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Targeting CXCR2+ Neuroendocrine-like Cells for the Treatment of Castration-resistant Prostate Cancer

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Targeting CXCR2+ Neuroendocrine-like Cells

for the Treatment of Castration-resistant Prostate Cancer

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

In Molecular Biology

by

Yanjing Li

2017
ABSTRACT OF THE DISSERTATION

Targeting CXCR2+ Neuroendocrine-like Cells for the Treatment of Castration-resistant Prostate Cancer

by

Yanjing Li

Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2017

Professor Lily Wu, Chair

Prostate cancer (PCa) is the most commonly diagnosed non-cutaneous malignancy in men and the second leading cause of cancer-related deaths in the US. Men with PCa that has recurred after surgery or radiation therapy usually respond to androgen deprivation therapy (ADT); however despite initial responses rate of 80 to 90 percent, nearly all men eventually develop progressive disease following ADT; this is referred to as castration-resistant prostate cancer (CRPC), which remains an incurable disease. It has been reported that neuroendocrine (NE) cell type in prostate cancer is highly associated with castration-resistant disease. However, the exact role of NE cells in promoting the transition from castration-sensitive cancer to castration-
resistant disease is not fully understood. The goal of this study was to investigate the gene expression profile, cellular function and therapeutic resistant properties of NE cells purified through CXCR2 surface marker from prostate adenocarcinoma. We further investigated the role of CXCR2 in mediating CRPC carcinogenesis and explore the use of CXCR2 inhibitor combine with Enzalutamide as a potential therapy for advanced prostate cancer. In this research, we have identified a novel cross-talk between neuroendocrine cells in hormone naïve prostate cancer with both prostate cancer progression and hormonal therapy resistance. We have demonstrated that C-X-C motif chemokine receptor 2 (CXCR2) is a unique surface marker of neuroendocrine cells in prostate cancer and it is over-expressed in metastatic castration resistant, high grade, and NEPC patient cases. Importantly, we discovered that CXCR2 plays a critical role in facilitating this intercellular communication and NEPC transformation from typical prostate adenocarcinoma. CXCR2 overexpression led to enzalutamide resistance, loss of AR expression, and lineage plasticity with acquisition of stem cell-like properties. We further demonstrated that CXCR2 inhibition can prevent/delay enzalutamide resistance and restore AR function, leading to reductions in tumor burden. This research provides the mechanism for the first time of therapeutic resistance mediated by NE cells and raises the possibility to the final cure of lethal prostate cancer through CXCR2 blocker.
The dissertation of Yanjing Li is approved.

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2017
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INTRODUCTION

Prostate cancer (PCa) remains to be the number one cancer diagnosis in United States, accounting for about one-quarter of new diagnosis in men every year\(^1\). Primary treatment for men with localized prostate cancer includes radical prostatectomy and radiation therapy. 5-year relative survival rate approaches 100%\(^2\). For men whose cancer remains or relapsed after earlier treatment, hormonal therapy will be given to lower the level of androgen which is responsible for the growth of prostate cancer cells\(^3\). However, patients will develop resistance to androgen deprivation therapy (ADT) and progress to a more aggressive form of the disease called castration-resistant prostate cancer (CRPC)\(^4\). Second generation of anti-androgen drugs have been developed to target CRPC through blocking androgen receptor (AR) signaling or inhibiting androgen synthesis, including Enzalutamide\(^5\) and Abiraterone\(^6\). Despite the 4.8-month survival benefits they brought to the patients with CRPC\(^7\), the development of resistance is inevitable and leads to the death of patients who suffer from advanced prostate cancer.

Neuroendocrine (NE) cells, which constitute less than 1% in primary prostate cancer, are found in greater numbers in CRPC\(^8\). The accumulation of NE phenotype or NE differentiation in CRPC has been reported to correlate with PC progression and poor diagnosis\(^9,10\). Unlike luminal cells, which account for the major population of prostate adenocarcinoma, NE cells lack of the expression of both AR and prostate specific antigen (PSA)\(^11\), suggesting that they are hormone/AR-independent. In addition, NE cells do not proliferate\(^10\), indicating that they do not respond to chemo-drug including Docetaxel, which targets on proliferative cells. Although only accounts for a small population in prostate adenocarcinoma, NE cells play a regulatory role on surrounding tumor cells through secreting peptide hormones and growth factors in a paracrine manner to support tumor progression\(^12\). It has been reported that therapeutic interventions, including hormonal therapies and chemotherapy, can enrich NE-like cells which have superior...
anti-apoptotic properties\textsuperscript{13}. However the molecular properties of these NE-like cells and the mechanism behind their therapeutic resistance were not well characterized.

