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14. ABSTRACT TMPRSS2-ERG fusion transcripts have been shown to be expressed in a majority of prostate cancer (PC) patients due to chromosomal translocations or deletions involving the TMPRSS2 gene promoter and the ERG gene coding sequence. These alterations cause androgen dependent ERG transcription factor expression in PC patients. We and others have shown that chemokine receptor CXCR4 expression is upregulated in PC tumor cells and its ligand, CXCL12, is expressed in bone stromal cells. The CXCL12/CXCR4 axis functions in PC progression to enhance invasion and metastasis. To address the regulation of CXCR4 expression, we identified several putative ERG consensus binding sites in the promoter region of CXCR4. We hypothesized that androgen dependent regulation of the ERG transcription factor could induce CXCR4 expression in PC cells. Results of the current study show that (a) ERG protein expressed from TMPRSS2-ERG fusion gene binds selective ERG binding elements in CXCR4 promoter in VCaP cells; (b) Upstream ERG binding sites in CXCR4 promoter are active in ERG induced CXCR4 promoter activation; (c) R1881 induced ERG gene mediates CXCR4 expression in androgen responsive prostate cancer cells. These findings demonstrate biochemical interaction between ERG factor and CXCR4 gene promoter and link these activities with TMPRSS2-ERG translocations and enhanced metastasis of tumor cells via CXCR4 function in PC cells.					
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INTRODUCTION:

Specific chromosomal alterations were recently discovered in prostate cancer (PC) patients using bioinformatics analysis of micro-array data (1, 2). Prevalent chromosomal alterations due to interstitial deletion or translocations result in the fusion of the androgen responsive TMPRSS2 gene promoter with Ets transcription factor coding sequences. Ets family of transcription factors ERG, Etv1 and Etv4, were shown to be involved in chromosomal alterations. Among these transcription factors, ERG was identified as being commonly fused with the TMPRSS2 gene in a majority of PC patients (1-3). Some reports suggest that the presence of fusions is associated with a poor outcome (4-6) and that specific ERG isoform expression correlates with aggressive disease characteristics (7). Other studies suggest that these chromosomal alterations alone are not associated with patient outcome, but that copy number increase of the alterations results in poor outcomes (8). Previous reports demonstrate that prostate specific overexpression of the ERG gene in transgenic mouse models results in the development of prostate intraepithelial neoplasia (PIN) without progression to carcinoma (9, 10). TMPRSS2-ERG translocations have also been identified in the low grade PIN lesions adjacent to cancer suggesting that ERG expression contributes to PIN development (11). Further, two recent reports demonstrate that ERG overexpression alone is not sufficient for prostate cancer progression; additional loss of PTEN co-operates in the development of highly invasive prostate adenocarcinoma (12, 13). Studies with patient tumor tissues confirmed *in vivo* findings that alterations in ERG and PTEN genes in prostate cancer patients results in the development of aggressive disease (14). The molecular targets related to androgen mediated activation of TMPRSS2-ERG are currently unknown; herein, we provide evidence that chemokine receptor CXCR4 is one such target of androgens in PC cells.

CXCR4 is a chemokine receptor that has been shown to function as a key receptor for homing of circulating tumor cells to secondary sites; its ligand CXCL12 is highly expressed at these metastatic sites (15-17). CXCL12/CXCR4 signaling has been shown to be involved in the adhesion, migration, invasion, and metastasis of PC cells in laboratory model systems (16, 17). We have recently shown that CXCL12/CXCR4 signaling transactivates members of the Epidermal Growth Factor Receptor (EGFR) family in membrane microdomains of prostate cancer cells, and this transactivation contributes to the expansion of intraosseous metastatic deposits (18). CXCR4 has been shown to be deregulated in tumor cells through transcriptional mechanisms. Prostate tumors and metastases express higher levels of CXCR4 compared to non tumor tissue (19-21) and this overexpression is associated with aggressive disease in patients (20, 22).

BODY:

Specific Aim 1: To test hypothesis that the ERG transcription factor promotes PC progression via regulation of CXCR4 expression and that this process is driven by androgens

1.1 Characterize the role of ERG mediated CXCR4 expression and function in PC cells:

1.1 (A) Determine androgen dependent regulation of CXCR4 promoter in prostate cancer cells.

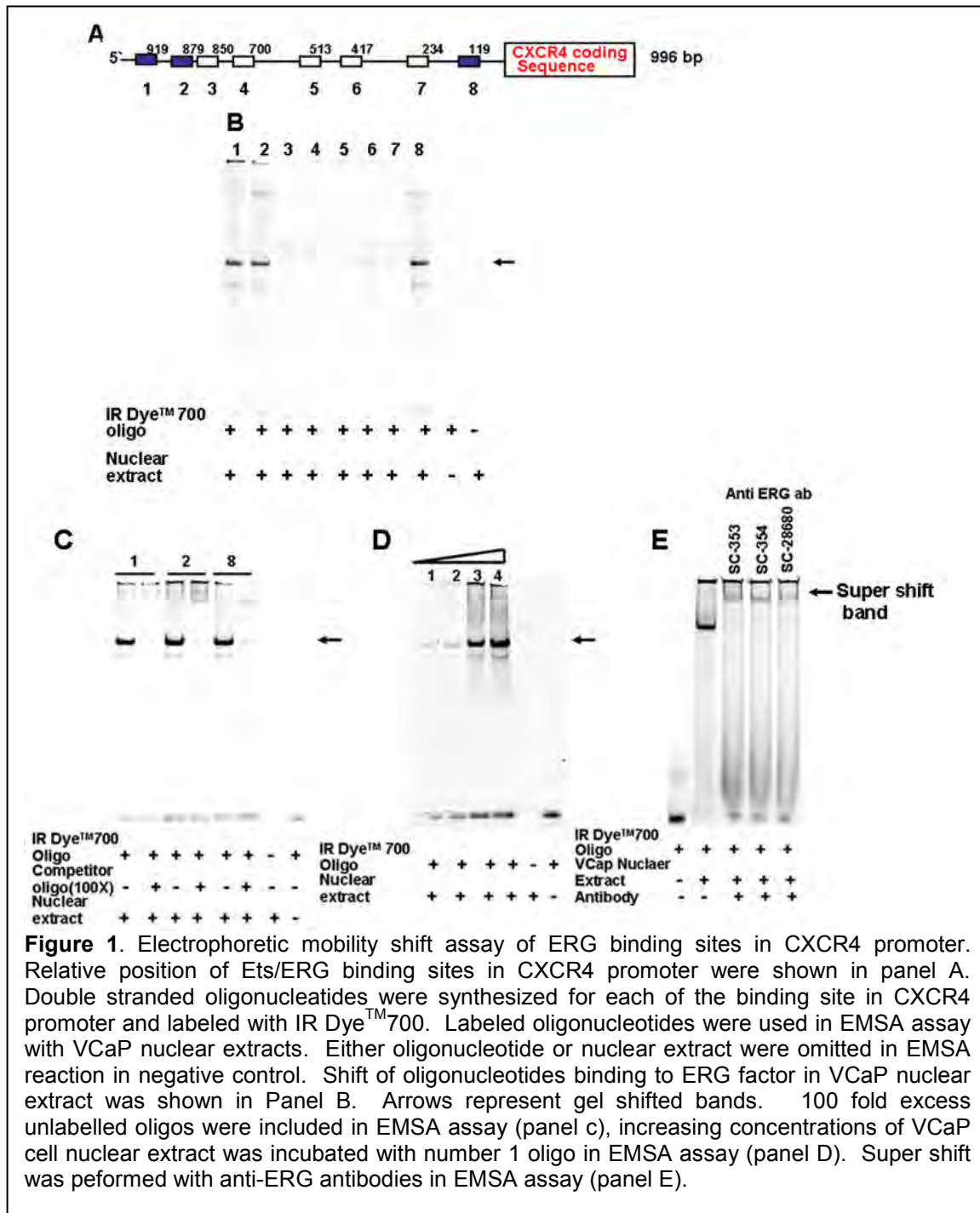
CXCR4 promoter transfection experiments: These experiments are in progress.

SiRNA mediated down-regulation of the ERG transcription factor in PC cells: These experiments are in progress.

