Targeting the MIF/CXCR7/AKT Signaling Pathway in Castration-Resistant Prostate Cancer

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Abstract

Although androgen deprivation therapy (ADT) is an effective treatment for metastatic prostate cancer, incurable castration-resistant prostate cancer (CRPC) inevitably develops. Importantly, androgen receptor (AR) continues to be critical for prostate cancer growth and progression after ADT. One of the underlying molecular mechanisms is derepression of AR-repressed genes involved in cell cycle and proliferation after ADT. Here, the data demonstrate that C-X-C chemokine receptor type 7 (CXCR7), a seventransmembrane G-protein-coupled chemokine receptor, is an AR-repressed gene and is upregulated after ADT. AR directly regulates CXCR7 using clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) gene editing. Macrophage migration inhibitory factor (MIF) was identified as a ligand for CXCR7, which induces expression of cell-cycle genes through activating AKT signaling pathway. Previous studies have been focused on chemokine CXCL12 and its

Introduction

Prostate cancer remains the second leading cause of cancerrelated death among American men behind only lung cancer (1). Androgen deprivation therapy (ADT) has been considered the standard of care for metastatic patients with metastatic prostate cancer since 1941 when Huggins and Hodges demonstrated significant remissions in prostate cancer after castration (2). Although ADT is initially effective, prostate cancer cells tend to survive and proliferate under androgen-deprived conditions and castration-resistant prostate cancer (CRPC) inevitably develops. Despite the development of next-generation anti-androgen therapies, including enzalutamide and abiraterone, acquired resistance to these drugs is nearly universal over time. Metastatic CRPC remains a lethal disease and further treatments are palliative. Castration resistance is often medi-

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receptor CXCR4 in mediating metastasis of various cancer types, including prostate cancer. The critical roles of CXCL12/CXCR4 axis in the interaction between cancer cells and their microenvironment render it a promising therapeutic target in cancer treatment. The data suggest that the MIF/CXCR7/AKT pathway drives CRPC growth and metastasis independent of the CXCL12/CXCR4 axis. Furthermore, CXCR7 blockade in combination with antiandrogen enzalutamide inhibits CRPC tumor growth and potentially prevents metastasis. Notably, both MIF and CXCR7 are overexpressed in CRPC patient specimens and therefore are attractive therapeutic targets for these patients.

Implications: This work suggests that CXCR7 plays more important roles than CXCR4 in CRPC progression; thus, targeting CXCR7 in combination with anti-androgen is a promising therapeutic approach for metastatic CRPC.

ated by restoration of androgen receptor (AR) activity through AR mutations, AR variants, AR amplification, and overexpression. Interestingly, recent studies revealed a subset of genes that are normally suppressed by AR are upregulated in patients with CRPC (3, 4). It has been suggested that one of the molecular mechanisms for prostate cancer cells to survive ADT is derepression of AR-repressed genes. However, it is unclear which and how AR-repressed genes contribute to prostate cancer growth and progression after ADT.

It is well known that chemokines and their receptors not only are key mediators of inflammation, but also play a crucial role in tumor growth and more importantly metastasis because tumor cell migration share many similarities with leukocyte trafficking. CXCR4 is one of the most studied chemokine receptors in a variety of cancers, including prostate cancer. CXCR4 and its respective ligand CXCL12 have been increasingly demonstrated to regulate tumor progression. This is achieved by metastatic spread of CXCR4-positive tumor cells to organs (such as bone) expressing high levels of CXCL12. CXCR4 and CXCL12 are the key factors in the link between cancer cells and their microenvironment. The critical role of CXCR4/CXCL12 interaction in determining the metastatic destination was initially discovered in breast cancer (5). Further studies have shown that CXCR4 is overexpressed in at least 20 different cancers, including prostate cancer (6). CXCL12 functions as a chemotactic factor involved in prostate cancer cell migration through activation of the CXCR4 (7). For a long time, CXCR4 was thought to be the only receptor for CXCL12, with CXCL12 being its only ligand. However, interaction between



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chemokines and their receptors is promiscuous. CXCR7 (also known as atypical chemokine receptor 3, ACKR3) was identified as an alternative receptor for CXCL12 based on structural similarity and experimental evidence (8). Studies have shown that upregulation of CXCR7 is associated with invasive activities and growth of prostate cancer cells (9). CXCR4 and CXCR7 may form homo- and heterodimers and mediate G-protein signaling, contributing to prostate cancer progression (10). Thus, CXCR4 and CXCR7 are considered as emerging targets for prostate cancer.

CXCR7 was recently identified as an AR-repressed gene (11), implying its unique role in prostate cancer, although it remains unclear whether AR directly regulates CXCR7 expression. The importance of CXCL12/CXCR4/CXCR7 signaling in prostate cancer progression has also been elucidated (12). Targeting CXCR7 in combination with anti-androgen treatment inhibits tumor growth and angiogenesis in preclinical androgen-dependent prostate cancer models (13). However, it is unclear whether and how CXCR7 plays a role in metastatic CRPC progression. In this study, we identified CXCR7 as one of the top AR-repressed genes at a genomic level and demonstrated that AR directly regulates CXCR7 using a CRISPR/Cas9 gene editing approach. We further explored the molecular mechanism by which CXCR7 mediates CRPC progression. We identified MIF is a ligand for CXCR7 in CRPC cells. The MIF/CXCR7 pathway appears to play a crucial role in CRPC growth and progression by regulating gene expression closely associated with cell cycle through AKT activation. Finally, combination treatment of CXCR7 inhibitor with enzalutamide not only inhibits or delays CRPC tumor growth, but also prevents the development of metastases, which may potentially result in a more profound, long-lasting remission in patients with CRPC

Materials and Methods

Cell lines and materials

LNCaP, PC-3, DU145 (from ATCC), and C4-2B (from ViroMed Laboratories, Minneapolis, MN) cells were maintained in RPMI1640 (Gibco) medium supplemented with 5% FBS as previously described (14). The AR-expressing PC-3 stable cell line was a gift from Dr. Baruch Frenkel, University of Southern California, Los Angeles, CA (15). All cell lines were authenticated using high resolution small tandem repeats (STR) profiling at Dana-Farber Cancer Institute (DFCI) Molecular Diagnostics Core Laboratory. Cells were grown for 20 passages and then replaced with fresh stocks. Cells are free of mycoplasma examined by MycoSensor PCR Assay Kit (Catalog No. 302108; Agilent Technologies). Human Recombinant MIF (rMIF) and CXCL12 (rCXCL12) were purchased from PeproTech. The CXCR7 small molecule inhibitor, CCX771, was a gift from ChemoCentryx (16). The CXCR4 inhibitor, AMD3100, was obtained from Sigma-Aldrich.