Several markers have been established to distinguish neuroendocrine cells in PCa through immunohistochemistry (IHC) staining. Among these NE markers, chromogranin A (CgA) has been widely accepted as an excellent marker of NE cells\textsuperscript{14,15,16}. Neuron specific enolase (NSE) and Synaptophysin (Syn) also belong to NE markers, however their correlation with NE differentiation in PC is not as strong as CgA\textsuperscript{17}. There hasn’t been any cell surface marker to distinguish NE cells in PC, as CgA, NSE and Syn are expressed in cytoplasm. Neural cell adhesion molecule (NCAM) or CD56 antigen has been reported as a surface marker to diagnose NE tumor in lung cancer, liver cancer and Merkel cell carcinoma\textsuperscript{18}, but no reports on the expression of CD56 in prostate NE tumor. Our previous discovery suggested that the rare NE population in prostate adenocarcinoma uniquely express a type of interleukin-8 (IL-8) receptor CXCR2, whereas the rest luminal population is CXCR2 negative\textsuperscript{12}. CXCR2, as one of the G-protein couple receptors (GPCRs), binds to angiogenic CXC chemokine family members, including IL-8, CXCL1,2 and 3 (GPO\(\alpha\), \(\beta\) and \(\gamma\)), CXCL5(ENA-78), CXCL6 (GCP2) and CXCL7 (NAP2) and mediates chemokine-induced angiogenic activity\textsuperscript{19}. Apart from being part of the leukocyte chemotaxis and being involved in the initiation and amplification of inflammatory responses\textsuperscript{20,21}, CXCR2 is also shown to promote growth, progression and metastasis of various types of cancer, including colon cancer\textsuperscript{22}, pancreatic cancer\textsuperscript{23}, ovarian cancer\textsuperscript{24}, gastric cancer\textsuperscript{25}, breast cancer\textsuperscript{26,27}, lung cancer\textsuperscript{28} and melanoma\textsuperscript{29}. However, the role of CXCR2 in the progression of primary PC to CRPC hasn’t been reported. Regarding the molecular mechanisms, it has been shown that CXCR2 is able to activate Erk, Akt signaling pathways\textsuperscript{30}, mediate epithelial-mesenchymal transition\textsuperscript{31} and reinforce cellular senescence\textsuperscript{32}. 
In the current study, we explored the gene expression profile, cellular function and therapeutic resistant properties of NE cells purified through CXCR2 surface marker from prostate adenocarcinoma. We further investigated the role of CXCR2 in mediating CRPC carcinogenesis and investigate the use of CXCR2 blocker combine with Enzalutamide as a potential therapy for advanced prostate cancer.
EXPERIMENTAL PROCEDURES

Primary Human Cell Preparation

De-identified human prostate tissues were obtained from UCLA translation Pathology Core Laboratory and Duke Biospecimen & Processing Core and are exempt from UCLA and Duke Institutional Review Board approval. The processing of human prostate tissue was performed as previously described\textsuperscript{33}.

Cell Separation

Dissociated cells were stained with primary antibodies: PE-Cy7-conjugated CD49f (Clone GoH3, BioLegend), APC-conjugated Trop2 (FAB650A, R&D Systems), APC-eF760-conjugated CD45 (47-0459-42, eBioscience) and PerCP-eFluor710-conjugated CXCR2 (5E8-C7-F10, eBioscience) for 15 minutes on ice. Sorting was performed on a BD FACS Aria II (BD Biosciences).

Intracellular flow cytometry

Dissociated cells were stained with CXCR2-PerCP for 15 minutes on ice. Cell were washed with PBS and fixed in 1ml of 1% paraformaldehyde (Electron Microscopy Sciences) in PBS for 15 minutes on ice. Cells were washed with PBS and permeabilized in 1ml of permeabilization buffer (Sigma) for 20 minutes at room temperature in the dark. Cells were resuspended in 50ul of permeabilization buffer and 1ul of 1mg/ml goat IgG (Jackson ImmunoResearch) for 15 minutes on ice. Anti-Chromogranin A antibody (clone LK2H10, Millipore) was added for 15 minutes on ice. Cells were washed with washing buffer then stained with goat anti-mouse Alexa Fluor 488 secondary antibody
Cells were washed again with washing buffer and resuspended in PBS and analyzed on a BD FACSCanto (BD Biosciences).

**Cell Lines**

LNCaP, C4-2, C4-2-MDR (gift from Allen Gao, UC Davis) and CWRR1 (ATCC) were grown in RPMI with 10% FBS. LNCaP-CXCR2 was established by transfection of CXCR2 cDNA (Origene) into LNCaP cells and selection by G418. LNCaP-D4 and LNCaP-D6 were established by transfection of CXCR2 Crispr plasmid DNA. Sequences were listed below.

**Immunohistochemical and Immunofluorescence Analysis**

Paraffin-embedded tissue was analyzed as previously described. Primary antibodies used: CXCR2 (BD, 555932), AR (ThermoFisher, AR441), PSA (Dako, A0562), CD44 (Biolegend, IM7), CgA (Novus, NB120-15160), CD31 (abcam, ab28364). Secondary antibodies used: Goat-anti-mouse IgG conjugated to Alexa Fluor 594, Goat-anti-Rabbit IgG conjugated to Alexa Fluor 499 (ThermoFisher). Fluorescent images were counterstained with DAPI (Vector Laboratories).