1.1 (B) Identify which of the putative ERG binding elements activate ERG dependent CXCR4 promoter activation:

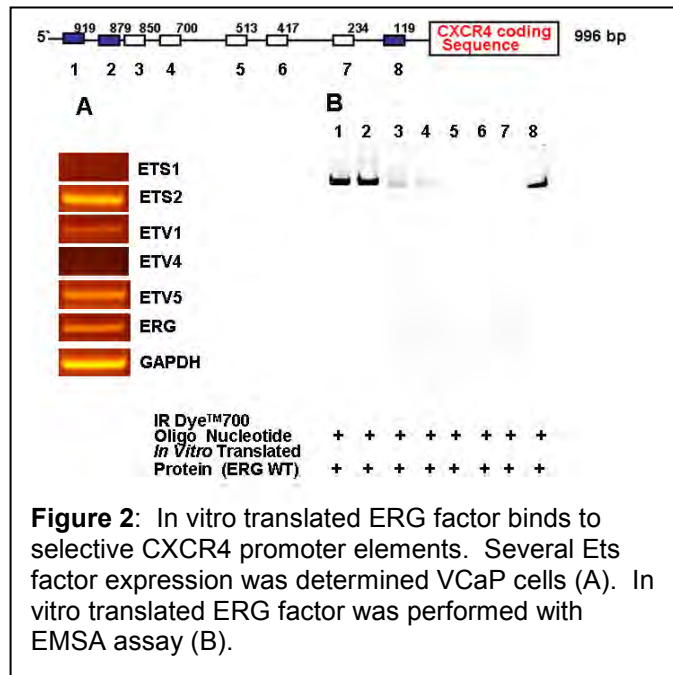
EMSA experiment with CXCR4 promoter sequences: We performed electrophoretic mobility shift assay to determine which of the putative ERG binding sites in the CXCR4

promoter binds with the ERG factor expressed in VCaP cells. Relative locations of these sites to CXCR4 coding sequence were shown in Figure 1A. Single stranded oligonucleotides were prepared and labeled with IR Dye™ 700 to each of the putative ERG site in CXCR4 promoter (IDT Inc., Skokie, IL). Single stranded nucleotides were annealed to prepare double stranded oligonucleotides. Nuclear extracts from VCaP cells were incubated with IR Dye™ 700 labeled oligonucleotides corresponding to putative ERG



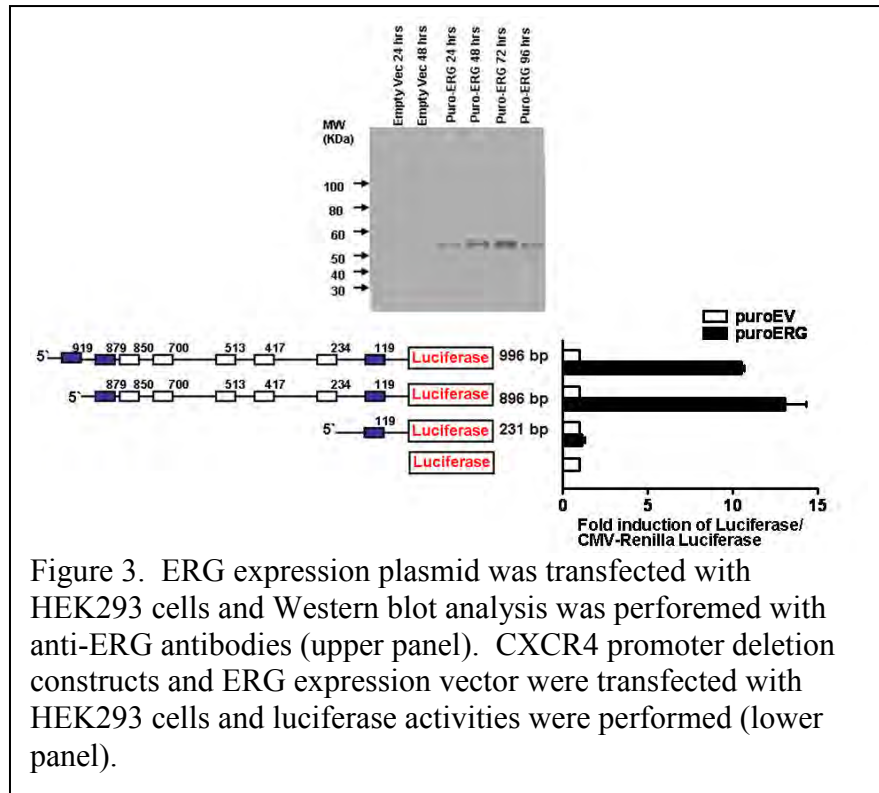
binding sites. As a negative control either nuclear extract or IR Dye™ 700 was omitted in binding reaction. Strong binding of oligonucleotides with nuclear proteins was observed with elements 1, 7, and 8 of CXCR4 promoter (Figure 1B). Specificity experiments show that excess unlabelled oligo abrogated the shifted band (Figure 1C) and increased VCaP nuclear extract protein in assay enhanced the intensity of bands (Figure 1D). Inclusion of anti-ERG antibodies super shifted the band (Figure 1E).

Several Ets family members are expressed in VCaP cells (Figure 2A) and ERG factor DNA binding sites contain a core Ets factor binding site. To rule out these possibilities and determine ERG factor binding to CXCR4 promoter sites, we cloned ERG gene into pT7CFE1-cHis (Thermoscientific, Rockford, IL) in vitro translation/transcription vector system. EMSA assay with in vitro translated ERG factor show that 1,7 and 8 elements of CXCR4 promoter was active in binding with ERG transcription factor (Figure 2B). Together this data represent that ERG factor expressed in VCaP cells selectively binds to CXCR4 promoter.



ERG factor activates CXCR4 promoter:

We have cloned 996 bp (contains 1-8 elements), 896 bp (contains 2-8 elements) and 231 bp (contains only 8th element) CXCR4 promoter fragments in pGL3 basic vector. We cloned full length ERG into pCMV-IRES-puromycin vector (Clontech). We transfected CXCR4 promoter deletion-reporter constructs and ERG expression vector into HEK293 cells and performed luciferase reporter gene assay (Figure 3). Both 996 and 896 bp CXCR4 promoters activated 10 to 15 fold in ERG transfected cells. Deletion of these two binding sites leads to abrogation of ERG induced CXCR4 promoter activation. These data demonstrate that ERG factor not only binds with upstream CXCR4 elements but also activates promoter.



1.1 (C) Determine the role of ERG factor in CXCR4 expression: These experiments are in progress.

1.1 (D) Determine the role of androgen regulated CXCR4 expression via ERG transcription factor in PC cell chemoinvasion. Completed in previous year.

1.2 Test the hypothesis that androgens regulate prostate cancer progression via ERG expression

Cloning of ERG in ARR2Pb-IRES-Luc plasmid and Transient transfection of ARR2Pb-ERG-IRES-Luc plasmid with LNCaP cells:

We have cloned full length ERG transcription factor into ARR2Pb-IRES-Luc vector and created a model system to study androgen induced ERG factor in androgen responsive cells. We transfected ARR2Pb-IRES-Luc and ARR2Pb-ERG-IRES-Luc plasmids into androgen responsive LNCaP cells. Upon R881 stimulation both ERG and CXCR4 were overexpressed in LNCaP cells (Figure 4). This data suggest that androgen induced ERG transcription factor regulates CXCR4 gene transcription via binding and activating selective promoter elements.

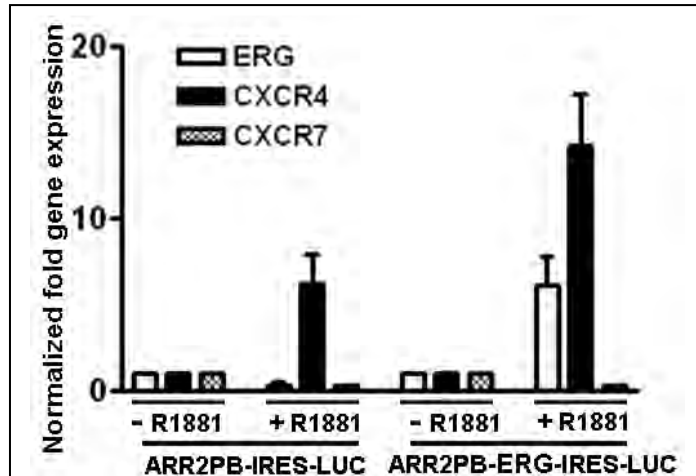


Figure : Androgens induce ERG dependent CXCR4 expression in LNCaP cells: LNCaP cells were transfected with ARR2PB-IRES-LUC and ARR2PB-ERG-IRES-LUC plasmids and treated with 5 nM of R1881. ERG, CXCR4, CXCR7 and GAPDH expression was measured with QPCR analysis. ERG, CXCR4 and CXCR7 message levels were normalized for GAPDH and R1881 induced gene expression were shown.

Specific Aim 2: To test hypothesis that CXCR4 activation promotes PC progression via transactivation of certain EGFR family members: These experiments are in progress.

