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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 transactivates EGFR family members in in PC cells and functions in PC progression to enhance invasion and metastasis. To address the mechanism of CXCL12/CXCR4 transactivation of EGFR family members, we evaluated the location of transactivation at cellular, cell surface and lipid raft microdomains. To determine the impact of CXCL12/CXCR4 activation on initial colonization of bone tissue, we targeted CXCL12/CXCR4 axis with a small molecule CXCR4 inhibitor AMD3100. Results of the current study show that (a) CXCL12/CXCR4 transactivation of EGFR members is confined to lipid raft membrane microdomains and (b) targeting CXCL12/CXCR4 axis with AMD3100 resulted in delayed growth of bone tumors. These findings demonstrate CXCL12/CXCR4 transactivation of EGFR members in lipid raft membrane microdomains contribute to initial colonization and growth of PC cells to bone metastatic site.

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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.

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^{TM} 700 to each of the putative ERG site in CXCR4 promoter (IDT Inc., Skokie, IL). Single stranded nucleotides were annealed to prepare double stranded oligonuclotides. Nuclear extracts from VCaP cells were incubated with IR Dye^{TM} 700 labeled oligonucleotides corresponding to putative ERG binding sites. As a negative control either nuclear extract or IR Dye^{TM} 700 was omitted in binding reaction. Strong binding of oligonucleotides with nuclear proteins was observed with elements 1, 7, 8 and 9 of CXCR4 promoter. Weaker binding was observed with 2,3,4 and 10 oligonucleotides with CXCR4 promoter (Figures 1,2 in Molecular Cancer Research journal (in press) appended with report). These data demonstrate that VCaP cell expressed Ets/ERG nuclear factors interact with multiple binding sites in 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 in Molecular Cancer Research journal (in press) appended with report). 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.

Androgens regulate CXCR4 expression in VCaP cells: To determine whether androgens regulate CXCR4 expression, we treated VCaP cells with different concentrations of synthetic androgen 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 surface expression (Figure 1 and 2 in Translational Oncology manuscript 2010).

ERG mediated CXCR4 expression regulates PC cell chemoinvasion. We have previously shown that bone tissue associated CXCL12 is active in CXCR4 dependent prostate cancer cell chemoinvasion via the expression of MMP-9 (17). To assess the functional significance of ERG mediated CXCR4 expression in VCaP cells, we performed *in vitro* chemoinvasion assays (Figure 3A). CXCL12 induced chemoinvasion of VCaP cells, which suggests that the CXCR4 expressed in these cells was active in chemoinvasion towards the CXCL12 gradient. Treatment of VCaP cells with R1881 also induced chemoinvasion compared to 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 towards a CXCL12 gradient. To determine, if R1881-induced CXCR4 enhances VCaP cell chemoinvasion, we treated VCaP cells with the CXCR4 antagonist AMD3100. AMD3100 downregulated CXCL12/CXCR4 mediated VCaP cell chemoinvasion similar to the levels of R881 treatment (Figure 6 Translational Oncology manuscript 2010). Together, these data suggest that the androgen activation of TMPRSS2-ERG translocations contributes to PC cell chemoinvasion via CXCR4 expression and activation.

Specific aim 2: To test the hypothesis that CXCR4 activation promotes PC progression via transactivation of certain EGFR family members:

Identify the individual EGFR family members transactivated by CXCL12/CXCR4: To determine the cellular location of CXCL12/CXCR4 transactivation of EGFR family members, we treated PC-3 and C4-2B cells with CXCL12 and performed Western blot analysis for HER2, pHER2, EGFR and pEGFR. CXCL12 did not significantly altered total cellular HER2 or EGFR phosphorylation in in PC cells. Then, we analyzed HER2 and EGFR phosphorylation in cell surface populations with biotinylation methodology. CXCL12 did not significantly change HER and EGFR phosphorylation in cell surface expressed receptors. We analyzed HER2 and EGFR phosphorylation in lipid raft microdomains and cytosol and membrane fractions, CXCL12 induced both HER2 and EGFR phosphorylation in lipid raft membrane microdomains in PC-3 cells (Figure 1). Thus, CXCL12/CXCR4 axis in lipid raft membrane microdomains transactivate EGFR and HER2 in prostate cancer cells.

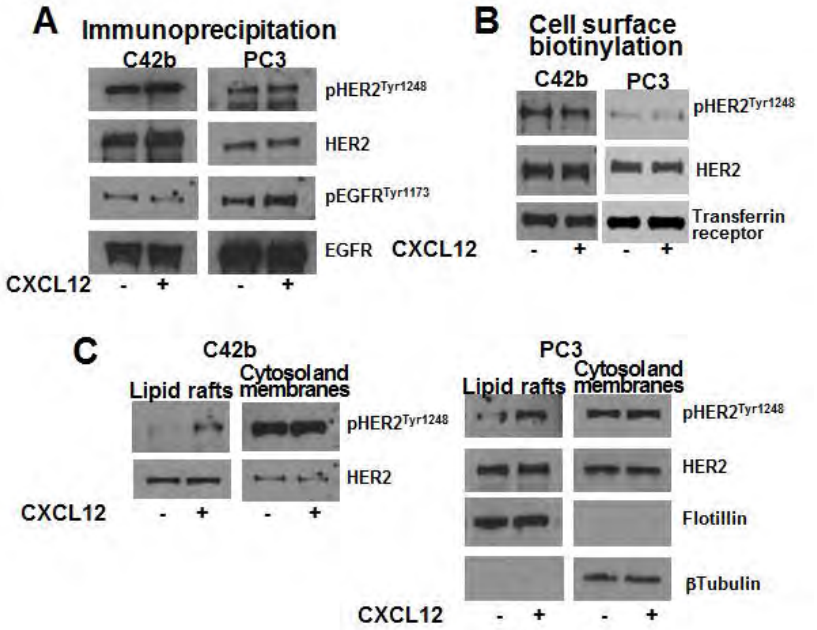

Figure 1: CXCL12/CXCR4 transactivates HER2 and EGFR in lipid raft membrane microdomains: Panel A. C4-2b and PC-3 cells were untreated or treated with CXCL12 at 200ng/ml for 15 min and total cellular lysates were immunoprecipitated with anti-HER2 and anti-EGFR antibodies and immunoblottd with pHER2^{Tyr1248}, HER2, pEGFR^{Tyr1173} and EGFR. Panel B. C4-2b and PC-3 cells were untreated or treated with CXCL12 at 200ng/ml for 15 min. Cells were biotinylated and cell extracts were affinity captured with anti-streptavidin beads and immunobolted with pHER2^{Tyr1248}, HER2 and cell surface protein marker transferrin receptor. Panel C. C4-2b and PC-3 cells were untreated or treated with CXCL12 at 200ng/ml for 15 min and lipid raft microdomains and cytosol and membrane fractions were isolated. Both fractions were immune blotted with pHER2^{Tyr1248} and HER2. In PC-3 cells both fractions were immunobloted with lipid raft marker Flotillin and cytosol marker beta Tubulin.