**Immunoblot analysis**

Cells were lysed in RIPA buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS) containing a Complete protease inhibitor cocktail tablet (Roche) for 15 minutes on ice. Samples were spun down for 15 minutes at maximum speed and the supernatant was collected. 10 micrograms were loaded per lane, and membranes were probed for anti-PSA (Dako), anti-AR (ThermoFisher), anti-p-Akt-S473 (CST), anti-p-p70S6K-T389 (CST), anti-p-Erk1/2 (CST), anti-CgA (Novus),...
anti-Snail (abcam), anti-vimentin (abcam), anti-e-cadherin (abcam), and loading control anti-GAPDH (ThermoFisher) followed by HRP-conjugated antibodies against rabbit or mouse IgG (Pierce).

**RNA Isolation, Reverse Transcription and Quantitative Real Time-Polymerase Chain Reaction**

Total RNA was isolated from freshly-sorted prostate populations using the RNeasy Micro Kit (Qiagen) and reverse transcription was carried out using SuperScript III first-strand synthesis system (Invitrogen). qRT-PCR and subsequent analysis was performed using the iQ SYBR Green Supermix for Real-Time PCR (Bio-Rad) on a Bio-Rad iCycler and iQ5 2.0 Standard Edition Optical System Software. Data was analyzed using the Pfaffl method. Target gene expression was normalized to human b-actin. Statistics were performed using GraphPad Prism software, analyzed with ANOVA and Newman-Keuls Multiple Comparison Test. RT-PCR was carried out for 35 cycles on a BioRad Thermo Cycler. Primer sequences are listed below.

**Whole Transcriptome Sequencing Analysis**

cDNA libraries were prepared from isolated RNA using the TruSeq RNA Sample Prep Kit v2 (Illumina). High-throughput sequencing with 75 bp paired-end reads was performed using an Illumina HiSeq 2500 in rapid run mode. Reads were mapped to human genome reference HG19 using MapSplice\[^{34}\]. Gene expression was quantified using RSEM\[^{35}\] and quantile normalized. Gene set enrichment analysis was performed using GSEA software from Broad Institute\[^{36}\] with a pre-ranked list of genes differentially
expressed (>4-fold) among CXCR2+ NE cells, CXCR2- luminal cells and basal cells in each tumor sample.

**Transcriptome Analysis of TCGA database**

Genome-wide measurements of mRNA expression profile for prostate adenocarcinoma (PRAD) was downloaded from TCGA Data Portal. The PRAD data contains 489 patients’ samples with measurements available of transcriptome profile. For expression data, processed RNA-seq data were used, which record the RPKM (read per kilobase of exon per million mapped reads) values for mRNA. The data were further log2-transformed and mean-centered.

**Human Cytokine Array**

Screening for cytokines secreted from LNCaP, LNCaP-CXCR2 and LNCaP-D4 were performed by hybridizing conditioned medium with antibody-coated membranes (Cytokine Human Membrane Antibody Array Kits, Abcam, Cambridge, MA) according to the manufacturer’s instructions. Briefly, the culture supernatants of cells culture with or without androgen were collected by centrifugation and then hybridized to the array membrane. A biotin conjugated second antibody was used and cytokines were detected by HRP-conjugated streptavidin. Signals were quantified by UN-SCAN-IT gel 6.1 software (silk Scientific, Orem, UT).

**Measurement of secreted ANG by ELISA**

Cells cultured with or without androgen were plated onto 10cm plate at density of 1x10^6 cells/well. After 48h, the conditioned media was collected and centrifuged to remove any dead or floating cells. Conditioned media was analyzed by ELISA for ANG
(ab99970; Abcam, Cambridge, MA, USA) using a FLUOstar OPTIMA microplate reader (BMG Labtech, Cary, NC, USA).

**In vitro Testing of CXCR2 Inhibitors**

Cell viability studies were performed by seeding 10,000 C4-2, C4-2-MDR, CWRR1 cells in 2ml of RPMI1640+10%FBS media in each well of a 6-well plate. Cell numbers after drug treatment were counted by using hemacytometers. The percentage of CXCR2+ cells after treatment were stained by CXCR2-PerCP antibody and analyzed by flow cytometry.

**In vivo MK7123 Studies**

For MK7123 treatment studies, 1,000,000 dissociated cells from C4-2 cell line were resuspended in 100 μl of 1:1 cold Matrigel and RPMI1640 mixture and subcutaneously xenografted in NSG mice. Once tumors achieved a volume of 50 mm³, mice were administrated with MK7123 50 mg/kg or vehicle (0.5% methyl cellulose) through oral gavage daily for three weeks. Tumor dimensions were measured by calipers and tumor volumes were calculated using the following equation, \( V = (L \times W \times H) / 2 \). In vivo administration of MK7123 and treatment monitoring were performed in accordance with a protocol approved by the Animal Research Committee at Duke.
RESULTS