KEY RESEARCH ACCOMPLISHMENTS:

1. TMPRSS2-ERG fusion protein binds and activates selective ERG factor binding sites in CXCR4 promoter.
2. Upstream ERG binding sites in CXCR4 promoter mediate CXCR4 promote activation.
3. Androgen induced ERG in express CXCR4 and functionally active CXCR4 expression.

REPORTABLE OUTCOMES:

Abstracts:

Singareddy R, St. John J and **Chinni, SR**. Molecular characterization of ERG mediated CXCR4 transcriptional regulation. 102end annual American Association for Cancer Research. Orlando, FL. April 2-6, 2011.

Chinni, SR. Singareddy R, and St. John J. Role of ERG and CXCR4 in prostate cancer progression. Innovative Minds in Prostate Cancer Today. Orlando, FL. March 9-12, 2011.

Podium Presentation:

The role of ERG and CXCR4 in Prostate Cancer Progression. Innovative Minds in Prostate Cancer Today. Orlando, FL. March 9-12, 2011.

Publication:

Cai, J., Kandagatla, P., Singareddy, R., Kropinski, A., Sheng, S., Cher, M.L., and **Chinni, S.R.** Androgens Induce Functional CXCR4 via ERG Factor Expression in TMPRSS2-ERG Fusion Positive Prostate Cancer Cells. *Translational Oncology*. 3(3):195-2003, 2010.

CONCLUSION:

Our results suggest that androgens activate CXCR4 expression in TMPRSS2-ERG fusion positive prostate cancer cells via ERG transcription factor expression. Biochemical studies indentified upstream ERG binding sites in CXCR4 promoter are active in ERG binding and transactivation of promoter. Further, androgens can induce CXCR4 gene expression via induced ERG factor expression. These studies identify a specific pathway involving selective ERG binding sites in CXCR4 promoter mediating CXCR4 gene transcription.

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APPENDICES

Molecular characterization of ERG mediated CXCR4 transcriptional regulation. Rajareddy Singareddy, Jason St. John and Sreenivasa R. Chinni

CXCR4 is a chemokine receptor, which has been shown to be expressed in several types of tumor cells, and mediate invasion and metastasis. CXCR4 expression is transcriptionally regulated in cancer cells and is associated with aggressive phenotypes of prostate cancer. Previously, we and others have shown that ERG transcription factor regulates CXCR4 expression in prostate cancer cells. In prostate cancer patients ERG is expressed via chromosomal alterations resulting in fusion of androgen responsive TMPRSS2 promoter with the coding sequence of ERG transcription factor and systemic androgens regulate ERG expression. We further show that in TMPRSS2-ERG fusion positive prostate cancer cells androgens regulate CXCR4 expression via activating ERG transcription factor expression. The CXCR4 promoter contains several putative ERG/Ets transcription factor binding sites. We hypothesize that ERG binds to selective ERG/Ets binding sites in the CXCR4 promoter and activates CXCR4 expression. Using electrophoretic mobility shift assay experiments with each of the individual ERG/Ets binding sites with VCaP cell nuclear extracts and *in vitro* translated ERG protein, sequential ERG/Ets binding site deletions in CXCR4 promoter reporters, chromatin immunoprecipitation experiments with anti-ERG antibodies in TMPRSS2-ERG positive VCaP cells, and chemoinvasion studies with VCaP cells we show that: (a) ERG expressed in VCaP cells selectively interacts with specific ERG/Ets bindings sites in the CXCR4 promoter; (b) several Ets factor family members are expressed in VCaP cells; (c) *in vitro* translated ERG binding with ERG/Ets elements in CXCR4 promoter further confirms that selective ERG/Ets sites in CXCR4 promoter are active in binding with ERG; (d) ERG binds with CXCR4 gene promoter in VCaP cells; (e) androgens regulate CXCR4 expression in TMPRSS2-ERG fusion positive cells; and (f) androgen induced CXCR4 is active in chemoinvasion of prostate cancer cells.

These data suggest that androgens regulate CXCR4 expression via ERG transcription factor expression and ERG factor may regulate CXCR4 expression by binding to the specific ERG/Ets responsive elements and activating CXCR4 transcription. These findings may provide a link between TMPRSS2-ERG translocations and enhanced metastasis of tumor cells via CXCR4 function in PC cells.

ROLE OF ERG AND CXCR4 IN PROSTATE CANCER PROGRESSION.

Chinni, SR. Singareddy R, and St. John J

TMPRSS2-ERG fusion transcripts have been shown to be expressed in a majority of Prostate Cancer (PC) patients due to chromosomal translocations or deletions between the TMPRSS2 gene promoter and the ERG gene coding sequence. The TMPRSS2 promoter contains androgen receptor binding sites, and these alterations cause androgen dependent expression of ERG transcription factor in PC patients. Overexpression of ERG factor confers growth and invasive advantage to PC cells.

We and others have shown that chemokine receptor CXCR4 expression is upregulated in PC tumor cells and its ligand CXCL12 is highly expressed in bone stromal cells. The CXCL12/CXCR4 axis functions in PC progression to enhance cell invasiveness and metastatic growth. Towards this end, we recently identified CXCL12/CXCR4 transactivation of the epidermal growth factor receptor system to be an upstream signaling pathway for PC cell invasion and metastatic growth at secondary

sites. To address the regulation of CXCR4 expression, we identified several putative ERG consensus binding sites in the promoter region of CXCR4. We hypothesized that androgen dependent regulation of the ERG transcription factor could induce CXCR4 expression in PC cells. Subsequent CXCR4 localization to raft membrane microdomain and signaling contributes to PC cell invasion and metastasis.

Using a variety of methods including RT-PCR, chromatin immunoprecipitation, Western Blot Analysis, siRNA transfection, and chemoinvasion assay, we show that (a) prostate tumor cells co-express higher ERG and CXCR4 compared to benign tissue; (b) CXCR4 expression is increased in the TMPRSS2-ERG fusion positive cell line; (c) ERG transcription factor binds to the CXCR4 gene promoter; (d) synthetic androgen (R1881) upregulates both ERG and CXCR4 in TMPRSS2-ERG fusion positive VCaP cells; (e) siRNA mediated downregulation of ERG resulted in a loss of androgen dependent regulation of CXCR4 expression in VCaP cells; (f) R1881 activated TMPRSS2-ERG expression functionally activates CXCR4 in VCaP cells; (g) CXCR4 activation in raft membrane microdomains leads to Src and EGFR family member activation. These findings identify CXCR4 as a target for androgen activated TMPRSS2-ERG fusions in PC cells. Subsequent CXCR4 function in raft membrane microdomains confers invasive and metastasis phenotype to cancer cells. Together these results may provide a link between TMPRSS2-ERG translocations and enhanced metastasis of tumor cells via CXCR4 function in PC cells.

Androgens Induce Functional CXCR4 through ERG Factor Expression in TMPRSS2-ERG Fusion-Positive Prostate Cancer Cells^{1,2}

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Abstract

TMPRSS2-ERG fusion transcripts have been shown to be expressed in a majority of prostate cancer (PC) patients because of chromosomal translocations or deletions involving the TMPRSS2 gene promoter and the ERG gene coding sequence. These alterations cause androgen-dependent ERG transcription factor expression in PC patients. We and others have shown that chemokine receptor CXCR4 expression is upregulated in PC tumor cells, and its ligand, CXCL12, is expressed in bone stromal cells. The CXCL12/CXCR4 axis functions in PC progression to enhance invasion and metastasis. To address the regulation of CXCR4 expression, we identified several putative ERG consensus-binding sites in the promoter region of CXCR4. We hypothesized that androgen-dependent regulation of the ERG transcription factor could induce CXCR4 expression in PC cells. Results of the current study show that 1) prostate tumor cells coexpress higher ERG and CXCR4 compared with benign tissue, 2) CXCR4 expression is increased in the TMPRSS2-ERG fusion-positive cell line, 3) ERG transcription factor binds to the CXCR4 gene promoter, 4) synthetic androgen (R1881) upregulates both ERG and CXCR4 in TMPRSS2-ERG fusion-positive VCaP cells, 5) small interfering RNA-mediated down-regulation of ERG resulted in the loss of androgen-dependent regulation of CXCR4 expression in VCaP cells, and 6) R1881-activated TMPRSS2-ERG expression functionally activates CXCR4 in VCaP cells. These findings provide a link between TMPRSS2-ERG translocations and enhanced metastasis of tumor cells through CXCR4 function in PC cells.

