was detected by RT-PCR. Bottom: Cell surface expression level of CXCR7 was evaluated by FACS analysis using mouse anti-human CXCR7 antibody, and mouse anti-human IgG served as isotype control.

(B) FACS analysis of cell surface CXCR7 expression in lentivirus-transfected bladder cancer cell lines. A mouse anti-human CXCR7 antibody was used to detect the overexpressing CXCR7 (J82\textsuperscript{control}/J82\textsuperscript{CXCR7} and T24\textsuperscript{control}/T24\textsuperscript{CXCR7}) or in which CXCR7 expression is reduced using shRNA (J82\textsuperscript{shcontrol}/J82\textsuperscript{shCXCR7} and RT4\textsuperscript{shcontrol}/RT4\textsuperscript{shCXCR7}). A GFP sequence was incorporated into the shRNA as a vector control. Mouse anti-human IgG served as isotype control.
(C) Proliferation of bladder cancer cell lines in response to altered CXCR7 levels.

After a 24-h serum withdrawal, bladder cancer cells were digested and washed three times in PBS, and $1 \times 10^4$ cells were plated into 96-well flat-bottomed tissue culture plates in 0.1 ml in complete growth medium. Proliferation was evaluated by XTT assay over a 4-day period. * denotes significant difference from controls ($p < 0.05$, ANOVA) for means ± S.E. of $n = 5$ samples per condition.

(D) Restoring expression of CXCR7 is responsible for cell migration. Serum was withdrawn before analysis to avoid effect of cell proliferation. The migration status was assessed by measuring the movement of cells into a scraped area created by a 10 μl pipette tube, and the spread of wound closure was observed at indicated times after scratching the surface of a confluent layer of cells. Scale bar: 100 μm. This experience was performed three times.

(E) CXCR7 regulates bladder cancer cell invasion. Bladder cancer cells were placed in the top chamber of invasion plates containing a reconstituted extracellular matrix in serum-free medium, and complete medium and CXCL12 (200 ng/ml) were added to the lower chambers. Invasion was determined at 48 h by MTT staining and the data were read on a multiwell scanning spectrophotometer (Thermo) at A580 and presented as % invasion binding ± standard deviation for $n = 5$. * denotes significant difference from controls ($p < 0.05$, ANOVA).

Figure. 3. CXCR7 expression regulates VEGF and IL-8 secretion.

(A) Detection of cytokines for angiogenesis in array. Detection of cytokines from CM derived from J82, and T24 cells overexpressing CXCR7 and their respective control cells. CM from cells was collected after culturing for 24 h. The signals were visualized by chemoluminescence. Normalization was done by positive control (Pos) according to the manufacturer protocol. The data indicates common alterations in VEGF and IL-8 levels, following induced CXCR7 expression.
(B) CXCR7 regulates secretion of VEGF and IL-8. VEGF and IL-8 levels were evaluated by ELISA for bladder cancer cells with altered expression of CXCR7 at 48 h. The data are presented as mean ± std. dev. for triplicate determinations and normalized against total protein. *Denotes significant difference from respective controls ($p < 0.05$, ANOVA). $n = 6$ in groups

(C) Left, effect of bladder cancer expression of CXCR7 on blood vessel formation. HDMECs were plated in growth factor reduced Matrigel™ in the presence or absence of CM from bladder cancer cells as a functional *in vitro* assay of blood vessel formation. CM from cells was collected after culturing for 24 h. The cultures were then fixed and stained. As a negative control, only HDMECs were plated. Vessel sprout formation was quantified by direct microscopic counting. Original magnification $20 \times$, where the black bars represent 100 µM. Right, the data are presented as mean ± std. dev. for triplicate determinations. * indicates significant difference from controls ($p < 0.05$, ANOVA).

Figure. 4. CXCR7 is involved in regulating the expression of EMT markers.

(A) Western blots were used to assay expression of epithelial marker (E-cadherin) and mesenchymal markers (N-cadherin and $\beta$-catenin). Whole cell lysates were immunoblotted with antibody to E-cadherin, N-cadherin and $\beta$-catenin. The blots were stripped and reprobed with GAPDH antibody to confirm equal protein loading.

(B) Potential target gene identification by cDNA microarray analysis. Clustering map of differentially expressed genes overlapped with cancer-associated genes in CXCR7 shRNA-targeted RT4 cells compared with the average expression of the control. Row represents gene, column shows experimental cells. Down-regulated genes were shown in red and up-regulated in green. Detailed data were set in Supplemental Table1.

(C) Selective fold-change map associated with EMT process in bladder cancer cells.
Up-regulated genes are listed on the right; and down-regulated genes are listed on the left.

**Figure. 5. CXCR7 mediates activation of AKT, ERK, and STA3 pathways.**

(A) Evaluation of the effect of CXCR7 on AKT, ERK and STAT3 signaling. After a 24 h serum withdrawal, whole cell proteins were extracted and lysates were separated on SDS-PAGE and immunoblotted with antibodies to p-AKT (Thr-308), p-ERK and p-STAT3 (Tyr-705). The blots were stripped and reblotted with antibodies against total AKT, ERK and STAT3.