2.1 Intratibial model to target the CXCR4 and EGFRHER2 functions PC cells.

CXCR4 targeting delays intratibial tumor growth in PC-3 cells: We implanted PC-3 cells expressing luciferase construct and treated mice with CXCR4 inhibitor AMD3100 with a subcutaneous pump system. Tumor growth was monitored with luciferase imaging. AMD3100 treatment significantly reduced the tumor growth compared to control mice as measured by luciferase signal. X-Ray analysis of bone tumors showed osteolysis of bone tumors in untreated mice and AMD3100 treated mice show no signs of osteolysis. CXCR4 inihibitor significantly inhibited tumor growth and bone tumor osteolysis in intratibial bone tumor growth model (Figure 2).

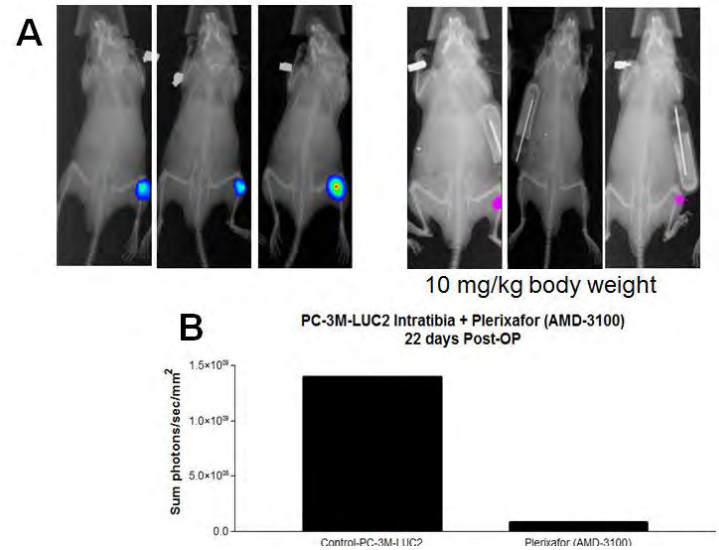

Figure 2. AMD3100 inhibits intratibial tumor growth of PC-3 cells.PC-3ML_Luc cells were implanted in tibiae of mice and treated with AMD3100. Panel A shows a representative 3 mice images of X-rays and luciferase imaging. Panel B is sum of photons which represents tumor burden in animals.

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. Androgens induce functionally active CXCR4 expression via ERG factor in TMPRSS2-ERG fusion positive cells.
4. CXCL12/CXCR4 trans activated HER2 and EGFR receptors in PC cells and lipid raft microdomains is the site for transactivation.
5. Targeting CXCL12/CXCR4 axis with AMD3100 inhibits PC bone tumor growth.

REPORTABLE OUTCOMES:

Abstracts:

Chinni, SR, Cai J, Sheng S, Kropinski A. CXCR4 is a target for androgen activated TMPRSS2-ERG fusions in prostate cancer cells. The 16th annual scientific retreat of Prostate Cancer Foundation. Lake Tahoe, NV. September 23-26 2009.

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 Metastasis. The 16th annual scientific retreat of Prostate Cancer Foundation. Lake Tahoe, NV. September 2009.

The role of ERG and CXCR4 in Prostate Cancer Metastasis. Protease group meeting, Wayne State University, Detroit, MI. October 7th, 2009.

The role of ERG and CXCR4 in Prostate Cancer Metastasis. Prostate Cancer Working Group meeting, Wayne State University, Detroit, MI. October 2nd, 2009.

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

Role of ERG and CXCR4 in Prostate Cancer Bone Metastasis. 7th Annual National Symposium on Prostate Cancer. Clark Atlanta University, Atlanta, GA. May 23-24, 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 Onclology*. 3(3):195-2003, 2010.

St. John, J*, Powell, K*, Conley-LaComb, M, and **Chinni, S.R.** *TMPRSS2-ERG* fusion gene expression in prostate tumor cells and its clinical and biological significance in prostate cancer progression. *Journal of Cancer Science and Therapy* 4(4):94-101, 2012.

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Conley-LaComb M.K, Saliganan A, Kandagatla P, Chen, YQ, Cher ML, **Chinni S.R**. PTEN loss mediated Akt activation promotes prostate tumor growth and metastasis via CXCL12/CXCR4 signaling. *Molecular Cancer*. In Press.

CONCLUSION:

Our results suggest that androgens activate CXCR4 expression in TMPRSS2-ERG fusion positive prostate cancer cells. ERG induced CXCR4 expressed in these cells is active in chemoinvasion in response to CXCL12 and sensitive to CXCR4 antagonist AMD3100. 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.

CXCL12/CXCR4 axis activates EGFR and HER2 members of EGFR family. Biochemical studies demonstrate that this transactivation is exclusively occurring in lipid raft membrane microdomains in PC cells. Targeting CXCR4 receptor with small molecule inhibitor AMD3100 show that tumor growth in bone site is inhibited. This inhibition of bone tumor growth is accompanied by reduced tumor mediated osteolysis at bone site. These studies identify that GPCR mediated activation of growth family factor receptors particularly CXCR4 transactivation of HER2 and EGFR is confined to lipid raft microdomains in PC cells and targeting the receptor activity inhibits bone tumor growth in intratibial animal model.

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APPENDICES

CXCR4 IS A TARGET FOR ANDROGEN ACTIVATED TMPRSS2-ERG FUSIONS IN PROSTATE CANCER CELLS.

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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 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 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 PC progression to enhance cell invasiveness and metastatic growth. Towards this end, we recently identified CXCL12/CXCR4 transactivation of 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. Using a variety of methods including RT-PCR, chromatin immunoprecipitation, Western Blot Analysis, siRNA transfection, and chemoinvasion assay, we show that (a) CXCR4 expression is increased in TMPRSS2-ERG fusion positive cell line; (b) ERG transcription factor binds to CXCR4 gene promoter; (c) Prostate tumor cells co-express higher ERG and CXCR4 expression compared to non tumor cells from cancer patients; (d) synthetic androgen (R1881) upregulated 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) both R1881 and CXCL12 enhanced VCaP cell chemoinvasion and a combination exposure to both reagents produced an additive effect on cellular chemoinvasion. These findings may provide a link between TMPRSS2-ERG translocations and enhanced metastasis of tumor cells via CXCR4 function in PC cells.

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.

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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 in-creased 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.