Translational Oncology (2010) 3, 195–203

Introduction

Specific chromosomal alterations were recently discovered in prostate cancer (PC) patients using bioinformatics analysis of microarray data [1,2]. Prevalent chromosomal alterations due to interstitial deletion or translocations result in the fusion of the androgen-responsive TMPRSS2 gene promoter with Ets transcription factor coding sequences. The Ets family of transcription factors ERG, ETS translocation variants 1 and 4 (ETV1 and ETV4), were shown to be involved in chromosomal alterations. Among these transcription factors, ERG was identified as being commonly fused with the TMPRSS2 gene in most PC patients [1–3]. Some reports suggest that the presence of fusions is associated with a poor outcome [4–6] and that specific ERG isoform expression correlates with aggressive disease characteristics [7]. Other studies suggest that these chromosomal alterations alone are not associated with patient outcome but that copy number increase of the alterations results in poor outcomes [8]. Previous reports demonstrate that prostate-specific overexpression

of the ERG gene in transgenic mouse models results in the development of prostate intraepithelial neoplasia (PIN) without progression to carcinoma [9,10]. TMPRSS2-ERG translocations have also been identified in the low-grade PIN lesions adjacent to cancer, suggesting that ERG expression contributes to PIN development [11]. Further, two recent

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²This article refers to supplementary material, which is designated by Table W1 and is available online at www.transonc.com.

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reports demonstrate that ERG overexpression alone is not sufficient for PC progression; additional loss of PTEN co-operates in the development of highly invasive prostate adenocarcinoma [12,13]. Studies with patient tumor tissues confirmed *in vivo* findings that alterations in ERG and PTEN genes in PC patients result in the development of aggressive disease [14]. The molecular targets related to androgen-mediated activation of TMPRSS2-ERG are currently unknown; herein, we provide evidence that the chemokine receptor CXCR4 is one such target of androgens in PC cells.

CXCR4 is a chemokine receptor that has been shown to function as a key receptor for homing of circulating tumor cells to secondary sites; its ligand CXCL12 is highly expressed at these metastatic sites [15–17]. CXCL12/CXCR4 signaling has been shown to be involved in the adhesion, migration, invasion, and metastasis of PC cells in laboratory model systems [16,17]. We have recently shown that CXCL12/CXCR4 signaling transactivates members of the epidermal growth factor receptor family in membrane microdomains of PC cells, and this transactivation contributes to the expansion of intraosseous metastatic deposits [18]. CXCR4 has been shown to be deregulated in tumor cells through transcriptional mechanisms. Prostate tumors and metastases express higher levels of CXCR4 compared with nontumor tissue [19–21], and this overexpression is associated with aggressive disease in patients [20,22].

A recent study suggests that CXCR4 is one of the functional target genes for ERG transcription factor in PC cells [12]. To determine the link between TMPRSS2-ERG translocations and CXCR4 expression in PC cells, we investigated the role of androgens in the activation of TMPRSS2-ERG and the subsequent expression of CXCR4 in PC cells. Herein, we show that PC cells that exhibit TMPRSS2-ERG fusions have androgen-regulated CXCR4 expression and that knock down of ERG abrogates androgen-induced CXCR4 expression. Furthermore, the CXCR4 promoter contains several putative ERG binding sites, and the ERG factor binds to the CXCR4 promoter in TMPRSS2-ERG-positive VCaP cells. Androgens and CXCL12 independently induced chemoinvasion of VCaP cells, and in combination, they induced chemoinvasion in an additive manner. CXCR4 inhibition studies suggest that androgen-induced CXCR4 expression is functional in TMPRSS2-ERG-positive PC cells. These studies provide an important link between TMPRSS2-ERG chromosomal translocations and androgen-induced CXCR4-mediated metastasis formation.

Materials and Methods

Cell Culture

VCaP, PC-3, and LNCaP cells were purchased from American Type Culture Collection (Manassas, VA). PC-3 and LNCaP cells were cultured in RPMI 1640 medium, and VCaP cells were cultured in Dulbecco's modified Eagle medium. All cell lines were tested for *Mycoplasma* contamination before use in the experiments with VenorGem *Mycoplasma* detection kit from Sigma Biochemicals (St Louis, MO). The culture medium was supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. R1881 was purchased from NEN Life Sciences (Waltham, MA), flutamide and cycloheximide were purchased from Sigma, and CXCL12 was purchased from Peptide (Rocky Hill, NJ).

Quantitative Polymerase Chain Reaction

A total of 4×10^5 cells were seeded in six-well plates. Each plate was treated with a single agent of an androgen agonist R1881, antagonist

flutamide, or cycloheximide or with a combination of R1881 with flutamide or cycloheximide as shown in figure legends. Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA). For reverse transcription-polymerase chain reaction (PCR) studies, first-strand complementary DNA

was synthesized from 2 μ g of total RNA with an oligo(dT) primer and SuperScript II Reverse Transcriptase (Invitrogen). Forward and reverse

primers were designed using the Primer 3 program

(http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The forward

and reverse primers are as follows: for ERG, 5'-AAC GAG CGC AGA GTT ATC GT-3' and 5'-GTG AGC CTC TGG AAG TCG TC-3'; for CXCR4, 5'-GGC CCT CAA GAC CAC AGT CA-3' and 5'-TTA GCT GGA GTG AAA

ACT TGA AG-3'; for prostate-specific antigen (PSA), 5'-GGT GAT GAC TCC AGC CAC GA-3' and 5'-GCG CAC ACA CGT CAT TGG AA-3'; and

for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-AAG GTC ATC CCT GAG CTG AA-3' and 5'-TGA CAA AGT GGT CGT TGA GG-3'.

Real-time PCR analysis was performed with SYBR Green PCR core reagents (Stratagene, La Jolla, CA) in a Stratagene Mx4000 cycler, and data analysis was performed using Mx4000 v3.01 software. All primer sets were

tested in real-time PCR and found to produce no detectable peaks in

dissociation curves due to primer-dimer amplifications. Relative message

levels were calculated with a comparative C_t (threshold cycle) method [23].

Briefly, message levels were normalized to endogenous GAPDH message levels. In treated samples, relative quantitation was performed by the

comparative C_t method [23] using the formula 2^{-C_t} , where $C_t = [C_t \text{ test}$

gene (treated sample) - C_t GAPDH (treated sample)] - [C_t test gene

(control sample) - C_t GAPDH (control sample)]. For each sample, real-

time PCR was performed in triplicate samples. C_t represents the mean C_t

value of each sample, and GAPDH is the endoge-

nous control used to normalize the quantification of a test gene.

Secondary Data Analysis for ERG and CXCR4 Expression in Human Benign Prostate and Prostate Cancer Tissue

Expression profile data sets for human benign and PC tissue were queried for ERG and CXCR4 expression using the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>). This record was deposited by Yu et al. as previously described [24]. We extracted the gene expression values for ERG and CXCR4 for benign prostate ($n = 18$) and PC tissue ($n = 65$) from GDS2546 record. ERG and CXCR4 expression values were analyzed with GraphPad Prism software version 3.0 (GraphPad, San Diego, CA).

Western Blot Analysis

Subconfluent cultures of VCaP cells were washed with phosphate-buffered saline, and total cellular proteins were extracted with buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 1 mM PMSF, and $1 \times$ Protease inhibitor cocktail (Roche, Indianapolis, IN). Protein content was quantified with a BCA protein assay (Pierce Biotechnology, Inc, Rockford, IL), and equal amounts of protein were resolved by 10% SDS-PAGE. Immunoblot was performed with antibodies to ERG (Santa Cruz Biotechnology, Santa Cruz, CA), CXCR4 (Millipore, Billerica, MA), and GAPDH (Trevigen, Gaithersburg, MD). After secondary antibody incubation, chemiluminescence reaction was performed with SuperSignal Western Femto or Pico Substrate (Pierce Biotechnology, Inc). The band intensities were determined by quantitation of pixel intensities using Un-Scan-It software (version 5.1; Orem, UT). Apparent molecular weights of ERG forms were also determined by Un-Scan-

It software using a reference position of molecular weight markers in the radiographs.

Fluorescence-Activated Cell Sorting Analysis

Fluorescence-Activated Cell Sorting Analysis (FACS) was performed on VCaP cells as previously described [17]. Briefly, VCaP cells grown in culture plates were scraped and counted on hemocytometer. A total of 5×10^5 cells were resuspended in phosphate-buffered saline supplemented with 5% fetal bovine serum and incubated with either phycoerythrin (PE)-conjugated anti-CXCR4 antibody (BD Pharmingen, San Diego, CA) or isotype-matched IgG_{2a} (BD Pharmingen) for 15 min-utes on ice. Antibody-bound cancer cells were washed three times and analyzed on fluorescence-activated cell sorter (Becton Dickinson, San Diego, CA). CXCR4-positive cells were enumerated using the cell quest software (Becton Dickinson). Data shown are percent of total gated cells that are positive for anti-CXCR4-PE antibody binding.