Chromatin immunoprecipitation

LNCaP and C4-2B cells were grown in RMPI1640 with 5% charcoal stripped FBS (CSS) for 3 days followed by DHT (10 nmol/L) treatment for 4 hours. AR ChIP was performed as previously described (17). ChIP DNA was quantified by quantitative PCR (qPCR). The primer sequences are listed in Supplementary Table S1.

Identification of AR-repressed genes

To assess the AR-repressed genes, we performed binding and expression target analysis (BETA) with default parameters by integrating androgen-regulated gene expression microarray dataset (GSE7868) with six AR ChIP-seq datasets (GSM759658, GSM980657, GSM980662, GSM699631, GSM969565, and GSM696842), respectively (18–23). Rank products (*P*-value) from individual dataset were calculated by BETA, and were combined with Fisher's method to rank the gene list. Genes that were down-regulated by androgen [with log₂ (fold change) < -0.58 and *P*-value < 0.01] and with an AR binding site nearby (combined rank product <0.01) were defined as AR-repressed genes.

Western blot analysis

Whole cell lysates were prepared from prostate cancer cells as indicated. Western blot analysis was performed as previously described (14). Antibodies are: anti-ERK (#4695), anti-p-ERK (#4370), anti-AKT (#4691), anti-p-AKT (#4051), anti-JNK (#9252), anti-p-JNK (#4668), anti-p38 (#8690), and anti-p-p38 (#4511) from Cell Signaling Technology; anti-β-actin (sc-47778) and anti-β-tubulin (sc-80011) from Santa Cruz Biotechnology; anti-MIF (AF-289-PB) from R&D Systems.

Cell viability assay

Prostate cancer cells were seeded in 96-well plates (1×10^4 cells/well) and treated as indicated. The viability of cells was measured using Alamar Blue Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Transwell migration assay

C4-2B and PC-3 cells were grown in RMPI1640 with 5% CSS and 5% FBS respectively, and pretreated with or without CCX771 (5 μ mol/L) for 24 hours. C4-2B (2 × 10⁵ cells/well) and PC-3 (1 × 10⁵ cells/well) cells were then suspended in 200 µL RPMI1640 with 0.5% CSS (or FBS) and added into 8.0 µm pore-sized transwell inserts (Catalog No. 353097; BD Falcon). The inserts were subsequently placed in a 24-well plate containing 600 µL RPMI1640 with 15% CSS (or FBS), or 0.5% CSS with or without rMIF (10 ng/mL). After 18 hours of incubation, the cells that had migrated through the membrane were stained with crystal violate. The cell images were captured at ×20 magnification and counted in four representative fields. Quantification of migrated cells was performed using ImageJ software.

Flow cytometry

Cells were treated as indicated. Approximately 1×10^{6} cells/mL were washed with ice-cold PBS containing 0.5% BSA and 1% sodium azide. Cells were stained with 10 µg/mL phycoerythrin (PE)-conjugated anti-CXCR7 (Catalog No. 331103; Biolegend) on ice for 40 minutes in the dark. Nonspecific isotype-matched IgG was used as a control. The stained cells were washed three times with PBS, resuspended and fixed in 1% (w/v) paraformal-dehyde for analysis. Ten thousand cells from each sample were evaluated using FACSCanto II (BD Biosciences) or EPICS ALTRA (Beckman Coulter). The fluorescence intensity was analyzed with FlowJo software (FlowJo LLC) and was presented in arbitrary units.

Human serum specimens

Clinical serum samples were obtained from the Authur and Linda Gelb Center for Translational Research at DFCI. The study protocol was approved by the institute. Localized patients with prostate cancer (n = 20) had been treated with radical prostatectomy at the time of blood collection. Patients with metastatic hormone-naïve prostate cancer (n = 20) had not been treated with ADT at the time of blood collection. Patients with metastatic CRPC (n = 20) had been treated with ADT at the time of blood collection and had evidence of progression of disease with either rising PSA or evidence of radiographic progression. Normal individuals (n = 20) without cancer were used as controls.

ELISA

Serum MIF levels were determined with the MIF ELISA Kit (R&D Systems) according to the manufacturer's instructions. To measure secreted MIF from cell culture, conditioned medium was collected and measured with ELISA. Values were normalized to protein concentration from whole cell lysates.

RNA interference

Cells were transfected with CXCR7 siRNA, MIF siRNA, or nonspecific siRNA at a final concentration of 20 nmol/L using Lipofectamine RNAiMAX transfection reagent (Life Technologies) according to the manufacturer's protocol. The siRNA information is listed in supplementary Table S1.

Reverse transcription qPCR

Total RNA was extracted from cells treated as indicated using TRIzol reagent (Invitrogen). cDNA synthesis was performed using the iScript cDNA Synthesis Kit (Bio-Rad). Reverse transcription qPCR (RT-qPCR) reactions were performed using SYBR Green PCR master mix reagents (Bio-Rad). Each measurement was performed in triplicate and the mRNA levels of each gene were normalized to GAPDH levels. The primer sequences are listed in Supplementary Table S1.

Generation of knockout cell line with clustered regularlyinterspaced short palindromic repeats/CRISPR-associated protein 9

To perform genome editing via CRISPR-associated protein 9 (CRISPR/Cas9) approach, specific guide RNAs (gRNA) targeting *CXCR7* AR binding site (ARBS) or *CXCR7* exon were designed using http://crispr.mit.edu/ and http://crispr.dfci. harvard.edu/SSC/ (24, 25). gRNAs with a 20 nucleotides spacer were cloned into lentiGuide-Puro vector (#52963, Addgene). The vector containing gRNA targeting GFP was obtained from Dr. William C. Hahn (Dana-Farber Cancer Institute, Boston, MA) as a gift, and used as a control in parallel. The lentiCas9-Blast vector that expresses Cas9 was obtained from Addgene (#52962). Lentiviruses that carry each gRNA or Cas9 were packaged in 293T cells. All gRNA sequences are listed in Supplementary Table S1.