CXCR2 is Expressed on Prostate Cancer Cells with Neuroendocrine Cells Feature

We previously demonstrated that neuroendocrine (NE) cells in human prostate adenocarcinoma (PCa) express CXCR2 whereas the rest luminal cells are CXCR2 negative through immunohistochemistry (IHC) staining\(^1\). To determine if CXCR2 can be used as a reliable surface marker to purify NE cells from PCa tumors, we performed flow activated cell sorting (FACS) to separate CXCR2⁺ cells and CXCR2⁻ luminal cells from fresh human PCa tumors collected from UCLA Ronald Reagan Hospital and Duke University Hospital. Since infiltrating inflammatory cells in PCa tumor tissue also express CXCR2\(^3\), we used leukocyte marker CD45\(^3\) to exclude CD45⁺ intraepithelial inflammatory cells before sorting by CXCR2 marker (Figure 1A). We collected CD45⁻/CXCR2⁺ PCa cells and further sorted the rest CD45⁻/CXCR2⁻ population by Trop2 and CD49f to exclude stroma cells and basal cells\(^3\). We collected CD45⁻/CXCR2⁻/Trop2⁺/CD49f⁻ population which is luminal PCa cells (Figure 1A) and compared the expression of NE markers Chromogranin A (CgA) and Neuron specific enolase (NSE) between CXCR2⁺ PCa cells and CXCR2⁻ luminal cells. We found that CXCR2⁺ PCa population consistently express higher level of NE markers CgA (Figure 1B) and NSE (Figure 1B) than CXCR2⁻ luminal cells collected from 5 patients’ PCa tumor, suggesting that CXCR2 can be used as NE cells surface marker to purify NE-like cells from human fresh PCa tumor. To confirm this finding, immunofluorescence double staining of CgA and CXCR2 were performed on human prostate tissue microarray slide and CRPC tissue slide. The results showed co-localization of CgA and CXCR2 in the identical cancer cells in both primary prostate cancer (Figure 1C) and castration-resistant disease (Figure 1D), confirming that CXCR2 positive cells were NE cells. Furthermore, CXCR2⁺ and CXCR2⁻ prostate cancer cells sorted from patients’ fresh tumor were collected for whole genome RNA-sequencing. We identified the significantly enriched gene networks for CXCR2⁺ and CXCR2⁻ populations through gene set enrichment analysis (GSEA).
Among the gene signature pathways revealed by GSEA, we found that CXCR2+ cancer population is enriched for genes that are involved in neuronal differentiation, including CHGA, NRXN1, NGFR, DCX, NRP2, NHLH2, NEFL etc.; whereas CXCR2- cancer population is enriched for genes that are luminal markers and are involved in AR signaling (Figure 3B). Thus, we concluded that CXCR2 can be used as a reliable surface marker for purifying NE cancer cells from human prostate cancer.

To determine if CXCR2+ population could represent NE-like cells in established human prostate cancer cell lines, we sorted CXCR2+ cells and CXCR2- cells from C4-2 cell line and compared the expression of NE markers. We found that CXCR2+ cells express significantly higher level of CgA, NSE, Synaptophysin (Syn) and CD56 (NCAM) than CXCR2- cells in C4-2 cell line at mRNA level (Figure 1E). To confirm this finding, we performed double staining of CXCR2 and CgA on both LNCaP and C4-2 cell line through flow cytometric analysis. In LNCaP cell line, almost 100% of CXCR2+ cells are CgA positive whereas CXCR2- cells do not express CgA (Figure 1F). In C4-2 cell line, although 50% of CXCR2- cells express CgA at relative low level, 100% CXCR2+ cells are CgA positive and show much stronger expression of CgA than CXCR2- cells (Figure 1F). These data suggest that NE-like cell population exists in established human prostate adenocarcinoma cell line which can be distinguished by CXCR2. Based on the analysis on both in vitro cell line and patients’ fresh tumor, CXCR2 was identified as a novel surface marker for NE cancer cells in human prostate cancer.

**CXCR2 Expression is Associated with Tumor Grade, Stage and Disease Progression in Prostate Cancer**
To investigate the clinical relevance of CXCR2 expression in prostate adenocarcinoma, we analyzed the RNA sequencing data of prostate cancer tumors from 185 patients (131 primary tumors, 19 metastatic tumors) based on the genomic data set generated by Taylor et al. Patients were grouped by tumor stage, including low grade primary tumor (Gleason score=6/7), high grade primary tumor (Gleason score =8/9) and metastatic tumor. Although CXCR2 expression didn’t show differences between normal group and low grade primary tumor (Gleason score=6/7), CXCR2 was significantly up-regulated in high grade primary tumor (p-value=0.0008) and metastatic tumor (p-value=0.004) compared with low grade primary tumor (Figure 2A). Similar results have been confirmed by immunohistochemistry (IHC) staining of CXCR2 on the prostate tissue from untreated prostate cancer patients of various Gleason score (Figure 2B) and patients with castration-resistant prostate cancer (CRPC) (Figure 2C) and patients with small cell neuroendocrine carcinoma (SCNC) (Figure 2D). Low grade primary tumor (Gleason score =6) contains very few CXCR2+ cells whereas high grade primary tumors (Gleason score=7/9) (Figure 2B), CRPC (Figure 2C) and SCNC (Figure 2D) are significantly enriched of CXCR2+ cells. Comparing the distribution of CXCR2+ cells between high grade primary tumors and CRPC, we noticed that CXCR2+ cells are normally located on the outer layer of prostate gland in high grade primary tumors (Figure 2B) but are evenly distributed in CRPC (Figure 2C) and SCNC (Figure 2D). Combining data from genomic analysis and pathology staining, we demonstrated that CXCR2 expression is associated with disease progression and recurrence in prostate cancer.