Chemoinvasion Assay

VCaP cells were serum-starved for 4 hours. A total of 1.5 to 2.0×10^5 cells were seeded onto inserts in the upper chamber of transwell culture plates (Becton Dickinson). Before seeding, the inserts were pre-coated with Matrigel. To activate the androgen receptor (AR), 0.5 nM of R1881 was added to the upper chamber. CXCL12 was placed in the bottom chamber for CXCR4-mediated chemoinvasion. Cell invasion was allowed to proceed for 24 hours. Later, the upper chambers were cleaned with cotton swabs to remove nonmigrated/invaded cells, and the inserts were stained with Diff-Quik stain set (Dade Behring, Inc, Newark, DE). The total number of migrated cells in a high-power field was counted under a microscope, and the data presented are based on three independent experiments.

Chromatin Immunoprecipitation

The experiment was performed using a kit from Active Motif, Inc (Carlsbad, CA). As per the manufacturer's recommendations, sub-confluent cultures of VCaP cells were fixed with 1% formaldehyde solution, sonicated to shear the chromatin, and incubated with anti-ERG or isotype IgG antibodies and protein G magnetic beads. The immunoprecipitates were washed to remove nonspecific complexes. Chromatin was then eluted by reverse cross-linking and was treated with proteinase K. PCR was performed with primers designed with 5' sequences of CXCR4 and GAPDH promoters. The forward primer 5'-GGA TCC CCA ACG CCT AGA AC-3' and reverse primer 5'-CAG CCC ATT CAG GAG GTA AA-3' were used for CXCR4, and the forward primer 5'-TAC TAG CGG TTT TAC GGG CG-3' and reverse primer 5'-TCG AAC AGG AGG AGC AGA GAG CGA-3' were used for GAPDH in the PCR. PCRs were analyzed on 2% agarose gels.

Statistical Analysis

For CXCR4 and ERG expression, the Mann-Whitney test was performed between benign and PC tissue data. The Pearson correlation test was performed for CXCR4 and ERG expression in tumor samples, and the correlation coefficient, r , was determined using GraphPad Prism software version 3.0 (GraphPad). For *in vitro* chemoinvasion study, statistical significance was determined by the nonparametric analysis of

variance test followed by the Tukey posttest to compare all pairs of a column. $P \leq .05$ was considered statistically significant.

Results

ERG and CXCR4 Coexpressed in PC Cells

CXCR4 expression in PC cells has been shown to contribute to secondary metastasis formation in bone tissue [18,25]. We identified several Ets transcription factor binding sites, including several consen-sus ERG transcription factor binding sites [26] in the promoter region of CXCR4 (Table W1). Binding of Ets factors to these sites potentially contribute to CXCR4 expression. To determine the role of TMPRSS2-ERG translocations on CXCR4 expression, we assessed ERG and CXCR4 expression in TMPRSS2-ERG fusion-positive VCaP cells, TMPRSS2-ERG fusion-negative PC-3 cells, and human prostate tumor tissues.

Secondary analysis of public domain expression array profile data of benign prostate and prostate tumor tissue [24] shows that both ERG and CXCR4 are expressed significantly higher in prostate tumor tissue samples (Figure 1A). Correlation studies with ERG and CXCR4 in prostate tumor samples show a moderate association between ERG and CXCR4 gene expressions ($r = 0.4238$ and $P < .001$). Similar ERG and CXCR4 expression patterns have been observed in the limited number of PC patient tumor tissues and the adjacent nontumor tissue available for investigation (data not shown). Together, these data suggest a concerted up-regulation of ERG and CXCR4 in tumor cells. Gene expression studies with cell lines show that both ERG and CXCR4 transcript levels are higher in VCaP cells compared with PC-3 cells (Figure 1B). Western blot analysis showed that ERG expression is not detectable in PC-3 cells, whereas in VCaP cells, ERG is expressed in two different forms. Similarly, the level of CXCR4 expression is significantly higher in VCaP cells compared with that in PC-3 cells (Figure 1C). To address whether ERG can regulate CXCR4 gene expression, we performed chromatin immunoprecipitation experiments with VCaP cells. These studies demonstrated that in VCaP cells anti-ERG antibodies immunoprecipitated ERG and CXCR4 gene promoter fragment complexes, whereas IgG failed to immunoprecipitate such complexes in VCaP cells (Figure 1D). Together, these data suggest that in TMPRSS2-ERG-positive cells, ERG and CXCR4 are highly expressed, and ERG binds with the CXCR4 promoter sequences in VCaP cells.

Androgens Regulate CXCR4 Gene through ERG Transcription Factor Expression in PC Cells

To determine whether R1881 regulation of CXCR4 gene expression was mediated through the activation of TMPRSS2-ERG fusions, gene expression studies with VCaP, LNCaP, and PC-3 cells were performed. In the absence of R1881 stimulation, CXCR4 was expressed in all cell types, whereas ERG expression was higher in VCaP cells compared with PC-3 and LNCaP cells (Figure 2A). R1881 treatment of VCaP cells induced both ERG and CXCR4 messenger RNA expression (Figure 2B). As expected, synthetic androgens induced PSA expression in AR-positive VCaP and LNCaP cells but not in PC-3 cells that lack a functional AR. Synthetic androgen treatment enhanced both ERG and CXCR4 expression in VCaP cells but not in AR-positive LNCaP cells. As expected, the antiandrogen flutamide abrogated the synthetic androgen induction of PSA in VCaP and LNCaP cells. Similarly, flutamide treatment also abrogated the androgen-induced ERG and CXCR4 expression in VCaP cells and CXCR4 expression in LNCaP cells

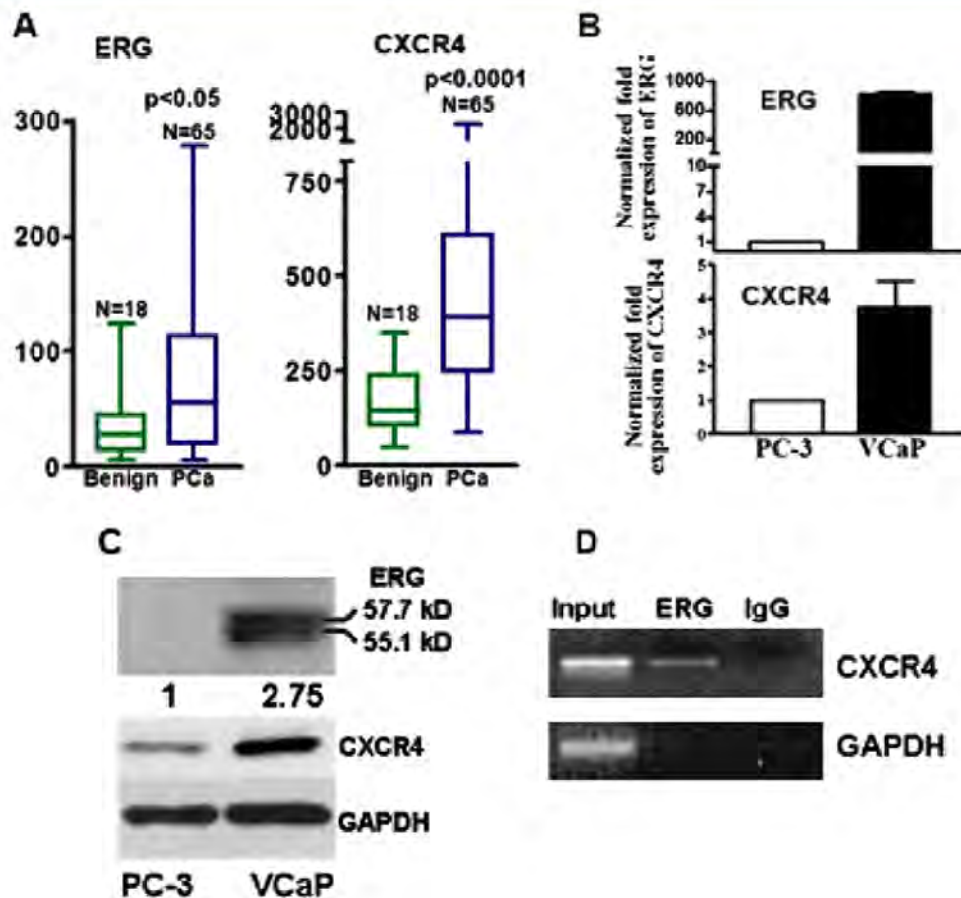


Figure 1. ERG and CXCR4 were highly expressed in TMPRSS2-ERG fusion–positive cell and prostate tumor cells, and ERG binds to CXCR4 promoter. (A) Expression array data for ERG and CXCR4 were obtained from GDS2546 record from Gene Expression Omnibus database. Mann-Whitney test was performed between samples to determine statistical significance. (B) Quantitative PCR analysis of ERG and CXCR4 genes was performed with messenger RNA prepared from PC-3 and VCaP cells. The relative expressions of genes were shown after normalization with the housekeeping gene GAPDH. (C) Total cellular proteins were isolated from PC-3 and VCaP cells and immunoblotted with anti-ERG, anti-CXCR4, and anti-GAPDH antibodies. A representative radiograph of chemiluminescence detection is shown with multiple independent Western blot analyses. Apparent molecular weights of ERG forms are shown for VCaP cells. (D) Chro-matin immunoprecipitation assay was performed with VCaP cell DNA with anti-ERG and isotype antibodies. Immunoprecipitated chro-matin was amplified with CXCR4 and GAPDH gene primers in the 5' region. Ethidium bromide–stained gel analysis of PCR-amplified DNA fragments is also shown.