(B) Expression of N-cadherin and β-catenin upon inhibiting activation of AKT and ERK signaling. Cell extracts were prepared from J82\(^{\text{CXCR7}}\) cells with the pretreatment of DMSO (10 μl) or PI3 Kinase Inhibitor LY294002 (5 μM) or MEK1/2 Inhibitor U0126 (10 μM) or two inhibitors combined for 24 h, and were used to detect the expressions of total ERK, p-ERK, total AKT, p-AKT (T-308), N-cadherin, β-catenin and GAPDH by Western blot assays.

(C) Expression profile of N-cadherin and β-catenin after block by CCX733 and CCX771, selective CXCR7 antagonists. Cell extracts were prepared from J82\(^{\text{CXCR7}}\) cells with the pretreatment of DMSO (0.5 μl) or negative control CCX704 (0.5 μM) or CXCR7 inhibitor CCX771 (0.5 μM) and CCX733 (0.5 μM) for 24 h, and were then used to detect the expressions of N-cadherin, β-catenin and GAPDH by Western blot assays.

**Figure. 6. CXCR7 promotes tumor growth, angiogenesis and EMT in vivo.**

(A) Effect of CXCR7 on J82 cell growth in vivo. 8-week-old SCID mice were implanted subcutaneously with \(8 \times 10^5\) J82 cells expressing various levels of CXCR7. Tumor volume (mm\(^3\)) was evaluated at indicated times. * indicates significant difference from the controls (\(p < 0.05\)).
(B) Effect of CXCR7 on tumor weight. Representative macroscopic appearance of tumor growth in J82control (bottom) and J82^{CXCR7} (top). Tumor weight was measured at sacrifice. Arrows indicate that abundant blood vessels are observed in tumors bearing J82^{CXCR7} cells. The data are presented as mean ± S.D. for triplicate determinations. N = 8 in groups. Representative 4 tumors are shown. * indicates significant difference from the controls (p < 0.05).

(C) Histologic evaluation of microvessel growth, and expression of EMT markers in tumors with altered CXCR7 levels. Subcutaneous tumors expressing various levels of CXCR7 were harvested and processed for immunohistochemistry staining for p-ERK, p-AKT (Thr308), N-cadherin, β-catenin, and human CD31 (1:100 anti-rabbit IgG). The arrows indicate positive areas. Original magnification 20 ×, where the black bars represent 100 µM.

(D) Quantification of microvessel formation in tumors with altered CXCR7 levels. The numbers of stained microvessels were blindly evaluated in 10 random fields per implant at 200 magnifications. * indicates significant difference from the controls (p < 0.05,
Hao et al. Figure 1
**Figure 2**

**A.**

RT4, J82, T24

CXCR7 ➔

GAPDH ➔

**B.**

RT4

IgG

CXCR7

Counts

J82

IgG

CXCR7

Counts

T24

IgG

CXCR7

Counts

**C.**

- RT4 control
- J82 control
- J82 shCXCR7
- T24 control
- T24 shCXCR7

**D.**

J82 control

J82 CXCR7

0h

24h

36h

**E.**

- T24 control
- T24 CXCR7

CXCL12

200 ng/ml
Hao et al. Figure 3
Figure 4

A. Immunoblot analysis showing changes in protein levels of E-Cadherin, N-Cadherin, and Beta-catenin across different cell lines.

B. Heatmap depicting the expression levels of various genes in RT4 control and RT4 siCXCR7 conditions. The color key indicates the row Z-score for gene expression.

C. Bar chart showing the fold change in expression levels of selected genes: FGFBP1, RPL13A, SERPINE1, SNAI2, MST1R, TGFBI, CDH3, CXCR7, and IGFBP6. Genes are classified as down-regulated or up-regulated based on their fold change.

Hao et al. Figure 4
A. Akt

- p-Erk1/2
- Erk1/2
- p-AKT(T308)
- AKT
- p-STAT3
- STAT3

B. J82 Control J82 CXCR7

- U0126
- LY294002
- p-Akt (473)
- p-Akt (308)
- Akt
- p-ERK
- ERK
- N-Cadherin
- β-catenin
- p-Akt(308)
- Akt
- p-ERK
- ERK
- N-Cadherin
- GAPDH

C. J82 Control J82 CXCR7

- DMSO
- CCX704
- CCX717
- CCX733
- N-cadherin
- β-catenin
- p-Akt(308)
- Akt
- p-ERK
- ERK
- N-Cadherin
- GAPDH

Hao et al. Figure 5
A. Tumor Volume (mm³)

B. Tumor Weight (mg)

C. CD31

D. CD31 Staining

Hao et al. Figure 6
CXCR7 promotes bladder cancer growth, angiogenesis and EMT \textit{in vivo}.