Acquisition of NE phenotype by PCa cells positively correlated with progression to CRPC, which failed to respond to androgen deprivation therapies (ADTs). To determine if CXCR2+ population is therapeutic resistant, we exposed both LNCaP cell line and C4-2 cell line to ADT, anti-androgen drug Enzalutamide or chemo-drug Docetaxel and compared the percentage of
CXCR2+ population before and after the treatments. In C4-2 cell line, CXCR2+ population reached 15.0% after 2-week Enzalutamide treatment, 9.64% after 2-week ADT, 14.1% after 3-day Docetaxel treatment, compared with basal 2.75% in parental cell line (Figure 2E). Similar result was observed in LNCaP cell line, where CXCR2+ population reached 6.42% after Enzalutamide treatment, 7.67% after ADT and 15.4% after Docetaxel treatment, compared with the basal 1.31% in parental cell line (Figure 2E). These data indicate that CXCR2+ population is more therapeutic resistant than CXCR2- population in human PCa cell lines. To determine if CXCR2+ cells are also enriched in castration-resistant cell line models, we examined CXCR2 expression in Enzalutamide-resistant LNCaP-MDR (multi-drug resistant) and C4-2-MDR cell lines. Both MDR cell lines show CXCR2 overexpression compared with their parental cell line (Figure 2F). Thus, we concluded that CXCR2 expression is associated with prostate cancer progression and therapy resistance.

**CXCR2+ PCa Cells are enriched for Cancer Stem Cell Gene Signatures**

Since CXCR2+ PCa cells show resistance to conventional therapies compared to CXCR2- PCa cells, we investigated if CXCR2+ PCa cells possess cancer stem cell properties. We acquired prostate tissue from three patients that had undergone radical prostatectomy and sorted Basal cells from benign tissue, CXCR2+ NE cells and CXCR2- Luminal cells from cancer tissue. In total, we collected all three populations for each patient. Each sample was subjected to paired-end RNA sequencing (RNA-seq) and averaged 1.0 x 10^8 paired reads that uniquely mapped to the human genome (Table S1 and Dataset S1). To explore the molecular differences among the three populations, we performed hierarchical clustering on all 9 samples. To our surprise, CXCR2+ NE population from cancer region was more closely clustered with basal cell population from benign tissue than CXCR2- Luminal population from cancer region (Figure 3A).
Using gene set enrichment analysis (GSEA), we looked at differentially expressed genes between CXCR2+ NE and CXCR2- Luminal populations. A total of 3590 genes were differentially expressed between the CXCR2+ NE and CXCR2- Luminal populations, with 2339 genes up-regulated in the CXCR2+ NE population and 1251 genes up-regulated in CXCR2- Luminal population. To gain more biological insight into gene networks specific for each population, we ran gene ontology analysis (GO biological_process) on the differentially expressed genes that could identify CXCR2+ NE and CXCR2- Luminal populations. The CXCR2+ NE population overexpressed a number of genes that are involved in EMT, stemness, extracellular matrix disassembly and collagen catabolic process, cell invasion and migration, angiogenesis. Other up-regulated genes are found in Myc signaling pathway, TGFB1 signaling and integrin 1 splicing (Figure 3B). The CXCR2- Luminal population overexpressed genes commonly associated with luminal cell type, AR signaling pathways and sterol biosynthetic process (Figure 3B).

To further demonstrate the CXCR2+/Hi population is enriched for the cancer stem cell (CSC) gene signature, we performed linear regression analysis to illustrate the correlation between CXCR2 with CSC markers, CRPC markers, EMT markers and NE markers through the database of organ-confined prostate adenocarcinomas from The Cancer Genome Atlas (TCGA). We found that CXCR2 expression is positively correlated with its ligand IL-8 (p-value=2.15E-13) and the expression of cancer stem cell genes CD133 (p-value =2.07E-37), CD44 (p-value=9.15E-7), Oct3/4 (p-value=0.002), Sox9 (p-value=8.37E-5) and Klf4 (p-value=6.66E-15) (Figure 3C). In addition, CXCR2 also shows strong correlation with genes responsible for CRPC development, including Bcl-2 (p-value=2.09E-31), EGFR (p-value=2.04E-25), STAT3 (p-value=1.50E-8), Sox2 (p-value=4.61E-5) and TGFBR1 (p-value=0.001) (Figure 3C). These data indicate that CXCR2 expression may correlate with PCa progression. Besides, CXCR2 show negative correlation with
PSA (p-value=0.004, beta=-0.127) and proliferative marker Ki67 (p-value=0.01, beta=-0.11) (Figure 3C). A linear regression model was generated to show the significant positive correlation between CXCR2 and cancer stem cell marker CD133 based on RNA sequencing data of 489 patients with prostate adenocarcinoma (Figure 3C).