(Figure 2C). The absence of androgen-induced ERG expression in LNCaP cells suggests that androgen-induced ERG transcriptionally regulates CXCR4 in VCaP cells.

To determine whether androgens regulate CXCR4 expression, we treated VCaP cells with different concentrations of the synthetic andro-gen R1881 and measured cell surface CXCR4 expression through FACS analysis. R1881 treatment upregulated cell surface CXCR4 expression, and a higher concentration of R881 further enhanced CXCR4 cell sur-face expression (Figure 3).

To further determine that CXCR4 is an indirect target of androgens, we treated VCaP cells with the translational inhibitor cycloheximide. Vehicle- and cycloheximide-treated cells were analyzed for R1881 induction of CXCR4 and PSA expression in VCaP cells. As expected, cycloheximide did not abrogate R1881 induction of PSA but inhibited the R1881 induction of CXCR4 expression in VCaP cells (Figure 4). These data imply that R1881-induced new protein synthesis is required for CXCR4 expression in VCaP cells. Together, these data support the notion that R1881 activation of TMPRSS2-ERG translocations in-duces CXCR4 expression in PC cells.

ERG Is Required for CXCR4 Gene Expression in VCaP Cells

To confirm that the androgen-induced ERG transcription factor regu-lates CXCR4 gene expression, we tested the effect of small interfering RNA (siRNA)–mediated down-regulation of the ERG gene. SiERG transfection resulted in the down-regulation of both ERG and CXCR4 gene expression compared with scrambled siRNA transfection (Fig-ure 5A). Western blot analysis show that a 60% inhibition of ERG protein expression compared with scrambled siRNA transfection (Fig-ure 5B). To assess the role of androgens in the regulation of CXCR4 expression, we treated the scrambled and siERG-transfected VCaP cells with synthetic androgens and measured the CXCR4 gene expression (Figure 5, B and C). Synthetic androgens upregulated the CXCR4 gene expression in scrambled siRNA-transfected cells but were un-able to upregulate the CXCR4 gene in siERG-transfected cells. As ex-pected, synthetic androgens upregulated PSA expression in both cells (Figure 5D). These data imply that, although PSA is not a target for R1881-induced ERG expression, CXCR4 is regulated by this mecha-nism. Taken together, these data demonstrate that the androgen-induced expression of ERG transcription factor regulates CXCR4 expression.

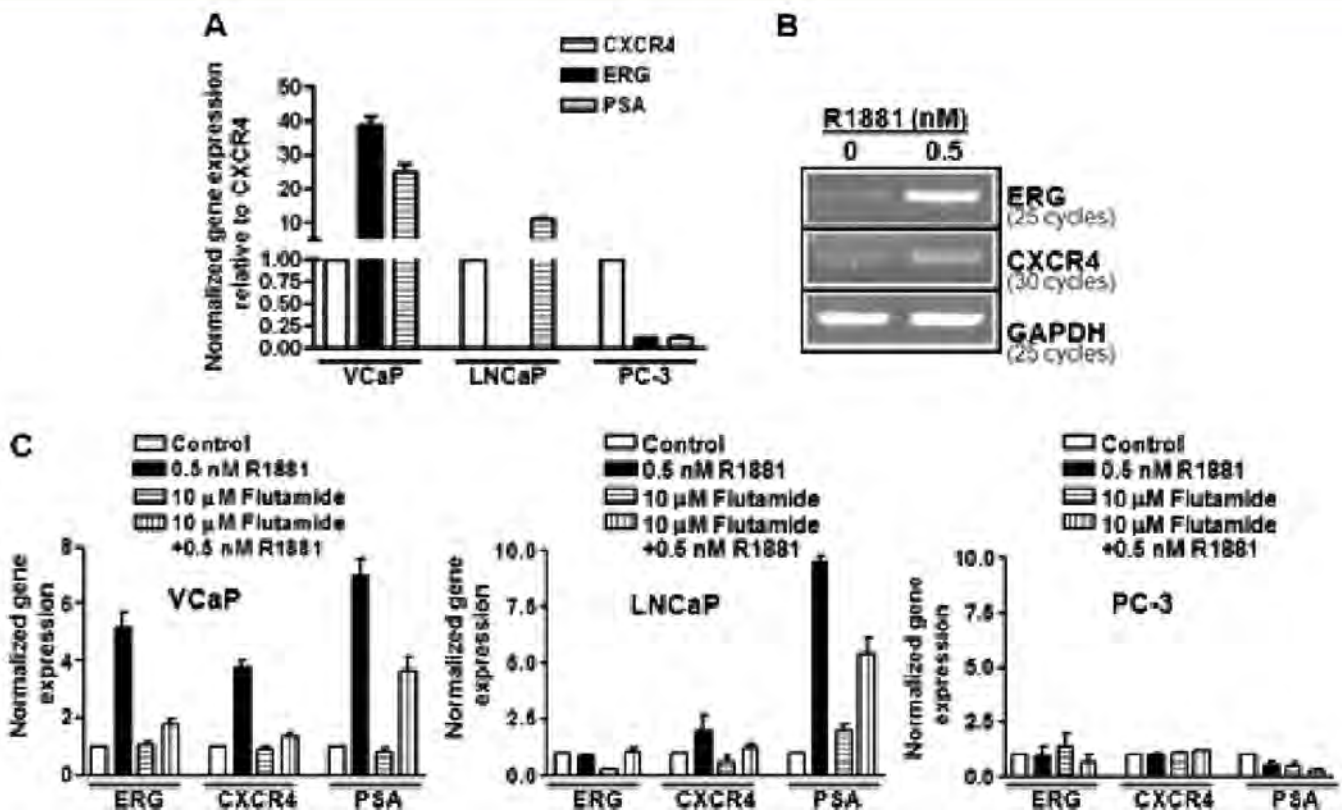


Figure 2. The synthetic androgen, R1881, induces ERG and CXCR4 expression in PC cells. (A) Relative gene expressions of ERG, CXCR4, and PSA are shown in PC-3, LNCaP, and VCaP cells. (B) R1881- and vehicle-treated VCaP cells were analyzed for ERG, CXCR4, and GAPDH gene expression. The PCR-amplified gene products were analyzed on ethidium bromide agarose gel. (C) PC-3, LNCaP, and VCaP cells treated with vehicle, R1881, flutamide, and a combination of both reagents were analyzed for ERG, CXCR4, PSA, and GAPDH gene expressions.

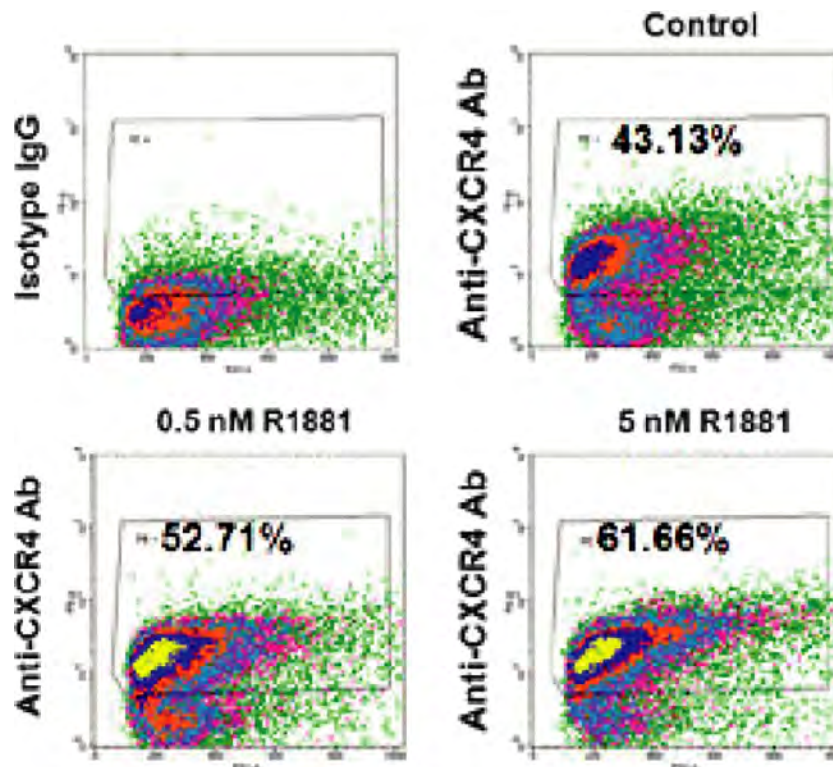


Figure 3. CXCR4 is an androgen-responsive gene in VCaP cells. VCaP cells were treated with different concentrations of R1881, and cell surface expression of CXCR4 was determined by FACS analysis.