To generate *CXCR7* ARBS KO cell lines, C4-2B cells were transected with lentiCas9-Blast and dual gRNAs targeting *CXCR7* ARBS (A1/B1 or A2/B2). Transfected cells were selected using 2 μ g/mL puromycin and 10 μ g/mL blasticidin for 3 days before plated at clonal density, and single-cell-derived colonies were generated by picking clones into 96-well plate. For genotyping, genomic DNA was extracted using QuickExtract (Lucigen), and PCR was performed with screening primers (listed in Supplementary Table S1). Selected KO clones were then expanded and further characterized.

To generate *CXCR7* gene KO cell lines, C4-2B cells were transfected with lentiCas9-Blast and four pooled gRNAs (CXCR7 KO A/B/C/D) that targeting *CXCR7* second exon. Single-cell-derived colonies were generated as above and validated by flow cytometry for CXCR7 protein expression.

To search for potential off-target sites of gRNAs in KO cell lines, we used the CRISPR/Cas9 target online predictor (CCTop) with default settings (26). The genomic regions of on-target and top two off-target sites for each gRNA were PCR-amplified and analyzed by Sanger sequencing (Supplementary Table S2). The sequence traces were analyzed and the frequency of mutations generated by CRISPR/Cas9 (or gene editing efficacy) was determined by TIDE (27).

RNA-seq analysis

RNA-seq was performed as previously described with modifications (17). Briefly, RNA-seq libraries were prepared using NEBNext Ultra RNA Library Prep Kit (New England Biolabs). The libraries were sequenced in the NextSeq 550 system (Illumina) according to the manufacturer's instructions. RNA-seq reads were mapped to the human genome GRCh37, using Tophat2 v2.0.14 and raw read counts were generated with Htseq (0.6.1) using the union method (28, 29). Genes that did not have at least three raw mapped counts in the sum of all six samples were filtered prior to differential testing. Differentially expressed genes (P < 0.01, FDR < 0.01, and fold of change >2) were identified using EdgeR (3.12.0; ref. 30). Gene ontology analysis was performed by David online analysis tools using all genes identified by our RNA-seq as a background (31). Gene expression is reported in counts per million.

Animal studies

The animal protocol was approved by the institutional Anima Care and Use Committee (IACUC). C4-2B cells (1×10^6 cells/site mixed with Matrigel at a 1:1 ratio, v/v) were injected subcutaneously into 6-week-old male ICR-SCID intact mice (Taconic Biosciences). After tumor formation ($\sim 100 \text{ mm}^3$), mice were randomized into four groups (9 mice/group) and treated with vehicle, enzalutamide (25 mg/kg, orally), CCX771 (30 mg/kg, s.c.), or enzalutamide + CCX771 in combination daily for 5 weeks. DMSO was used as the vehicle for enzalutamide. A special vehicle for CCX771 was provided by ChemoCentryx. The tumor growth was monitored bi-weekly using caliper measurement. Tumor volume was compared between the groups. The expression of CXCR7 mRNA in tumor tissues was analyzed using RT-qPCR. To detect metastasis, genomic DNA was isolated from bone marrow and liver tissues using Puregene DNA purification system (Qiagen), and the presence of tumor cells was analyzed by quantification of human Alu sequence as previously described (32, 33). Human Alu-specific TagMan qPCR was performed using the primers and probe listed in Supplementary Table S1.

Clinical expression data analysis

Two gene expression microarray datasets from primary and metastatic tumors (GSE21034 and GSE32269) were acquired from Gene Set Omnibus (GEO) using GEO2R (34, 35). The expression levels of CXCR7 and CXCR4 were isolated for each patient using "NM_020311/212977_at" or "NM_003467/211919_s_at," respectively. To study the association between the

expression levels of CXCR7 and CXCR4 and the disease-free time of patients with prostate cancer, expression data (*Z*-scores) for CXCR7 and CXCR4 were downloaded from The Cancer Genome Atlas (TCGA) dataset through cBioPortal (36). Patients were then split into two groups with high (>medium) and low (\leq medium) expression of CXCR7 and CXCR4, respectively. The Kaplan–Meier plots of biochemical relapse-free survival proportion were generated, and the statistical analysis was performed using log-rank (Mantel–Cox) test.

Statistical methods

All the experiments were performed at least three times. Values are shown as mean \pm SD of three replicates from one representative experiment. All statistical testing was done using two-tailed *t* test. *P*-value < 0.05 was considered as statistically significant.

Results

CXCR7 is a direct AR-repressed gene

Emerging evidence has suggested that AR-mediated gene repression contributes to the development of CRPC (3, 4). To elucidate the molecular mechanisms of AR-repressed genes in promoting prostate cancer growth and progression after ADT, we first defined such genes by analyzing AR cistrome in prostate cancer cell lines using BETA (18). We integrated a gene expression microarray dataset from LNCaP cells with six AR ChIP-seq datasets from three different prostate cancer cell lines (three from LNCaP, two from VCaP, and one from LNCaP-Abl cells). We identified 88 AR-repressed genes (Supplementary Fig. S1; Table S3), including well-known AR-repressed genes, OPRK1 and AMIGO2 (3, 4). These genes were suppressed by androgen treatment and had AR binding sites nearby. Gene ontology analysis revealed that ARrepressed genes were enriched in cell-cycle regulation (Supplementary Fig. S2), suggesting their important roles in prostate cancer cell growth.