To confirm this result in established human PCa cell lines, we sorted CXCR2+ and CXCR2- cells from C4-2 cell line and compared their gene expression level of stem cell markers, CRPC markers and EMT markers (Figure 3D). Prostate cancer stem cell markers CD44 and CD133 and stem cell markers Oct3/4, Sox9 and Klf4 are significantly over-expressed in CXCR2+ cells. Among the genes that are responsible for CRPC development, REG4, Bcl-2 and Sox2 are up-regulated in CXCR2+ cells compared to CXCR2- cells. Snail, which is responsible for epithelial-mesenchymal transition (EMT) also showed high expression in CXCR2+ cells (Figure 3D).

To show the correlation between CXCR2 expression and prostate cancer differentiation markers PSA and AR through IHC, we performed double staining of CXCR2 with AR or PSA on prostate tissues from patients with different Gleason scores (Figure 3E). The results showed that low grade primary prostate cancer express higher level of differentiation markers PSA and AR but low/no expression of CXCR2; whereas high grade primary prostate cancer is enriched of CXCR2 expression but lack of the expression of AR and PSA (Figure 3E). We further validated this result by performing immunofluorescence double staining of CXCR2 and PSA or CD44 on paraffin embedded human prostate cancer tissue. The results showed that CXCR2+ prostate cancer cells in untreated PCa do not express PSA (Figure 3F) but express stem cell marker CD44 (Figure 3G), whereas CXCR2- prostate cancer cells express luminal marker PSA but lack of the expression of CD44. Thus, we concluded that CXCR2+ NE cells in untreated primary PCa are
the pre-existing therapy resistant clones which are androgen-independent based on genomic profiling data.

**CXCR2 Expression is Responsible for Therapeutic Resistance to ADT, Enzalutamide and Docetaxel in LNCaP Cells and PCa Metastasis**

We have previously demonstrated that CXCR2 expression is highly associated with genes expression related to cancer stem cell (CSC) and CRPC development based on the analysis of TCGA database. To investigate if CXCR2 could be the master regulator of the both gene networks and if CXCR2 is responsible for therapy resistance, we established LNCaP-CXCR2 cell line by over-expressing CXCR2 in hormone-sensitive human prostate cancer LNCaP cell line, which contains very low basal CXCR2 level. We performed colony-formation assay to analyze the sensitivity of LNCaP-CXCR2 and LNCaP-EV (empty vector) in response to ADT, Enzalutamide 1uM or 10uM, or Docetaxel 0.2nM and 1nM, respectively. There were no differences of the colony formation rate of LNCaP-CXCR2 between untreated and ADT/Enzalutamide/Docetaxel treated groups, suggesting that CXCR2 over-expression led to the therapy resistance to castration, anti-androgen drugs and chemo-therapy. However, parental LNCaP-EV cells are hormone-naïve and sensitive to Docetaxel treatment (Figure 4A).

To illustrate the therapeutic resistance mediated by CXCR2 expression in LNCaP cell line, we compared the gene expression of AR and PSA in LNCaP-EV and LNCaP-CXCR2 cell lines. Surprisingly, we found that CXCR2 over-expression significantly inhibit the expression of AR and PSA at both mRNA (Figure 4B) and protein level (Figure 4C). This explains the hormone-independency of LNCaP-CXCR2 observed in the previous colony formation assay (Figure 4A). Whole genome RNA-sequencing of LNCaP-EV and LNCaP-CXCR2 was performed to further explore the possible genomic reprogramming mediated by CXCR2 over-expression. Gene
enrichment analysis of the significant enriched pathways showed that LNCaP-CXCR2 cells are enriched for genes in E2F3 gene network, EMT process and Angiogenesis; whereas LNCaP cells are particularly enriched for luminal markers or prostate cancer markers, including AR and PSA (Figure 4D). These results indicate that CXCR2 drives the lineage plasticity in prostate cancer cells with the acquisition of cancer stem cell like properties.

Since CXCR2 over-expression led to the therapy resistance in hormone-sensitive LNCaP cell, we further explored the differences and similarities between LNCaP-CXCR2 and LNCaP-MDR cell lines by comparing the expression of luminal markers, NE markers, EMT markers and the activation of survival pathways. It showed that LNCaP-CXCR2 and LNCaP-MDR shared striking similarities as both of them lack of the expression of luminal markers AR and PSA, express NE marker CgA, EMT marker Snail and Vimentin, and they both turned on survival pathways including PI3K/AKT, Erk and mTor (Figure 4C). This suggests that CXCR2 up-regulation during hormonal therapy is responsible for the development of multi-drug resistant properties in human prostate cancer cell line.