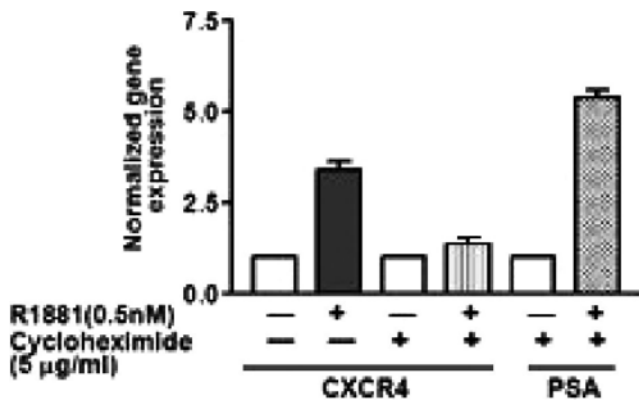


Figure 4. Cycloheximide abrogates R1881 induction of CXCR4 gene expression. VCaP cells were treated with vehicle, R1881 (0.5 nM), cycloheximide (5 µg/ml), and a combination of cycloheximide and R1881. In the combination experiment, cycloheximide was initially treated with cells, and 1 hour later, R1881 was added. CXCR4 and PSA gene expressions were determined by quantitative PCR. Fold differences in the gene expression were determined after normalization with GAPDH expression.

ERG-Mediated CXCR4 Expression Regulates PC Cell Chemoinvasion

We have previously shown that bone tissue associated CXCL12 is active in CXCR4-dependent PC cell chemoinvasion through the expression of matrix metalloproteinase-9 [17]. To assess the functional significance of ERG-mediated CXCR4 expression in VCaP cells, we

performed *in vitro* chemoinvasion assays (Figure 6A). CXCL12 induced chemoinvasion of VCaP cells, which suggests that the CXCR4 expressed in these cells was active in chemoinvasion toward the CXCL12 gradient. Treatment of VCaP cells with R1881 also induced chemoinvasion compared with that in vehicle-treated cells. Interestingly, simultaneous exposure to both agents additively enhanced chemoinvasion of VCaP cells, suggesting that androgen-induced CXCR4 is active in the chemoinvasion of VCaP cells toward a CXCL12 gradient. To determine whether R1881-induced CXCR4 enhances VCaP cell chemoinvasion, we treated VCaP cells with the CXCR4 antagonist AMD3100. AMD3100 down-regulated CXCL12/CXCR4-mediated VCaP cell chemoinvasion similar to the levels of R881 treatment (Figure 6B). Together, these data suggest that the androgen activation of TMPRSS2-ERG translocations contributes to PC cell chemoinvasion through CXCR4 expression and activation.

Discussion

Herein, we demonstrate that androgens induce CXCR4 gene expression in TMPRSS2-ERG-positive VCaP cells. To our knowledge, this is the first report identifying the CXCR4 gene as a target for TMPRSS2-ERG activation in PC cells. In this study, we show that androgen-responsive VCaP cell lines coexpress higher levels of CXCR4 and ERG compared with androgen-unresponsive PC-3 cells. ERG protein expression is absent in PC-3 cells, whereas it is expressed in two forms by VCaP cells (Figure 1B) as was previously shown by Tomlins et al. [1]. The two ERG species expressed in VCaP cells are most likely due to the alternative splicing of the fusion transcript. There is significant heterogeneity in the expression of fusion transcripts in tumor cells with TMPRSS2-ERG

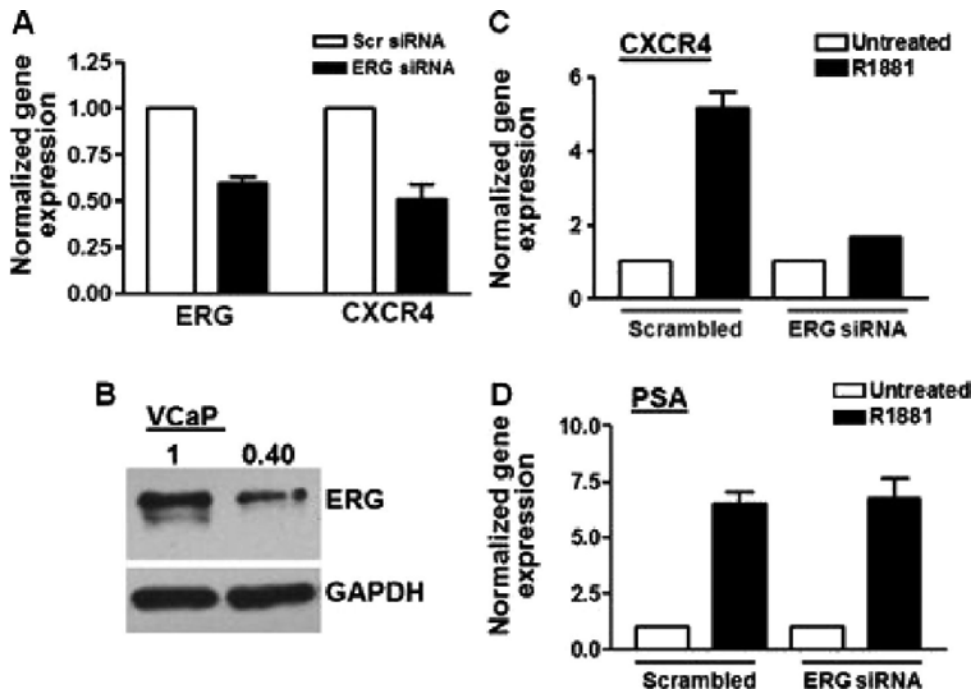


Figure 5. ERG factor mediates CXCR4 expression in VCaP cells. (A) ERG, CXCR4, and GAPDH gene expressions were determined in scrambled (Scr) and ERG siRNA-transfected VCaP cells, and ERG and CXCR4 gene expressions shown after normalization for GAPDH. (B) Scrambled and ERG siRNA-transfected VCaP cell lysates were immunoblotted with anti-ERG, anti-CXCR4 and anti-GAPDH antibodies. (C) Scr and ERG siRNA-transfected VCaP cells were treated with vehicle and R1881. Quantitative PCR analysis of CXCR4 and GAPDH was performed. (D) PSA gene expression was analyzed in scrambled and ERG siRNA-transfected VCaP cells.