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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 altera-tions. 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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reports demonstrate that ERG overexpression alone is not sufficient for PC progression; additional loss of PTEN co-operates in the develop-ment 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 acti-vation of TMPRSS2-ERG are currently unknown; herein, we provide evidence that the chemokine receptor CXCR4 is one such target of an-drogens 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 in-duced 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 impor-tant 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 con-tamination 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% peni-cillin and streptomycin. R1881 was purchased from NEN Life Sciences (Waltham, MA), flutamide and cycloheximide were purchased from Sigma, and CXCL12 was purchased from Peprotech (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 μg 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 for-ward 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 analy-sis 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 $C_t = [C_t$ 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 rep-resents the mean C_t value of each sample, and GAPDH is the endogenous 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 con-taining 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 1 mM PMSF, and 1 × 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, subconfluent 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 lim-ited 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 ex-pression, 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 com-plexes 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 expres-sion was mediated through the activation of TMPRSS2-ERG fusions, gene expression studies with VCaP, LNCaP, and PC-3 cells were per-formed. In the absence of R1881 stimulation, CXCR4 was expressed in all cell types, whereas ERG expression was higher in VCaP cells com-pared 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

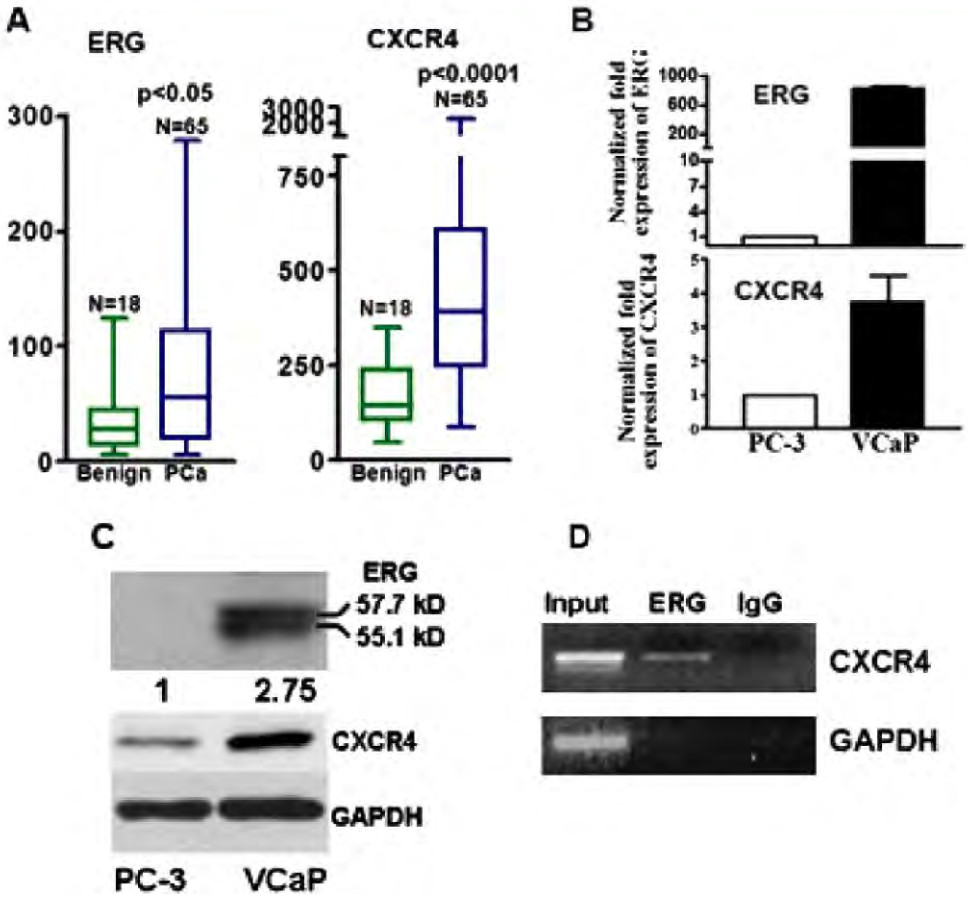

Figure 1. ERG and CXCR4 were highly expressed in TMPRSS2-ERG fusion–positive cell and prostate tumor cells, and ERG binds to CXCR4 promoter. (A) Expression array data for ERG and CXCR4 were obtained from GDS2546 record from Gene Expression Omnibus database. Mann-Whitney test was performed between samples 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 in-duction 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 regulates 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.