To prevent the development of therapy resistance during hormonal therapy, we performed CXCR2 gene knocked-out in LNCaP cell line by using Crispr/Cas9 system. We established LNCaP-D4 and LNCaP-D6 clones where the whole ~10000 base pair CXCR2 DNA were successfully removed from their genome (Figure 4E). Sox9 and E2F3 were demonstrated to promote the transformation to CRPC and their expression were induced by hormonal therapy. Therefore, we compared the gene expression of Sox9 before and after ADT in LNCaP, LNCaP-CXCR2, LNCaP-D4 and LNCaP-D6 cell lines. The results showed that Sox9 gene expression was induced in LNCaP cell line after ADT, which agrees with previous findings in the literatures41.
We further discovered that LNCaP-CXCR2 was able to induce a much higher level of Sox9 expression after ADT when compared to LNCaP cells; whereas CXCR2-knockout in LNCaP-D4 and D6 prevents the inducement of Sox9 gene expression during hormonal therapy (Figure 4F). This confirms our previous observation that CXCR2 regulates the gene networks of cancer stem cell and CRPC development. More importantly, we identified that inhibition of CXCR2 could prevent therapy resistance during hormonal therapy.

**CXCR2 Expression Promotes Angiogenesis in Prostate Cancer**

One of the most distinguishing feature of neuroendocrine cells is that they are involved endocrine, paracrine pathways by releasing secretory molecules to the microenvironment. To investigate if CXCR2+ NE cancer cells regulate the cancer microenvironment, we performed cytokine array for simultaneous detection of 42 human cytokines that are secreted by LNCaP, LNCaP-CXCR2 and LNCaP-D4. The results showed that ADT can significantly induce the expression of several cytokines in LNCaP and LNCaP-CXCR2 cell line, including Angiogenin, IL-8 and stem-cell factor (SCF or c-Kit) etc., suggesting that hormonal therapy could induce angiogenesis in prostate cancer (Figure 5A). However, there were no significant differences in the levels of angiogenin, IL-8 and SCF in LNCaP-D4 before and after ADT (Figure 5A). This indicates that the secretion of these cytokines was mediated by CXCR2 during hormonal therapy.

Angiogenin ELISA assay was performed for the quantification of secretory angiogenin during ADT. As shown in Figure 5B, ADT induced angiogenin secretion in both LNCaP and LNCaP-CXCR2 cells but not in CXCR2-knockout LNCaP-D4 clone (Figure 5B). This suggests that CXCR2+ NE cells can trigger angiogenesis in prostate cancer during hormonal therapy.
The CXCR2 Antagonist, MK7123, Suppresses the Growth of Castration-resistant Prostate Cancer in Preclinical Prostate Cancer Model

The accumulation of NE phenotype in CRPC is responsible for the failure of hormone therapy or second generation of anti-androgen drugs. Therefore, developing NE cell-targeted therapy has the potential to treat lethal prostate cancer that has failed AR-targeted therapies. Based on our previous findings, CXCR2 plays an essential role in the function of NE cells as it confers therapy resistance, promotes angiogenesis and drives lineage plasticity through inducing stem-cell like properties. Thus, we proposed to use CXCR2 blocker to inhibit the NE population in CRPC and restore cancer cells’ sensitivity to hormonal therapy.

The result of growth curve assay showed that CXCR2 antagonist MK7123 has significant inhibitory effect on both C4-2 (Figure 6A) and C4-2-MDR cell lines (Figure 5B), both of which are CRPC cells. The combination therapy of Enzalutamide and MK7123 also showed more significant therapeutic effect than applying Enzalutamide alone in both cell lines (Figure 6A and 6B). To demonstrate that MK7123 specifically target NE population in C4-2 cell line, which is a mix of luminal and NE cell types, flow cytometric analysis was performed to compare the percentage of CXCR2+ population before and after treatment. The results showed that CXCR2 antagonist MK7123 inhibits the NE cell component in C4-2 cells upon Enzalutamide treatment. As shown in figure, CXCR2+ population were dropped from 11.3% post-enzalutamide to 1.15% post-combination therapy (Figure 6C).

To apply CXCR2 antagonist MK7123 in pre-clinical trial model, we established C4-2 mice xenograft model by injecting C4-2 cell line subcutaneously into immuno-deficient NSG mice.
Then the mice were divided into four groups to receive different treatments, including vehicle, Enzalutamide, MK7123 or Enzalutamide and MK7123. We recorded the sizes of tumor burden and mice weight throughout the course of treatments. The result showed that MK7123 reduced tumor burden and enhanced the therapeutic effect of Enzalutamide during the combination therapy (Figure 6D). Detailed molecular analysis of xenograft tumors was performed through IHC staining. We identified that MK7123 restored AR function (Figure 6E), induced apoptosis (Figure 6F) and inhibited angiogenesis (Figure 6G) in CRPC xenograft model. More importantly, we identified that the combination therapy of MK7123 and Enzalutamide almost completely inhibited tumor growth in vivo and induced massive apoptosis and necrosis. Thus, we concluded that inhibition of CXCR2 can effectively inhibit NE cell component, disrupts the paracrine cycle, augmenting the efficacy of castration therapy and preventing tumor progression in CRPC.
DISCUSSION