Among these genes, we discovered CXCR7 as one of the top AR-repressed genes. More importantly, a small molecule CXCR7 antagonist, CCX771, became available for in vitro and in vivo studies (37, 38). Therefore, we decided to select CXCR7 for a further investigation. We next examined our previously published RNA-seq and ChIP-seq data in LNCaP (androgendependent) and C4-2B (LNCaP-derived CRPC) cells (17), and found that CXCR7 expression was inhibited upon dihydrotestosterone (DHT) treatment in both cells, but more so in C4-2B cells (Fig. 1A). Notably, androgen withdrawal dramatically elevated CXCR7 mRNA levels (about 20-fold) in CRPC C4-2B cells. Furthermore, our ChIP-seq analysis detected a strong ARBS about 100 kb downstream of the CXCR7 transcription start site. There are no annotated genes between the body of CXCR7 gene and the ARBS that contains an androgen response element (GGAACACTCTGTGGC), suggesting a AR cis-regulatory element. We validated DHT-induced AR occupancy at the ARBS in both LNCaP and C4-2B cells using sitespecific ChIP-qPCR (Fig. 1B). We further validated RNA-seq results by RT-qPCR (Fig. 1C). Notably, DHT-induced CXCR7 repression was completely abolished by AR antagonist, enzalutamide. In line with mRNA expression, flow cytometry analysis showed that CXCR7 protein levels on C4-2B cell surface were inhibited by DHT but enhanced by enzalutamide (Fig. 1D). In addition, we examined CXCR7 mRNA levels in AR-negative CRPC PC-3 cells and AR-expressing PC-3 cells (Fig. 1E). Overexpression of AR almost completely abolished CXCR7 expression in PC-3 cells.

Studies have shown that AR primarily binds distal enhancers that can be several kb to over 100 kb away from the transcription start site of its regulated genes (18-23). Interestingly, a previous study has reported an ARBS at the CXCR7 promoter (11). This ARBS defined by ChIP-qPCR is likely acquired from interaction between the distal AR-bound enhancer and the CXCR7 promoter through chromatin looping. To demonstrate whether CXCR7 transcription is directly regulated by the ARBS identified through our ChIP-seq result, we knocked out the ARBS in C4-2B cells using a CRISPR/Cas9 dual-guide approach. We used two independent sets of gRNAs to rule out the offtarget effect (Fig. 1F). Two single-cell-derived knockout colons from each gRNA set were used to assess the regulatory effect of the ARBS on CXCR7 expression. As shown in the PCR-based genotyping results (Fig. 1G), we were not able to generate a complete knockout clone. However, partially knocking out the ARBS, which presumably disrupt AR binding, significantly attenuated the androgen-mediated CXCR7 repression. This was confirmed by decreased AR binding at the ARBS (Fig. 1H) suggesting AR directly regulates CXCR7 expression by binding to the cis-regulatory element. We further analyzed potential offtarget sites that might be recognized by the gRNAs used for ARBS KO. We found very minimum mutations at the putative off-target sites (Supplementary Fig. S3).

CXCR7 is overexpressed in patients with metastatic prostate cancer and associated with high risk of recurrence

Next, we examined CXCR7 mRNA expression levels in clinical prostate cancer tissues using two publicly available microarray datasets (34, 35). Because CXCR4 is a well-studied chemokine receptor in prostate cancer and shares the same chemokine ligand with CXCR7, we analyzed CXCR4 expression in parallel. Meta-analysis showed that both CXCR4 and CXCR7 were upregulated in localized tumors compared with normal prostate tissues and CXCR7 levels were further elevated in metastatic tumors (Fig. 2A). The metastatic tumors used in this dataset were collected from different metastatic sites. including lymph node, brain, bone, lung, neck, testes, or bladder. Interestingly, in an independent dataset, both CXCR4 and CXCR7 expression levels were significantly increased in metastatic bone samples but not in lymph node samples, indicating their implication in bone metastasis (Fig. 2B). Furthermore, we plotted Kaplan-Meier curves between high and low CXCR7 expression with disease-free time using clinical follow-up data from 492 patients in TCGA dataset (Fig. 2C; ref. 36). Patients with low CXCR7 expression had significantly longer disease-free time compared with patients with high CXCR7 expression (P < 0.0379). In contrast, CXCR4 expression was not associated with biochemical recurrence. These results suggest that CXCR7 may play more important roles in CRPC progression than CXCR4.

CXCR7 is required for CRPC growth and migration in vitro

To determine whether CXCR7 is required for CRPC cell growth, we examined the growth of several CRPC cell lines after inhibition of CXCR7 through genetic and pharmacologic means. We initially knocked down CXCR7 in C4-2B cells using RNA interference (RNAi). CXCR7 knockdown efficiency was confirmed at mRNA levels using RT-qPCR (Fig. 3A) and at

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Figure 1.

CXCR7 is an AR-repressed gene. **A**, Genome browser view of RNA-seq and AR ChIP-seq results at the CXCR7 locus in the presence or absence of 10 nmol/L DHT in LNCaP and C4-2B cells. Fragment Per Kilobase of transcript per Million (FPKM) was used as RNA-seq expression unit. **B**, AR occupancy at the AR binding site (ARBS) was examined by ChIP-qPCR in the presence and absence of DHT (10 nmol/L) in both LNCaP and C4-2B cells. **C**, CXCR7 mRNA levels were measured by RT-qPCR in LNCaP and C4-2B cells after DHT (10 nmol/L) or vehicle treatment in the presence or absence of 10 µmol/L enzalutamide (ENZ). The values were normalized to GAPDH levels. **D**, Flow cytometry analysis of CXCR7 expression on C4-2B cell surface after treatment with DHT (10 nmol/L) or INZ (10 µmol/L). **E**, CXCR7 mRNA levels were measured by RT-qPCR in AR-negative PC-3 and AR-expressing PC-3 (PC-3-AR) cells. **F**, The ARBS at the *CXCR7* locus was knocked out using two sets of gRNAs (A1/B1 and A2/B2) as indicated. **G**, Four ARBS knockout (KO) cell lines were validated by PCR using genomic DNA from each cell line. Wild-type (WT) and KO bands were observed indicating partial ARBS KO. GFP-KO is the C4-2B control line using a gRNA against GFP. Parental C4-2B cells are also examined in parallel. CXCR7 mRNA expression (–DHT/+DHT) are represented. **H**, AR occupancy at the ARBS was examined in GFP-KO and ARBS-KO cells in the presence of DHT (10 nmol/L). Because the ARBS was partially deleted, the control region was used as input normalization for the ARBS.



Figure 2.