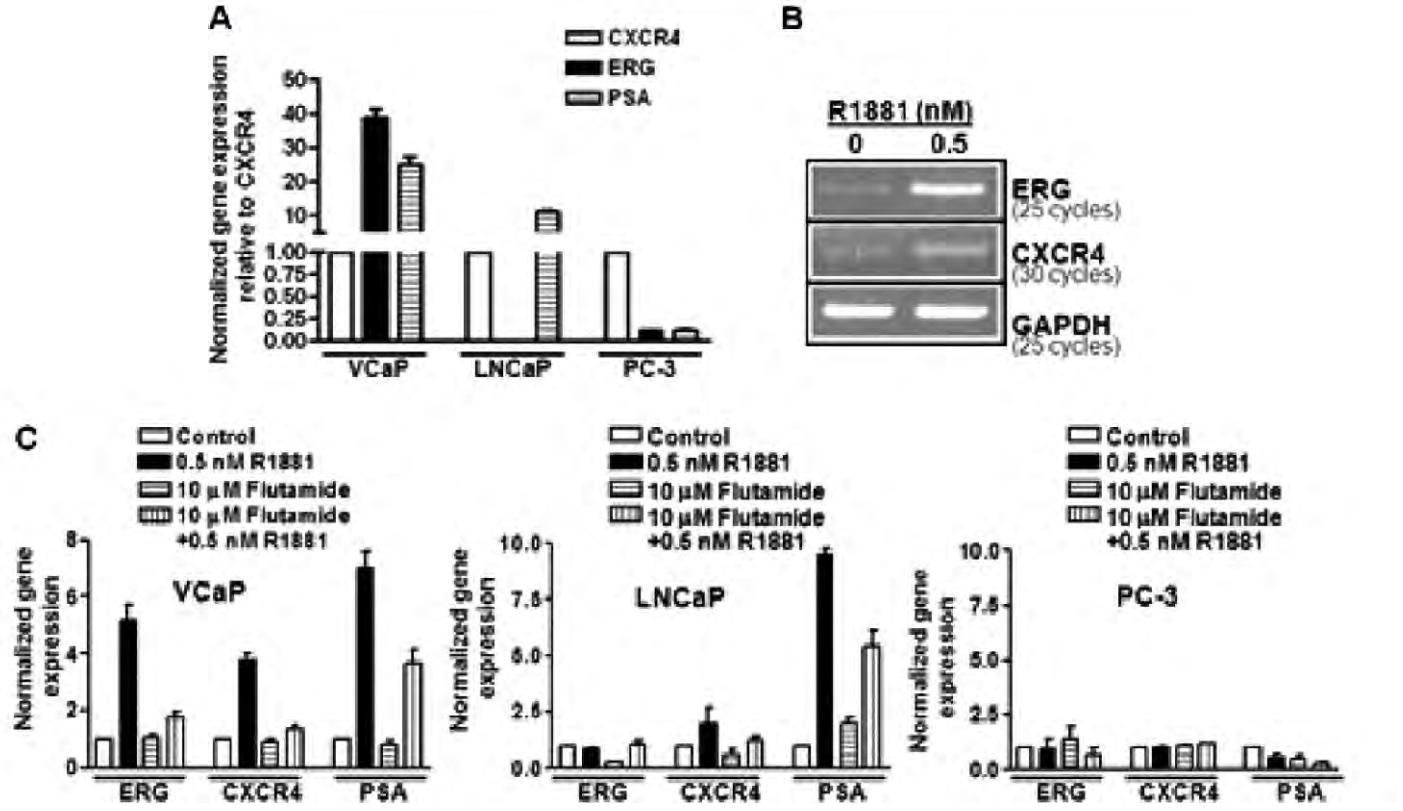

Figure 2. The synthetic androgen, R1881, induces ERG and CXCR4 expression in PC cells. (A) Relative gene expressions of ERG, CXCR4, and PSA are shown in PC-3, LNCaP, and VCaP cells. (B) R1881- and vehicle-treated VCaP cells were analyzed for ERG, CXCR4, and GAPDH gene expression. The PCR-amplified gene 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.

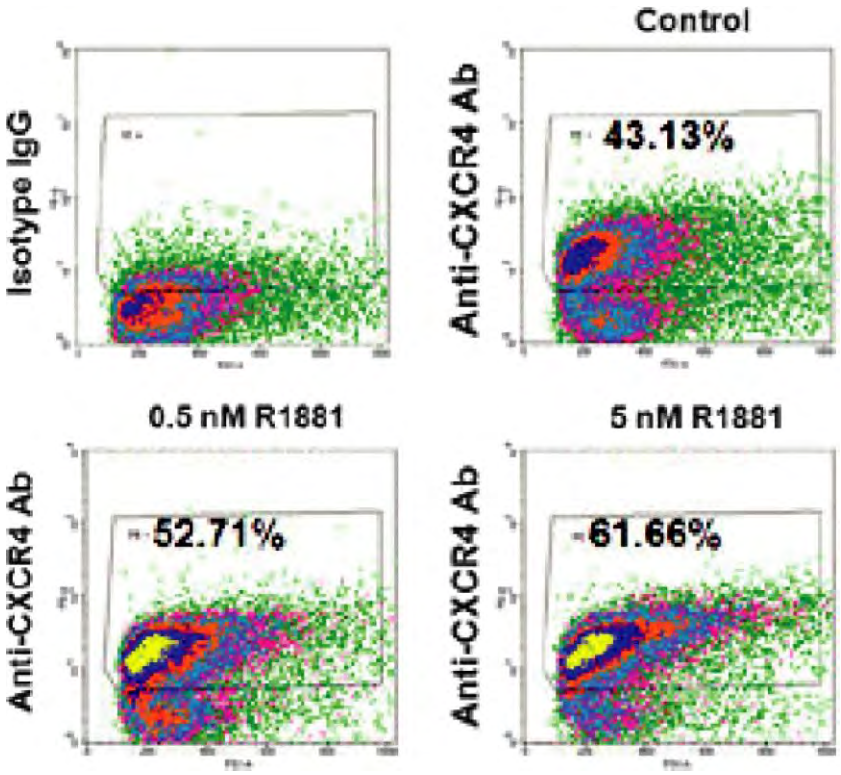

Figure 3. CXCR4 is an androgen-responsive gene in VCaP cells. VCaP cells were treated with different concentrations of R1881, and cell surface expression of CXCR4 was determined by FACS analysis.

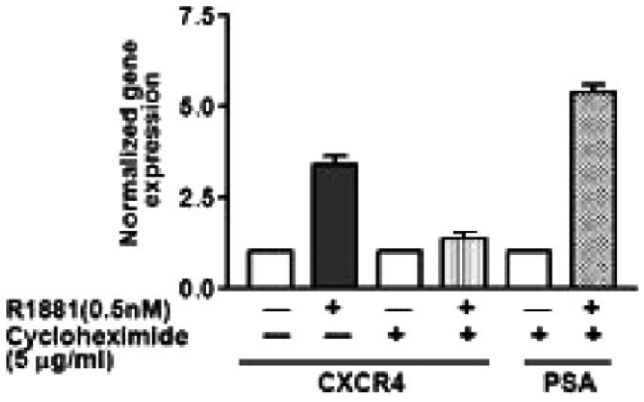

Figure 4. Cycloheximide abrogates R1881 induction of CXCR4 gene expression. VCaP cells were treated with vehicle, R1881 (0.5 nM), cycloheximide (5 μg/ml), and a combination of cycloheximide and R1881. In the combination experiment, cycloheximide was initially treated with cells, and 1 hour later, R1881 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 metallopeptidase-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 com-pared with that in vehicle-treated cells. Interestingly, simultaneous expo-sure to both agents additively enhanced chemoinvasion of VCaP cells, suggesting that androgen-induced CXCR4 is active in the chemoinva-sion 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 sug-gest 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 expres-sion 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 expres-sion 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

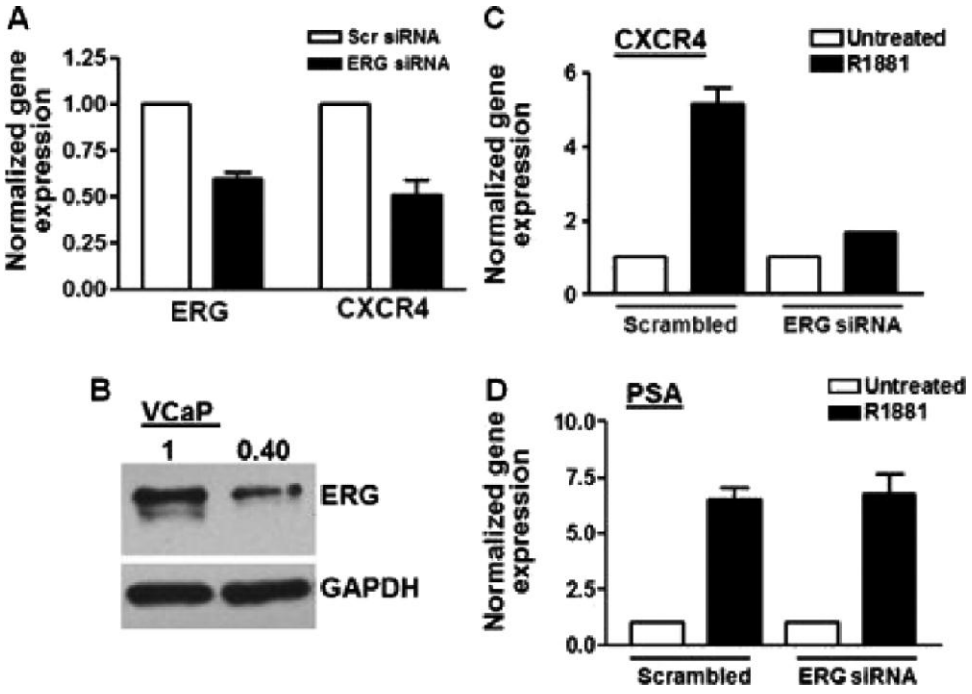

Figure 5. ERG factor mediates CXCR4 expression in VCaP cells. (A) ERG, CXCR4, and GAPDH gene expressions were determined in scrambled (Scr) and ERG siRNA-transfected VCaP cells, and ERG and CXCR4 gene expressions shown after normalization for GAPDH. (B) Scrambled and ERG siRNA-transfected VCaP cell lysates 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.