Neuroendocrine (NE) cells, which constitute less than 1% in primary prostate cancer (PCa), are found in greater numbers in metastatic castration-resistant prostate cancer (mCRPC), which remains an incurable disease. Current therapies for mCRPC, including hormonal therapy and anti-androgen drugs, have demonstrated to promote accumulation of NE phenotypes, which is responsible for therapeutic resistance and the death of patients who suffer from mCRPC. However, it is unclear how NE phenotype is evolved after receiving hormonal therapy and how to develop therapeutically specific drug against NE population due to the lack of knowledge of its underlying biology. Many groups have suggested that the increase of NE phenotype is the result of NE trans-differentiation or de-differentiation from luminal cell type after receiving hormonal therapy\textsuperscript{42,43,44,45}. However, due to the lack of reliable NE cell surface marker, very little is known about the rare NE population in untreated prostate adenocarcinoma rather than they are different from normal NE cells in the prostate\textsuperscript{46}.

Our group has identified that chemokine receptor CXCR2 is a unique surface marker for NE cells in prostate cancer and it is the driver of NE phenotype and therapeutic resistance. By detailed transcriptome analysis of human NE cells purified through CXCR2 surface marker of low grade, high grade PCA harvested from prostatectomy, we identified that CXCR2+ NE cells of hormone naïve prostate cancer were greatly enriched in gene signatures associated with basal stem cells of benign prostate, which has been demonstrated to share a common transcriptional program with aggressive prostate cancer-small cell neuroendocrine carcinoma (SCNC)\textsuperscript{47}. Distinguished from basal stem cells of benign prostate, CXCR2+ NE cancer cells are enriched for genes that are involved in extracellular matrix disassembly, angiogenesis and tumorigenesis, suggesting CXCR2+ NE cells possess unique endocrine or paracrine function. This observation also indicates that CXCR2+NE cells play a role in the early metastasis, supporting the previous
finding that the number of NE cells on initial PC biopsies is positively correlated with risk of distant metastasis. Importantly, we identified that CXCR2+ NE population from hormone naïve PCa are particularly resistant to ADT, enzalutamide and Docetaxel and they are highly tumorigenic. All these evidences indicate that the sub-clonal selection of the pre-existing resistant CXCR2+ NE cells by hormonal therapy contributes to the progression from hormonal naïve state to the castration-resistant disease. This observation challenges the current perspective that the therapy resistant NE population is derived from hormone-sensitive luminal cells during hormonal therapy rather than clonal expansion from pre-existing NE cancer cells.

The identification and characterization of CXCR2+ NE cells raises the possibility of developing cell-type-specific therapy for neuroendocrine cells in prostate cancer. Based on the traditional view of hormonal therapy resistance, the development of NE “trans-differentiation” is the result of a number of AR-dependent mechanisms, including AR amplification, AR genomic alterations, AR splice variants or even the loss of AR signaling that take place in luminal cells. As a result, second generation of anti-androgen drugs were developed to target those “bypass” AR signaling pathways in order to overcome the resistance to castration. However, further resistance to these therapies is usually developed within a short range of time. Key questions remain on whether resistant clones are derived from new mutation induced by therapeutic reagents or are rare subpopulations that are preexisting and selected after therapy. A recent study using single cell sequencing analysis to investigate genome evolution in metastatic prostate cancer suggested that the clonal selection and expansion of a pre-existing resistant population confers the therapy resistance. In this study, by identifying NE cells in untreated prostate adenocarcinoma as pre-existing therapy resistant clones, we developed NE cell target therapies which could eliminate the possibility of clonal selection during traditional hormonal therapies, therefore prevent and overcome the resistance to hormonal therapy.
CXCR2, the newly identified surface marker for NE cancer cells, was demonstrated to play a critical role in facilitating the cross-talk between NE cells in the prostate with both prostate cancer progression and hormone therapy resistance. We identified CXCR2 overexpression led to enzalutamide resistance, loss of AR expression, and lineage plasticity with acquisition of stem cell-like properties. Therefore, we nominated CXCR2 as a critical NEPC promoting factor that may be targetable therapeutically. In the study, we demonstrated that CXCR2 inhibition can prevent enzalutamide resistance and reduce tumor burden in pre-clinical trial model. Importantly, CXCR2 inhibition can reverse NE phenotype and restore AR function. This supports our finding that CXCR2 over-expression suppresses AR and PSA expression in LNCaP cell line model. Future work is needed to elucidate the molecular mechanism of CXCR2 mediated regulation of AR signaling pathway as well as the anti-neuroendocrine activity carried out by CXCR2 inhibitor.
REFERENCES