A and B, Meta-analyses of two publicly available microarray datasets showing CXCR7 and CXCR4 mRNA expression levels in normal prostate tissues vs. localized and metastatic (Met) tumors. LN, lymph node. C, Kaplan-Meier survival curves of biochemical recurrence-free survival were plotted between high and low CXCR4 and CXCR7 expression.

protein levels using flow cytometry (Fig. 3B). We observed a significant decrease of C4-2B cell viability after CXCR7 knock-down (Fig. 3C). The same inhibitory effect was observed in two

other CRPC cell lines, PC-3 and DU145 (Fig. 3D). Next, we generated CXCR7 gene KO C4-2B cell lines using CRISPR/Cas9 gene editing. Four knockout cell clones were selected and confirmed by flow cytometry (Fig. 3E), which showed abolished CXCR7 protein expression on cell surface. Analysis of mutations generated by CRISPR/Cas9 at on-target and putative off-target sites further confirmed CXCR7 gene knockout (Supplementary Fig. S4), although the gene editing efficacy at on-target site is around 40% to 70% estimated by sequence trace decomposition (27). Knockout of CXCR7 in C4-2B cells dramatically attenuated cell proliferation and colony formation compared with parental or GFP-KO C4-2B cells (Fig. 3F and G). Using transwell migration assay, we found that knockout of CXCR7 abolished C4-2B cell migration (Fig. 3H). Because the migration assay was performed within 18 hours, the reduction in cell migration was unlikely attributed to the reduced cells growth.

CCX771 is a small molecule CXCR7 antagonist and has been previously used *in vitro* and *in vivo* studies (37, 38). We treated three CRPC cell lines with CCX771 and observed its inhibitory

effect on cell growth in a dose-dependent manner (Fig. 4A). In contrast, treatment with CXCR4 inhibitor, AMD3100, rendered minor effect on CRPC cell growth under the same concentration (Fig. 4B). In addition, we found that CCX771 enhanced the inhibitory effects of enzalutamide and androgen withdrawal on C4-2B cell growth (Fig. 4C and D). This was likely due to elevated CXCR7 expression after AR suppression in these cells, indicating its critical role in promoting CRPC growth after anti-androgen treatment. Furthermore, CCX771 abolished C4-2B and PC-3 cell migration in transwell migration assays (Fig. 4E and F). We tested AMD3100 in parallel and observed little effect on cell migration even when we increased the concentration to 50 µmol/L (Supplementary Fig. S5). To determine the efficacy of AMD3100, we treated colorectal cancer HCT116 cells, which possess high CXCL12/CXCR4 activity (39), under the same concentration and observed reduced cell migration. In addition, we analyzed an independent RNA-seq dataset to compare CXCR4 and CXCR7 expression in 4 prostate cancer cell lines (LNCaP, C4-2B, PC-3, and DU145), which we used in the present study (40). By plotting RNA-seq reads directly to the genome browser, we found very high CXCR7 levels in LNCaP, C4-2B, and PC-3 cells and a low

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Figure 3.

CXCR7 knockdown (KD) or knockout (KO) decreases viability and migration in CRPC cells. A, CXCR7 mRNA expression levels in C4-2B cells were measured by RT-qPCR after CXCR7 siRNA KD. Gene expression with nonspecific (NS) siRNA is defined as 1. B, CXCR7 protein expression on C4-2B cell surface was measured by flow cytometry after siRNA KD. C4-2B cell viability was measured by Alamar Blue assay after CXCR7 siRNA KD. D, PC-3 and DU145 cell viability was measured by Alamar Blue assay after CXCR7 siRNA KD. E, CXCR7 KO C4-2B cells were established using a CRISPR/ Cas9 approach. Four cell clones (CXCR7-KO-1 and -2 on the left and CXCR7-KO-3 and -4 on the right) were selected for validation based on CXCR7 protein expression using flow cytometry. Nonspecific IgG staining was used as a negative control. Parental C4-2B and GEP-KO C4-2B cells were used as positive controls. F, Cell viability of CXCR7 KO C4-2B cell lines was measured by Alamar Blue compared with control cell lines. G. Representative images of colony formation assays on CXCR7 KO C4-2B cells compared with control cell lines. H, Representative images of transwell migration assays in CXCR7 KO C4-2B cell lines compared with control cell lines. The images were quantified using ImageJ software. Data are representative of three independent experiments. Mean \pm SD is plotted. P-value was determined by two-tailed Student t test. ***, P < 0.0001.



CXCR7 level in DU145 cells (Supplementary Fig. S6). In contrast, CXCR4 expression levels were either low in C4-2B and DU145 cells or undetectable in LNCaP and PC-3 cells.

Taken together, our results are consistent with high CXCR7 expression in clinical metastatic tumors, which is associated with biochemical recurrence.

Figure 4.

CXCR7 antagonist, CCX771, inhibits CRPC cell proliferation and migration. **A**, Cell viability of C4-2B, PC-3, and DU145 cells was measured by Alamar Blue 5 days after treatment with different concentration of CCX771. All cells were grown in the RPMI1640 with 5% FBS. **B**, Same experiments were performed after treatment with AMD3100. **C**, C4-2B cells were grown in the RPMI1640 with 5% CSS. Cell viability was measured by Alamar Blue 5 days after treatment with CCX771 in the presence or absence of DHT (10 nmol/L) **D**, C4-2B cells were grown in the RPMI1640 media with 5% FBS. Cell viability was measured in the presence or absence of 10 μ mol/L enzalutamide (ENZ). **E**, Representative images of transwell migration assays in C4-2B cells after treatment with CCX771 (5 μ mol/L) or AMD3100 (10 μ mol/L). The images were quantified using ImageJ software. **F**, Same experiments were performed in PC-3 cells. Data are representative of three independent experiments. Mean \pm SD is plotted. *P*-value was determined by two-tailed Student *t* test. ***, *P* < 0.0001.

CXCR7 enhances CRPC growth and metastasis in vivo

To examine the role of CXCR7 in CRPC growth and progression *in vivo*, we implanted C4-2B cells subcutaneously into severe immunodeficient mice. Mice bearing established tumors were randomly assigned into four groups (nine mice/group) and

treated with vehicle, enzalutamide, CCX771, or enzalutamide + CCX771 in combination for 5 weeks. Tumor volume was measured and compared between groups. Although enzalutamide and CCX771 initially showed marginal inhibitory effect on tumor growth, overall C4-2B tumors did not respond to

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Figure 5.