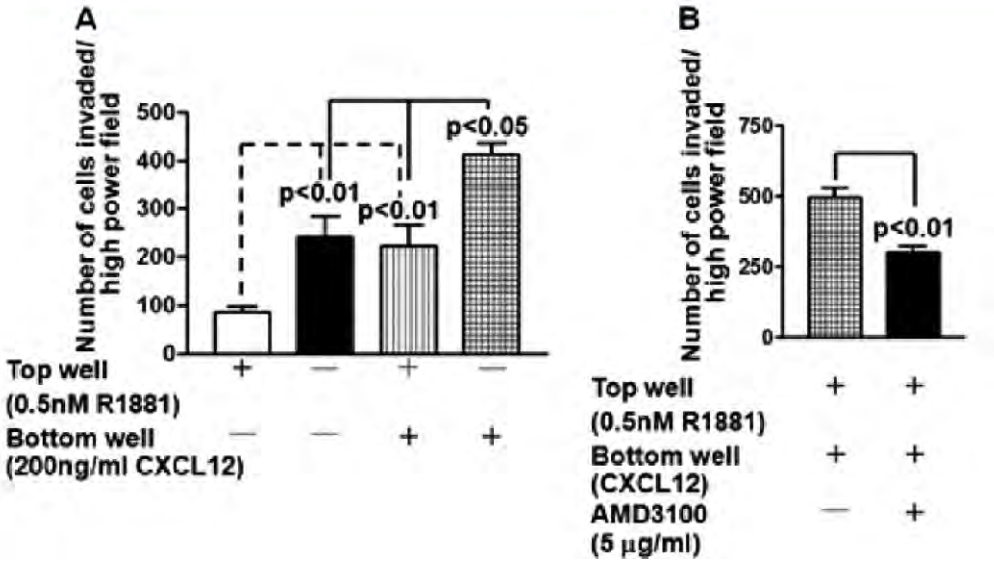

Figure 6. R1881 induced CXCR4 active in the chemoinvasion of VCaP cells: (A) VCaP cells were serum-starved, and chemoinvasion was performed using Matrigel-coated filters. CXCL12 was included in the bottom chamber as a chemoattractant. The cells were treated either with vehicle or with R1881 at 0.5 nM. (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 pres-ent 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 dis-semination 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 activa-tion 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 regulation 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 κB 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 downregulated 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 downregulation could be indirectly mediated by AR activation, which would be inde-pendent of ERG function in these cells, overexpression of ERG in TMPRSS2-ERG fusion–positive cells could override these inhibitory ef-fects 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 progres-sion 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 immunoprecip-itation 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-teracting 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 demon-strate that ERG knockdown attenuates androgen-dependent CXCR4 expression without significantly changing PSA expression. These re-sults suggest that the TMPRSS2-ERG fusion facilitates tumor cell inva-sion and metastasis through the regulation of CXCR4 expression and function in PC cells. To test this concept, our data with an in vitro in-vasion 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, suggest-ing 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. Tar-geting 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.

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Review Article Open Access

TMPRSS2-ERG Fusion Gene Expression in Prostate Tumor Cells and Its Clinical and Biological Significance in Prostate Cancer Progression

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Abstract

TMPRSS2-Ets gene fusions were identified in prostate cancers where the promoter of transmembrane protease, serine 2 (TMPRSS2) fused with coding sequence of the erythroblastosis virus E26 (*Ets*) gene family members. *TMPRSS2* is an androgen responsive transmembrane serine protease. *Ets* family members are oncogenic transcription factors that contain a highly conserved *Ets* DNA binding domain and an N-terminal regulatory domain.

Fusion of these gene results in androgen dependent transcription of *Ets* factor in prostate tumor cells. The *ERG* is the most common fusion partner with *TMPRSS2* promoter in prostate cancer patients. The high prevalence of these gene fusions, in particular *TMPRSS2-ERG*, makes them attractive as potential diagnostic and prognostic indicators, as well as making them a potential target for tailored therapies.

This review focuses on the clinical and biological significance of *TMPRSS2-ERG* fusions and their role in PC development and progression.

Keywords: TMPRSS2-ERG; Prostate Cancer; Clinical; Biological significance

Introduction

Prostate cancer (PC) is the most common form of cancer found in American men and the second leading cause of cancer death. The American Cancer Society [1] estimates that in 2012 there were 241,740 newly diagnosed cases of PC, with an estimated 28,170 men dying as a result of the disease. This means that approximately 28.5% of cancers and 3.5% of cancer related deaths in men are due to PC. Because of research that has generated improved treatments and earlier diagnosis, the five-year survival rate has significantly increased in PC patients [1]. However, the key molecular mechanisms responsible for the initiation and progression of PC remain largely unknown.

Gene fusions were known to be prevalent in liquid tumors, such as *BCR-ABL* in chronic myelogenous leukemia, but had not been identified in solid epithelial tumors. The first PC fusion genes, involving the promoter of transmembrane protease, serine 2 (*TMPRSS2*) fused with coding sequence of the erythroblastosis virus E26 (*Ets*) gene family members, were identified in 2005 by Tomlins et al. [2]. *TMPRSS2* is a prostate specific, androgen responsive, transmembrane serine protease. *Ets* family members are oncogenic transcription factors that contain a highly conserved *Ets* DNA binding region and an N-terminal regulatory domain. The *ETS* domain serves as a DNA binding recognition site, as well as a protein-protein interaction site commonly used for interactions with other transcription factors [3-5]. Therefore, the fusion of these genes leads to the production of *Ets* transcription factors under the control of the androgen sensitive promoter elements of *TMPRSS2*. This allows for a situation in which androgen-bound androgen receptor can bind these regions of *TMPRSS2*, resulting in the overexpression of *Ets* gene family members; these *Ets* members can then induce their target gene expression (Figure 1) [2]. The most common of these fusions is with *ERG* (*Ets* related gene), a member of the *Ets* family, resulting in the *TMPRSS2-ERG* fusion. The *TMPRSS2-ERG* fusion has been identified in approximately 50% of PC cases. *TMPRSS2* has also been identified in fusions with *Ets* family members *ETV1, ETV4,* and *ETV5* in PC. The prevalence of these gene fusions, in particular *TMPRSS2-ERG*, makes them attractive as potential diagnostic and prognostic indicators, as well as making them potential targets for tailored therapies.