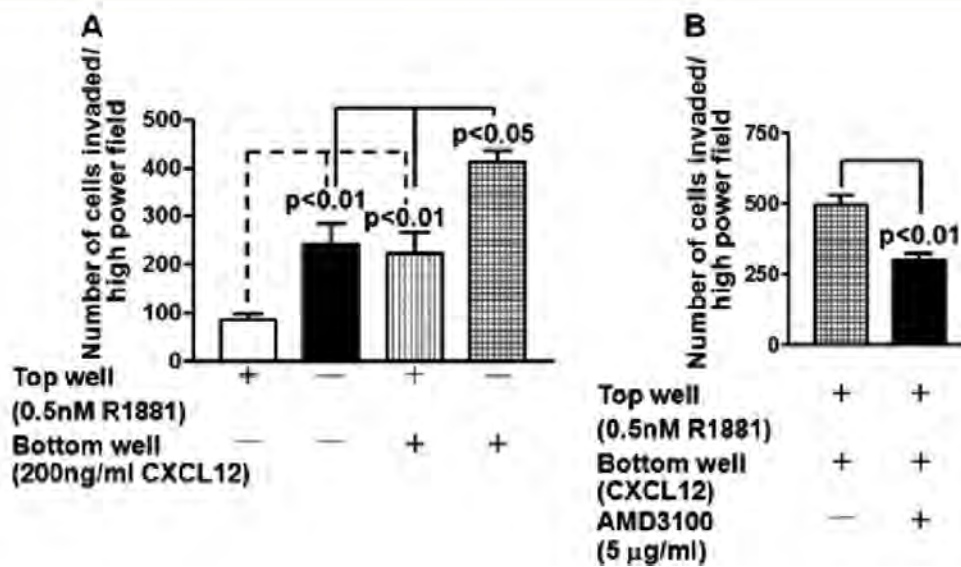


Figure 6. R1881 induced CXCR4 active in the chemoinvasion of VCaP cells: (A) VCaP cells were serum-starved, and chemoinvasion was performed using Matrigel-coated filters. CXCL12 was included in the bottom chamber as a chemoattractant. The cells were treated either with vehicle or with R1881 at 0.5 nM. (B) R1881-treated VCaP cells were further exposed to the CXCR4 antagonist AMD3100, and chemoinvasion was performed in the presence of CXCL12.

alterations [1,7,27,28], and the translation of these transcripts could give rise to several ERG species. For example, these ERG forms can lack 39 amino acids at the N-terminus [1], fusion with the first five amino acids of TMPRSS2 protein and lack the N-terminus of ERG [7], have an insertion of 24 amino acids in the central domain of ERG [29], or have a deletion of the C-terminus Ets binding domain [27]. A recent study by King et al. [13] has shown that fast migrating ERG species in the doublet has been the translation product from the first AUG codon in the fourth exon. This translation product lacks 39 N-terminal amino acids. Overexpression studies in cell culture and animal models with several of these ERG forms, with the exception of the C-terminus Ets domain deletion, demonstrate that they play a key role in PC cell proliferation [29], invasion [9,10], and progression [12,13]. Overall, the data strongly indicate that activation and alterations of TMPRSS2-ERG contribute to the lethal characteristics of PC development in patients.

Previous studies show that similar TMPRSS2-ERG deletions are present in both primary tumor cells and disseminated metastatic cells to several secondary sites [30], which suggest that downstream target genes of TMPRSS2-ERG fusions facilitate the tumor cell invasion and dissemination process. Recent studies suggest that the chemokine receptor CXCR4 in tumor cells and its ligand CXCL12 expressed in secondary metastatic sites play a key role in the metastasis of primary tumor cells [16–18,25]. Our data support the notion that TMPRSS2-ERG activation in PC cells regulates CXCR4 expression and subsequent metastasis to secondary sites.

Our study shows that the synthetic androgen upregulates the ERG expression in fusion-positive VCaP cells, which is in line with findings previously reported by Tomlins et al. [1]. Interestingly, we found that CXCR4 is regulated in parallel with ERG in a panel of PC cells. In LNCaP cells, the synthetic androgen induced a very modest degree of CXCR4 gene expression in (Figure 2C). Because LNCaP cells have very low levels of ERG in the absence of TMPRSS2-ERG translocations, this regulation could be mediated indirectly by additional AR-dependent processes. Alternatively, other Ets factors could contribute to CXCR4 expression in these cells. In support of the potential regula-

tion by other Ets factors, ETV1 has been shown to be expressed in LNCaP cells [1] and could mediate CXCR4 expression through Ets binding sites in the CXCR4 promoter. Studies are in progress to identify the specific ERG and Ets binding sites in the CXCR4 promoter. The moderate levels of CXCR4 expression induced by R1881, coupled with the presence of ETV1 in these cells, support the notion that ERG and ETV1 translocations could be mutually exclusive in prostate tumor samples and could mediate prometastatic CXCR4 gene expression, as well as the subsequent invasion and metastasis of tumor cells. In VCaP cells, androgen-induced CXCR4 expression is mediated by the overexpression of ERG rather than by the general growth effects of androgens because we did not observe changes in the CXCR4 expression in VCaP cells in the presence of serum (data not shown).

CXCR4 has been shown to be regulated at the transcriptional level by the growth factor through hypoxic [31] and nuclear factor κ B transcription factor activities [32]. In contrast with those models, our data with cycloheximide (Figure 3) suggest that androgen-mediated protein synthesis is required for CXCR4 expression in PC cells. Furthermore, such a requirement is only present in PC cells exhibiting the TMPRSS2-ERG translocations. Our analysis (data not shown) suggests that CXCR4 promoter does not contain consensus AR binding sites [33], and thus, this regulation is most likely to be mediated indirectly. Conversely, Akashi et al. [34] have shown that overexpression of AR in DU145 cells down-regulated CXCR4 expression, although it is not clear whether the overexpressed AR is active in these cells. Although the lack of an AR binding site in the CXCR4 promoter region suggests that such down-regulation could be indirectly mediated by AR activation, which would be independent of ERG function in these cells, overexpression of ERG in TMPRSS2-ERG fusion-positive cells could override these inhibitory effects on CXCR4 expression.

In agreement with our conclusions, Carver et al. [12] recently reported, while this article was in preparation, that ERG-transfected PC-3 cells have higher functional CXCR4 expression. Our data also demonstrate that CXCR4 is a target gene for the ERG transcription factor, and our results with the synthetic androgen regulation of CXCR4 (Figure 2) further

suggest that CXCR4 is a physiological target of androgens in prostate tumor cells and that this process could facilitate the pathological progression of tumor cell metastasis through the CXCL12/CXCR4 axis. Carver et al. reported that ERG binds to Ets binding sites in the -2683 to -2531 promoter of CXCR4, whereas the data from chromatin immunoprecipitation analysis in this study identified ERG binding sites in the CXCR4 promoter between the transcription start site and the -513 of the CXCR4 promoter. We identified eight potential ERG binding sites in the 1-kb CXCR4 promoter, and three of these putative sites were present in the transcription start site to the -513 of the CXCR4 promoter. Maroni et al. [35] reported that Ets1 factor binds to -397 to -412 and -478 to -481 sites in the CXCR4 promoter, suggesting that Erg could also bind to these sequences. Because ERG is under androgen control in TMPRSS2-ERG-positive cells, it is highly likely that ERG could be the relevant factor in interacting with the CXCR4 promoter in PC cells. The sequences between -513 and -996 have four potential ERG binding sites, but our attempts to design primers to amplify this region have been unsuccessful so far because of the high percentage of GC content at this region. Studies are in progress to determine the relative contribution of these putative ERG binding sites in the regulation of the CXCR4 promoter.

ERG knock down by siRNA has been shown to decrease invasive and proliferative functions in VCaP cells. Our previous data demonstrated that the CXCL12/CXCR4 axis promotes PC cell invasion through activation of signaling pathways leading to protease expression [17]. CXCR4 expression also has been shown to contribute to the growth of tumor cells in bone metastatic sites [18]. Our present data demonstrate that ERG knockdown attenuates androgen-dependent CXCR4 expression without significantly changing PSA expression. These results suggest that the TMPRSS2-ERG fusion facilitates tumor cell invasion and metastasis through the regulation of CXCR4 expression and function in PC cells. To test this concept, our data with an *in vitro* invasion assay (Figure 6) demonstrate that R1881 alone can induce VCaP cell invasion. This supports previously published reports that R1881-induced ERG expression contributes to *in vitro* invasion of VCaP cells and is mediated by the expression of proteases [9,10]. Interestingly, the R1881-treated cells invaded more efficiently in the presence of CXCL12. CXCR4 inhibition suppressed the CXCL12 effect, suggesting that androgen-induced CXCR4 expression is functional in VCaP cells and contributes to PC cell invasion. These data are in line with previous reports, demonstrating the role of ERG in CXCR4 function in PC cells [12].

In summary, we show that TMPRSS2-ERG activation in fusion-positive cancer cells induces the expression of the prometastatic gene CXCR4, which is functionally active in the chemoinvasion process. Targeting CXCR4, a relevant target for androgen activation of TMPRSS2-ERG, could be an advantageous strategy for lethal phenotypes associated with these chromosomal translocations in PC patients.

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Table W1. ETS/ERG Binding Sites in CXCR4 Promoter.

Nucleotide Position Relative to Transcription Start Site in CXCR4 Promoter	Nucleotide Sequence
-119 to -126	GAGGAAGC
-234 to -241	TAGGAAAT
-420 to -417	GCGGATGT
-513 to -520	AAGGAAGT
-700 to -707	TAGGATAA
-850 to -857	CAGGAAGT
-889 to -896	GCGGATCT
-919 to -926	CCGGAAGA
-2517 to -2525	CTGGAATT
-2664 to -2656	GGGGAATG

ETS core sequence: **GGA(A/T)**.

ERG consensus sequence: (C/A)GGAA(G/A)T.