Combination treatment with CCX771 and enzalutamide (ENZ) inhibits C4-2B tumor growth and metastasis *in vivo*. **A.** C4-2B cells (1 \times 10⁶ cells/site) were injected subcutaneously into ICR-SCID male mice. Mice were assigned into four groups (nine mice/group) and treated with vehicle, CCX771 (30 mg/kg), ENZ (25 mg/kg), or CCX771 + ENZ for 5 weeks. The tumor growth was monitored using caliper measurement. Values of tumor volume are mean + SE. *P*-value was calculated using two-tailed Student t test between groups. B, CXCR7 mRNA expression levels in tumor tissues were measured by RT-qPCR. P-value was calculated using two-tailed Student t test between groups, C and D. C4-2B tumor metastases were assessed by guantification of humanspecific Alu sequences in DNAs extracted from bone marrow from tibia and liver tissues

enzalutamide or CCX771 treatment as a single agent (Fig. 5A). In contrast, the combination treatment significantly inhibited C4-2B tumor growth. Analysis of CXCR7 expression in tumor tissues revealed significantly elevated CXCR7 mRNA levels after enzalutamide treatment (Fig. 5B), which likely contributed to tumor growth and progression. Interestingly, CCX771 appeared to inhibit enzalutamide-induced CXCR7 expression with combination treatment although statistical significance was not reached. To investigate whether enzalutamide-induced CXCR7 expression enhances C4-2B metastasis, we harvested bone marrows from mouse tibia and liver tissues. Because we did not visually observe any metastases, we examined human Alu DNA sequences using TaqMan qPCR to determine whether there are micrometastases in collected samples (32, 33). As shown in Fig. 5C and D, four of nine samples were found to contain high levels of human Alu DNA sequences in either bone marrow and/or liver tissues from the enzalutamide-treated group. In contrast, little Alu DNA signal was detected in other groups. Our data are consistent with the results from previous studies showing enzalutamide-enhanced metastasis in preclinical models (32, 41). In line with our in vitro data, these results indicate that enzalutamide-induced CXCR7 expression may promote the development of metastatic CRPC, which can be potentially delayed or prevented by CXCR7 blockade.

CXCR7 inhibition impacts M-phase cell-cycle gene expression and AKT signaling

To determine the impact of CXCR7 on global gene expression, we performed RNA-seq in C4-2B cells upon CCX771 treatment in the absence of androgen. AMD3100 treatment was performed in parallel. We identified 995 and 803 genes with altered expression levels in CCX771- and AMD3100treated cells, respectively (Fig. 6A; Supplementary Table S4). There are 653 common genes shared by both treatments. Gene ontology analyses revealed that genes involved in focal adhesion, extracellular matrix (ECM)-receptor interaction, and pathways in cancer are enriched in both treatments (Fig. 6B). Importantly, we discovered that blocking CXCR7 with CCX771 resulted in downregulation of cell cycle genes including many critical M-phase cell-cycle genes, such as AURKA, CDC6, CDC45, CDK1, and E2F1. We examined expression levels of these five genes using RT-qPCR and confirmed the RNA-seq results (Fig. 6C). We further validated this result by knocking down CXCR7 using two independent siRNAs (Fig. 6D). This alteration is unique to CXCR7 inhibition, indicating the critical role of CXCR7 in promoting CRPC cell proliferation through upregulation of cell-cycle genes.

To characterize the CXCR7-mediated signaling pathways involved in gene expression alteration, we analyzed the impact of CXCR7 on several mitogenic signaling pathways. We examined AKT, ERK, p38, and JNK activation using Western blot analysis in C4-2B cells after CCX771 treatment or CXCR7 siRNA knockdown in the absence of androgen (Fig. 6E). It was evident that AKT phosphorylation was significantly inhibited after targeting CXCR7 with small molecule inhibitor or RNAi. This result was consistent with a previous report, showing CXCR7 activates AKT signaling (9). ERK phosphorylation was also inhibited by CCX771, but was not confirmed by RNAi. Phosphorylation of JNK or p38 remained unchanged. We then treated C4-2B cells with the PI3K inhibitor, LY294002, and observed downregulation of AURKA, CDC6, CDC45, CDK1, and E2F1 expression (Fig. 6F). Our data suggest that CXCR7 affects cell-cycle gene expression at least partially through a PI3K/AKT-dependent mechanism.

MIF promotes CRPC growth and migration through CXCR7

Previously studies have demonstrated both CXCL12 and MIF are ligands for CXCR7 (42, 43). Both CXCL12/CXCR7 and MIF/CXCR7 physical interactions have been established. Here, we cocultured C4-2B cells with mouse bone marrow stroma ST2 cells in the presence or absence of androgen and measured secreted chemokine and cytokine concentrations in the medium. Surprisingly, we found that MIF had the highest protein concentration in the coculture medium. (Supplementary Fig. S7).

Figure 6.

CXCR7 induces M-phase cell-cycle gene expression through AKT signal transduction pathway A. Hierarchical clustering of gene expression alteration in C4-2B cells after treatment with CCX771 (5 μ mol/L) or AMD3100 (10 μ mol/L) for 16 hours in the absence of androgen. Venn diagram shows differentially expressed genes between the two groups. B, Heatmap showing the unsupervised clustering of the gene ontology (GO) terms enriched in CCX771- and AMD3100-altered genes. GO terms with -log₁₀ (P-value) greater than 5 in either gene list were selected for analysis. C, Gene expression levels of top five altered cell-cycle genes were examined using RT-qPCR after CCX771 (5 µmol/L) treatment. D, Downregulation of five cell-cycle gene expression was validated using RT-qPCR after CXCR7 siRNA knockdown (KD). E. Western blot analysis showing phosphorylation of AKT, p-38, ERK, and JNK after CCX771 (5 µmol/L) treatment for 24 hours or CXCR7 siRNA KD in C4-2B cells for 2 days. F, Expression levels of five cell-cycle genes after treatment with LY294002 (10 μ mol/L) were measured by RT-gPCR. Data are representative of three independent experiments. Mean \pm SD is plotted. P-value was determined by two-tailed Student *t* test. ***, *P* < 0.0001.