Due to its prevalence in PC, the primary focus of this review will be on the *TMPRSS2-ERG* fusion regarding its clinical significance, biological role in PC development, and progression. This review will therefore discuss the different forms of the *TMPRSS2-ERG* fusion found in PC patients, as well as the clinical associations found between fusion positive PCs and patient outcome and disease aggressiveness will be examined. Additionally, *in vivo*, *in vitro*, and gene expression studies will be evaluated to consider the biological role of the *TMPRSS2-ERG* fusion.

TMPRSS2-ERG Fusion Gene Express Alternatively Spliced Transcript Variants in Prostate Cancer

The first two fusion genes found in PC, *TMPRSS2-ERG* and *TMPRSS2-ETV1*, were discovered in 2005 by Tomlins et al. [2]. Shortly thereafter, *TMPRSS2-ETV4* was identified [6]. Other fusions involving *Ets* family members found in PC include: *HERV_K_22q11.23-ETV1, SLC45A3-ETV1, C15orf21-ETV1, HNRPA2B1-ETV1* [7], *KLK2-ETV4, CANT1-ETV4* [8], *TMPRSS2-ETV5, SLC45A3-ETV5* [9], *SLC45A3-ERG, DDX5-ETV4, FLJ35294-ETV1* [10], and *NDRG1-ERG* [11]

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(Figure 2). These rearrangements and translocations can occur due to an intrachromosomal deletion, such as with *TMPRSS2-ERG*, as well as from an interchromosomal translocation, such as with *TMPRSS2-ETV4*. In the case of *TMPRSS2-ERG,* the rearrangement occurs either by a ~3 Mb interstitial deletion on a single copy of chromosome 21, or by a chromosomal translocation. The high prevalence of *TMPRSS2-ERG* fusions suggests that this region is a hot spot for chromosomal rearrangements in PC. Prostate cancer targeted exome sequence studies also confirm the expression of fusion genes in PC patient tissue [12]. The other 5' fusion partners of *Ets* family members are only present in a small number of PC cases and tend to be located on different chromosomes.

Wild-type *ERG* has been shown to exist as multiple different mRNA transcript variants due to alternative splicing. This alternative splicing of native *ERG* mRNA transcripts results in the expression of different isoforms of the *ERG* protein [3,13]. In accordance with this, many different splice variants of the *TMPRSS2-ERG* fusion have been identified. The differences between these variants can be found in the exons included in the transcript of that particular variant. These include T1-E4 [2], T1-E2, T4-E4, T4-E5, T5-E4 [14], T1-E5 [15], T1-E3 [16], T3-E4, T2-E2, T1-E3,5, T1-E2,3,4,6, T2-E4, T1-E6, T1-E3a4, T1-E3b4, T1-E3c4 [17], T1-E6,4 [18], T2-E5 [19] (T represents the last exon of *TMPRSS2* in the fusion; E represents the first *ERG* exon included, as depicted in Figure 3). Among the *TMPRSS2-ERG* fusion transcript variants, T1-E4 is the most common. Additionally, a variably expressed 72-bp exon has been identified that can be expressed in several of the *TMPRSS2-ERG* isoforms [13].

The *TMPRSS2-ERG* fusion proteins are most commonly expressed as N-terminal truncated *ERG* proteins, due to the loss of the 5' *ERG* native exons. The 5' *TMPRSS2* exons included in the fusion transcripts are generally non-coding and are usually not translated into protein product. However, there are several transcript variants that result in both *TMPRSS2* and *ERG* exons being translated into protein. For example, fusion protein transcript variant T2-E4 is expressed as a true fusion protein product with part of *TMPRSS2* exon 2 being expressed in the N-terminus of the fusion protein fused to N-terminal truncated *ERG* [16,17]. The DNA binding *ETS* domain and transactivation domain of the *ERG* protein are located in the C-terminus region of the protein [3]. Therefore, it is likely that, despite the N-terminal truncation, the biological functions of DNA binding and protein-protein interactions via the *ETS* domain remain intact in *TMPRSS2-ERG* fusion proteins. Due to this, it is possible that the different *ERG* fusion isoforms have similar biological functions as their wild-type *ERG* counterparts, and mediate PC progression due to overexpression. Whether novel biological functions of the *ERG* fusion isoforms lend to their oncogenic phenotype remains to be further elucidated.

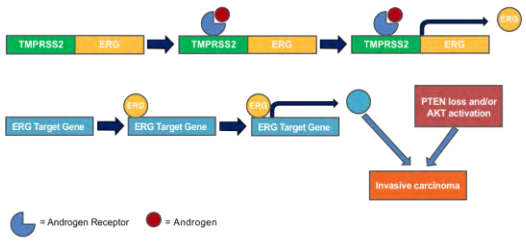

Figure 1: Biology of *TMPRSS2-ERG* gene fusions in prostate cancer. Association of bound androgen receptor with *TMPRSS2* in fusion genes results in the upregulation of *ERG* transcription (or other *Ets* gene family members, i.e. *ETV1*, *ETV4*, and *ETV5*). This over production of *ERG* can then exert its effects by binding target gene promoter regions, which results in their activation or inhibition, and the generation of a neoplastic phenotype. Known direct target genes of *ERG* in *TMPRSS2-ERG* fusion positive tissues include *MMP3*, *PLAU*, *LAMC2*, *KCNS3*, *PLA1A*, *C-MYC*, *GNMT*, *SARDH*, *CXCR4*, *ADAMTS1,TFF3*, *ERG*, *PLAT*, *MMP9*, *NDRG1*, *CUTL2*, *AR*, *KLK3 (PSA)*, *KLK2*, *SLC43A1*, *FKBP5*, *EZH2*, *ZBTB16*, *HPGD*, *ZEB1*, *SPINT1*, *IL1R2*, *PSMA*, and *OPN*. These and other up or down regulated genes in fusion positive cancers could facilitate PC progression. In addition, both *PTEN* loss and *AKT* overexpression facilitate the development of invasive carcinoma in *TMPRSS2-ERG* positive prostate tissues.