CXCL12 was also detectable at a relatively lower level. Androgen treatment had no effect on MIF and CXCL12 levels. Using humanand mouse-specific ELISA, we found that MIF was secreted from both cancer cells and stroma cells, and that stroma cells promoted MIF secretion from cancer cells when they were cocultured (Supplementary Fig. S8). This indicated that tumor microenvironment may contribute to MIF-induced effects. We then examined C4-2B cell growth after treatment with human rMIF and rCXCL12. We found that rMIF significantly enhanced cell growth only in the absence of androgen in consistent with upregulation of CXCR7 after androgen deprivation (Fig. 7A). However, rCXCL12 had little effect on cell growth even when we increased its

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B

Relative cell viability

1.2 -□ + 1.0 - ■ ,

0.8

0.4

0.2

0.0

Relative cell viability

1.2 1.0 0.8

0.6

0

0.0

С

- CXCL12 - CXCL12

Relative cell viability

1.6 1.4 1.2 1.0 0.8 0.6

CXCR7 Promotes Prostate Cancer Progression

- MIF

+ MIF

- MIF

273

P = 0.0479

— MIF

— HIF

Figure 7.

MIF enhances CRPC cell growth and migration through CXCR7. A and B, C4-2B cell viability was measured by Alamar Blue assay 5 days after treatment with rMIF (10 ng/mL) or rCXCL12 (100 ng/mL) in the presence or absence of DHT (10 nmol/L). C, Viability of CXCR7 knockout (KO) cells was measured after MIF (10 ng/mL) stimulation compared with parental C4-2B and GFP-KO C4-2B cells. D, MIF siRNA knockdown (KD) efficiency was determined by mRNA levels using RTqPCR and protein levels using Western blot and ELISA. E, C4-2B viability was measured after MIF siRNA KD. F, Representative images of transwell migration assays in rMIFtreated C4-2B cells in the presence or absence of CCX771 (5 µmol/L). Migration images were quantified using ImageJ software. G, Representative images of transwell migration assays in rMIF-treated CXCR7 KO cell lines compared with control lines. Migration images were quantified using ImageJ software. H, Expression levels of five cell cycle gene were examined using RT-qPCR after treatment with rMIF (10 ng/mL) for 16 hours. I, AKT phosphorylation was examined by Western blot after different concentration of rMIF stimulation for 6 hours. J, MIF levels in patient serum samples were determined by ELISA. P-value was determined by two-tailed Student t test. K. Schematic overview of a model depicting MIF/CXCR7/AKT signaling in CRPC cells leading to expression of cell-cycle genes and CRPC cell growth.

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rMIF-induced C4-2B cell growth was abolished when CXCR7 was knocked out (Fig. 7C). We then knocked down endogenous MIF in C4-2B cells using RNAi. MIF mRNA and protein levels were significantly reduced after MIF siRNA knockdown (Fig. 7D), which attenuated C4-2B cell growth (Fig. 7E). rMIF also promoted CXCR7 blockade with CCX771 (Fig. 7F). We did not observe increased cell migration after rCXCL12 stimulation (data not shown), indicating CXCR12 played only a minimal role in the C4-2B CRPC model. We further examined the migratory capacity of CXCR7 KO cell lines after rMIF stimulation. Cell migration was

C4-2B cell migration in a transwell assay, which was inhibited by

CRPC cel

enhanced by rMIF in parental and GFP-KO C4-2B cell lines (Fig. 7G). In contrast, rMIF had no effect on CXCR7 KO cell migration in transwell assays. Our results support the notion that MIF but not CXCL12 is the ligand for CXCR7 in CRPC cells and that MIF promotes CRPC cell growth and migration through CXCR7.

To further address the biological function of MIF/CXCR7 axis in CRPC cells, we examined MIF-induced gene expression. We found that rMIF treatment significantly upregulated the expression levels of AURKA, CDC6, CDC45, CDK1, and E2F1 genes (Fig. 7H). Western blot analyses showed MIF-induced AKT phosphorylation in a dose-dependent manner (Fig. 7I). To determine whether patients with CRPC have high levels of secreted MIF, we measured MIF protein levels in serum samples from normal individuals, localized prostate cancer, hormonesensitive prostate cancer, and patients with metastatic CRPC using ELISA. As shown in Fig. 7J, normal individuals and localized patients with prostate cancer had low levels of MIF in serum. A slight increase was observed in hormone-sensitive patients. In contrast, a significantly higher MIF level was detected in a subset of patients with CRPC compared with localized patients. Taken together, our results suggest that elevated MIF may stimulate CXCR7 in CRPC cells, leading to activation of PI3K/AKT signaling, upregulation of cell-cycle gene expression, and CRPC cell growth and progression.

Discussion

Despite the development of next-generation anti-androgens, resistance to these drugs inevitably develops and renders patients largely incurable. To prevent or overcome anti-androgen resistance, a combination therapy with drugs targeting alternative survival signaling pathways may be necessary. Toward this end, we have discovered a MIF/CXCR7-mediated signaling pathway by which CRPC cells continue to grow and metastasize after anti-androgen treatment. MIF/CXCR7 promotes CRPC progression by inducing M-phase cell-cycle gene expression at least partially through PI3K/AKT signal transduction pathway (Fig. 7K). Targeting CXCR7 by a small molecule inhibitor, CCX771, in combination with AR antagonist, enzalutamide, inhibits CRPC tumor growth and potentially prevents metastasis.

Chemokines and their receptors play a crucial role in cancer metastasis. Much attention has been focused on CXCR4. Interaction between CXCR4 and its ligand CXCL12 and their biological activities in prostate cancer has been extensively studied (7). The CXCL12/CXCR4 signaling plays a key role in homing of prostate cancer cells to the bone microenvironment. CXCR4 has been targeted by small molecule inhibitors in clinical trials, which are analogues to the amino-terminal region of the ligand, CXCR12. Although previous studies have showed that CXCL12 stimulates gene expression that leads to a more proliferative and invasive phenotype in prostate cancer, we did not observe any biological effect induced by CXCR12 in our CRPC cell models. Inhibition of CXCR4 with AMD3100 also showed little effect on CRPC cell proliferation and migration. Lack of active CXCL12/CXCR4 signaling is likely due to lack of CXCR4 expression and activities in our cell models. Our data suggest that CXCR4 and CXCR7 may act independently in different cancer cell types. MIF/CXCR7 signaling is highly active in a subset of CRPC patients independent of CXCL12/CXCR4 signaling. Analyses of CXCR4 and CXCR7 expression in publicly available datasets further indicate that CXCR7 may play more important roles in prostate cancer metastasis.

Previous studies have indicated that increased CXCR7 expression is associated with aggressiveness in a variety of cancers, including breast, prostate, lung, and pancreatic cancer, by enhancing cell survival, proliferation, migration, invasion, and angiogenesis (6). In prostate cancer, it was reported that proinflammatory chemokine IL8 upregulates CXCR7, which in turn promotes prostate cancer cell proliferation in a ligandindependent manner (44). Depletion of CXCR7 suppresses prostate tumor growth through cell-cycle arrest. More recently, It was demonstrated AR inhibition increases CXCR7 expression through transcriptional regulation, which enhances prostate cancer survival and proliferation under androgen-deprived conditions (11). Growth promoting is accompanied by enhanced EGFR-mediated mitogenic signaling. In line with these results, we have discovered a novel MIF/CXCR7/AKT pathway, which enhances CRPC growth and metastasis after anti-androgen treatment. Studies on different aspects of CXCR7 in prostate cancer have shown the complex processes of CXCR7-mediated oncogenic signaling. These results, however, have led to the conclusion that targeting CXCR7 in prostate cancer is a promising therapeutic approach. It should be noted that previous studies have shown the benefit of combination treatment with CCX771 and enzalutamide in preclinical prostate cancer models (13). The inhibitory effect on androgen-dependent VCaP and MDA 133-4 tumor growth are more dramatic compared with the C4-2B model used in our studies. This is likely because the VCaP and MDA 133-4 tumors are responsive to enzalutamide treatment as a single agent, whereas CRPC C4-2B cells derived from bone metastasis are more aggressive and enzalutamide resistant. Our results support the notion of targeting CXCR7 in enzalutamide- and abiraterone-resistant patients.

MIF is a proinflammatory cytokine with chemokine-like activities. The release of MIF from cancer or stroma cells in response to various deleterious stimuli, such as hypoxia, creates a microenvironment favorable to the development of tumor. As an autocrine/paracrine factor, MIF enhances tumor cell proliferation, migration, and tumor-induced angiogenesis. Overexpression of MIF has been observed in many different types of cancer including prostate cancer and associated with tumor aggressiveness (45). Early studies have showed elevated MIF levels in advanced prostate cancer, which correlates with cancer progression (46). Targeting MIF with neutralizing antibody inhibits prostate cancer growth in preclinical studies (47). CD74, CXCR2, and CXCR4 are three receptors for MIF that mediate its intracellular functions. More recently, MIF was demonstrated as an alternative ligand for CXCR7 with a functional role in lymphocyte migration (43). MIF induces cancer cell proliferation via sustained activation of several pathways such as MAPK and PI3K/AKT. In line with these results, we have provided evidence that CXCR7 is required for MIF-induced proliferation and migration in CRPC cells. We revealed upregulation of MIF and CXCR7 in patients with CRPC. Further analyses suggest MIF/CXCR7 axis promotes CRPC cell growth and progression likely through AKT activation. These results support the notion that CXCL12 and MIF may interact with their receptors, CXCR4 and CXCR7, independently under different cellular contexts.

PI3K/AKT signaling pathway is a pro-survival pathway and therapeutic target for many cancers including prostate cancer. A

reciprocal feedback activation loop is produced as the result of inhibition of AR or AKT (48). Therefore, cotargeting AR and PI3K/ AKT may restore CRPC sensitivity to anti-androgen therapy and prolong disease stabilization. In this study, we demonstrate increased CXCR7 expression after ADT induces AKT activation, leading to alteration of gene expression involved in M-phase cellcycle progression, which provides a mechanistic rationale for dual inhibition of AR and CXCR7. It remains unclear whether inhibition of AKT is equivalent to inhibition of CXCR7 as CXCR7 may activate other signaling transduction pathways. Further studies are needed to understand how CXCR7 activates AKT phosphorylation upon ligand binding because CXCR7 activation does not commonly lead to canonical signaling through heterotrimeric G-proteins. Instead, CXCR7 may activate signaling through recruitment of β-arrestins as an accessory protein/adapter molecule in a ligand-dependent manner (10, 49).

In conclusion, our results suggest that upregulation of CXCR7 after ADT is one of the underlying mechanisms for CRPC cell survival, growth, and metastasis. Inhibition of CXCR7 may afford therapeutic benefits in certain clinical settings. Similar to targeting CXCR4, inhibition of CXCR7 may remove prostate cancer cells from bone marrow niche so as to expose and sensitize them to chemotherapy. This is particularly important when prostate cancer cells have low CXCR4 expression but high CXCR7 expression as we have observed in clinical metastatic tumors. These patients may not be responsive to CXCR4 blockade. More importantly, targeting CXCR7 in combination with anti-androgen treatment may not only inhibit CRPC tumor growth but also have the potential to prevent or delay metastasis in these patients. Our results set the stage for a further investigation on the benefits of combination therapy for metastatic CRPC.

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Disclosure of Potential Conflicts of Interest

X.S. Liu is a guest professor at the Tongji University and Affiliated Pulmonary Hospital; has ownership interest (including stock, patents, etc.) in GV20 Oncotherapy; is a consultant/advisory board member for Genentech, Amgen, and 3D Med Care. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: S. Rafiei, B. Gui, L. Jia

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Rafiei, B. Gui, L. Jia

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Rafiei, B. Gui, J. Wu, X.S. Liu, A.S. Kibel, L. Jia Writing, review, and/or revision of the manuscript: S. Rafiei, B. Gui, A.S. Kibel, L. Jia

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Targeting the MIF/CXCR7/AKT Signaling Pathway in Castration-Resistant Prostate Cancer

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