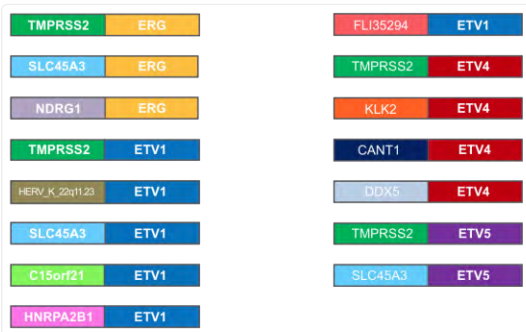

Figure 2: Additional *Ets* fusions identified in prostate cancer. Several *Ets* factors have been identified in fusion genes in prostate cancer, including *ERG*, *ETV1*, *ETV4*, and *ETV5*. In addition to *TMPRSS2*, numerous other 5' fusion partners of these *Ets* family members have been identified.

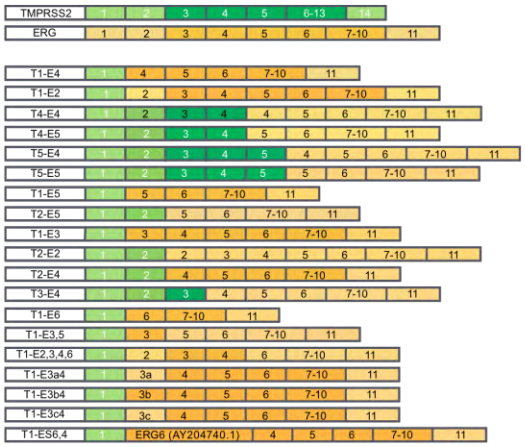

Figure 3: *TMPRSS2-ERG* fusion variants. Numerous variations of the *TMPRSS2-ERG* fusion have been identified, with the most common being T1-E4. As shown, these variants differ in the exons from *TMPRSS2* and *ERG* found in the fusion. T2-S6,4 is a variant in which the fusion is formed between exon 2 of *TMPRSS2* and 95 nucleotides that were shown to be identical to a portion of *ERG* splice form 6, which is then followed by *ERG* exon 4. The native transcripts of *TMPRSS2* and *ERG* are shown on top for reference, with *TMPRSS2* in green and *ERG* in gold. Light shading represents untranslated regions; dark shading represents open reading frame. T indicates the last exon of *TMPRSS2* in the fusion; E indicates the first *ERG* exon included.

The Role of *TMPRSS2-ERG* Fusion Genes in the Clinical Prognosis of Prostate Cancer Patients

Since the *TMPRSS2-ERG* fusion was identified in 2005, there have been conflicting reports regarding the effects of the fusion on PC development, progression, aggressiveness, and clinical outcome. Several groups found no association between *TMPRSS2-ERG* fusion positive cancers and stage, grade, Gleason score, PSA-indicated recurrence, progression, prognosis, and/or disease aggressiveness [20-28]. *TMPRSS2-ERG* fusion PCs have additionally been linked to favorable prognoses. One group found *TMPRSS2-ERG* fusion positive status to be associated with favorable outcomes for patients undergoing radical prostatectomy [29]. Another group found fusion positive tumors to be associated with better survival and lower Gleason scores [30]. Hermans et al. [31] found a novel *TMPRSS2* transcript involved in fusion that starts at an alternative site, resulting in the inclusion of what they designated to be exon 0, which when included in the fusion is found to be associated with less aggressive behavior and therefore more favorable prognosis.

Fusion status has also been linked to poor outcomes and prognostic indicators. Fitzgerald et al. [28] found copy number increases involving the fusion gene are associated with decreased survival. A study by Gopalan et al. [32] also found copy number increase of fusions generated by deletion to be linked with aggressive disease. Another group identified a similar group of fusion positive tumors, identified as 2+Edel (signifying duplicated copies of fusions generated by deletion), to be associated with only a 25% survival rate after 8 years [33]. An increase in fusion copy number is also associated with poor prognosis [34]. As mentioned above, there are also reports that have indicated fusion formed via deletion, rather than translocation, to be associated with aggressive disease. One such report, by Mehra et al. [35] found that fusion positive metastatic sites were uniformly generated via deletion. Deletion generated *TMPRSS2-ERG* fusions have also been associated with higher tumor stage and lymph node involvement in PC patients [36].

TMPRSS2-ERG fusions have also been associated with biochemical or PSA indicated recurrence [37-39]. One study found 58.4% of fusion positive, versus 8.1% of fusion negative, patients to have recurrence within 5 years of treatment [38]. Another found that those with fusion positive tumors had a 5-year recurrence rate of 79.5% compared to 37.5% for fusion negative patients [40]. Barwick et al. [37] also found *TMPRSS2-ERG* fusion positive cancers to be statistically associated with biochemical recurrence. Rostad et al. [41] found *TMPRSS2-ERG* fusions to be associated with high levels of PSA, advanced stage, and high Gleason scores. Another group also found a link between *TMPRSS2-ERG* positive tumors and high Gleason scores [42]. In addition, cancers with *TMPRSS2-ERG* fusions have been associated with high grade tumors [43], and are more prone to metastasis [44]. Another study showed that there is a link between *TMPRSS2-ERG* fusion positive tumors and prostate specific death [45].

Different *TMPRSS2-ERG* transcript variants have also been linked to poor prognostic indicators. Wang et al. [13] found a variably expressed 72 base pair region, that, when present, was found to increase proliferation, invasiveness, and mortality. This group also found T2-E4 fusion to be associated with aggressive disease [13]. It has also been found that having more full length *ERG* than truncated *ERG*, lacking the *Ets* domain, is associated with poor differentiation, higher Gleason scores, and biochemical recurrence [46]. Additionally, T1-E2, T1-E3, and T2-E4 fusions all are associated with more aggressive disease, seminal vesicle invasion, and early PSA recurrence [16].

The tumor suppressor *PTEN* (phosphatase and tensin homolog) is a critical regulator of growth factors and inhibitor of PI3K. Loss of *PTEN* is frequently observed in prostate cancer, resulting in the deregulation of cell survival, growth, and proliferation. Previous studies have found that *PTEN* is lost or mutated in 30-80% of primary prostate cancer, and 50% of prostate cancer bone metastases. Concomitant *PTEN* loss and *TMPRSS2-ERG* fusion are associated with poor outcome. Carver et al. [47] found that *PTEN* loss combined with *ERG* rearrangement are statistically independent predictors of biochemical recurrence. Another study showed that cancers with no rearranged *ERG* and normal *PTEN* expression were statistically associated with good prognoses [48]. A study by Mosquera et al. [49] also shows the *TMPRSS2-ERG* fusion to be associated with certain morphological features. These features, including cribriform growth, blue tinged mucin, macronucleoli, and collagenous micronodules, are characteristic of PC.

It seems that the majority of findings indicate that the presence of *TMPRSS2-ERG* fusion gene expression in PC patients is associated with poor clinical prognosis. Evidence suggests that different subclasses of the *ERG* fusion transcripts may yield different clinical outcomes, such as 2+Edel, T2-E4, and +72bp transcripts being associated with worse clinical outcome. If the latter is true, then future prognostic studies may reconsider grouping all *ERG* fusion transcript variants into one prognostic parameter when analyzing PC cohort samples, as this may skew statistical results. A better understanding of the biological mechanisms employed by the *ERG* fusion proteins in PC progression may help to divide large cohorts into more effective subsets for prognostic analyses. With emerging evidence suggesting the involvement of the *ERG* fusion proteins in metastatic and advanced PC, it seems likely that the presence of the fusion proteins in PC contributes to a poor clinical prognosis. However, the fact that several large cohort studies have revealed opposing results regarding the prognosis value of *TMPRSS2-ERG* fusion gene expression in PC warrants further investigation into this matter.

Role of *TMPRSS2-ERG* gene fusions in prostate cancer progression

It has been known since 2005 that gene fusions are found in the majority of PCs [2]. The role that these fusions play in the development and progression of PC is much less understood. Since the discovery of the gene fusions, several groups have identified a collection of pathways and effects associated with the overexpression of *ERG* found in *TMPRSS2-ERG* fusion positive PCs. Recent studies have revealed several of the protein-protein interactions and target genes of *ERG* fusions, lending insight into *ERG* transcriptional regulation. Many of these studies have elucidated a key involvement of *ERG* fusions with the androgen receptor (AR) in hormone naïve, androgen sensitive prostate cancers. Despite castration resistant prostate cancer (CRPC) displaying partial independence from AR regulation, the expression of *TMPRSS2-ERG* fusion proteins in castration resistant prostate cancer (hormone refractory PC) has been shown to persist.

ERG overexpression in mouse models induces prostate cancer progression

Tomlins et al. [50] was one of the first groups to study the biological effects of *TMPRSS2-ERG* fusions in PC. To this end, transgenic mice

expressing a truncated version of *ERG* (exon 2 thru the native stop codon) under the control of a probasin promoter were generated and identified as *ARR2Pb-ERG* mice. In these *ARR2Pb-ERG* mice, 3/8 developed murine prostatic intraepithelial neoplasia (mPIN) by 12-14 weeks of age. Klezovitch et al. [51] found that mice expressing high levels of *ERG* under probasin promoter control developed mPIN by five to six months of age, and mice expressing comparatively lower, but still higher than normal, levels of *ERG* developed mPIN in 10-12 months. In *ERG* overexpressing murine prostate, there was disruption of the basal cell layer when compared to benign glands, an indicator of early stages of PC development. These observations were further supported by tissue recombination studies with *ERG* overexpressing prostate epithelial cells implanted in renal capsule showing disruption of basal epithelial cells [52]. Subsequently, further studies showed that *ERG* overexpression in mouse prostate does not result in high grade PIN lesions but rather subtle changes in mice prostate epithelial cells [47,53]. These differences in observed phenotypes were attributed to the different genetic backgrounds of the mice used in these studies and different isoforms of *ERG* gene used in overexpression studies. Carver et al. [47] found that *ERG* levels in *PTEN* knockout mice to be significantly higher than in controls. This group additionally generated *PTEN* haploinsufficient mice with *ERG* expression driven by the probasin promoter. These mice developed HGPIN at approximately two months of age and multifocal adenocarcinoma by six months of age, whereas control *PTEN* heterozygous mice developed HGPIN at approximately eight months of age and did not develop adenocarcinoma [47]. Comparable results were found by King et al. [53] in a study in which *TMPRSS2-ERG* mice were crossed with *PTEN* haploinsufficient mice. All of these mice (8/8) showed PIN development by six months of age, compared with only 1/8 littermate controls. These studies demonstrate that *ERG* cooperates with *PTEN* loss leading to the development of adenocarcinoma. This observation was also supported by tissue recombinant studies, where *ERG* overexpression in combination with either *PTEN* knockdown or expression of constitutively activated Akt resulted in development of adenocarcinoma [52]. The studies considered so far have all examined *ERG* overexpression alone or in combination with other genetic alterations. Alternatively, Sun et al. [54] and Wang et al. [13] investigated the biological effects of *ERG* knockdown using *TMPRSS2-ERG* fusion positive VCaP cells in xenograft model systems. When *ERG* expression was knocked down using siRNA in VCaP cells in a xenograft model, only 2/9 of the *ERG* siRNA SCID mice developed tumors by day 42, compared to 5/5 SCID mice in the control group [54]. Wang et al. [13] similarly investigated *ERG* expression knockdown in a mouse orthotropic model using stably transfected shRNA (short hairpin RNA) VCaP cell lines. After four weeks the scrambled shRNA control animals had a luciferase signal and tumor weight 4-fold that of the *ERG* shRNA animals. Together, these xenograft studies show that *ERG* expression in VCaP cells promotes tumor growth.

The findings from these mouse studies indicate that *TMPRSS2-ERG* fusion is not, on its own, sufficient to induce the development of invasive carcinoma. However, these fusions can cause the formation of PIN lesions. These studies also indicate that *PTEN* loss and *ERG* overexpression cooperate in the formation of PIN and invasive carcinoma, indicating that *PTEN* loss could function as a "second hit" in *TMPRSS2-ERG* fusion positive PC. *TMPRSS2-ERG* fusions seem to further associate with the PI3K pathway through cooperation with active Akt, the combination resulting in the development of invasive carcinoma.

ERG overexpression in prostate cell culture models increases cell invasiveness

In addition to animal models, cell culture-based studies have also been used to elucidate the biological role of *ERG*. Knockdown of *ERG* by siRNA in VCaP cells significantly inhibits their invasiveness [13,50,54]. Blocking urokinase plasminogen activator (uPA) and plasminogen activator pathways, which have been associated with *ERG* overexpression, as well as siRNA knockdown of uPA, were also shown to significantly inhibit cellular invasiveness in RWPE, VCaP, and BPH-1 cells [50,51].

Conversely, *ERG* overexpression has also been found to have many effects in cell culture studies. *ERG* overexpression in RWPE and PrEC cells was found to significantly increase their invasion [50]. *ERG* overexpression was also shown to increase invasiveness in PNT1a cells [13]. Additionally, *ERG* overexpression in BPH-1 cells was shown to increase proliferation rates and invasiveness [51]. Proliferation rates were also shown to increase in *ERG* overexpressing PrEC cells [13]. The chemokine receptor CXCR4 increases cellular invasiveness in VCaP cells; gene expression of CXCR4 is directly upregulated in the presence of R1881 treatment via the *ERG* fusion protein binding to the CXCR4 promoter [55]. In an *in vitro* chemoinvasion assay, VCaP cells invaded through a matrigel chamber at a significantly higher rate in the presence of the CXCR4 ligand, CXCL12, as a chemoattractant. The chemoinvasion rate was enhanced further when VCaP cells were treated with synthetic androgen R1881, suggesting that AR activation and subsequent *ERG* fusion upregulation induced increased CXCR4 cell surface expression, leading to increased chemoinvasion [55]. This suggests another mechanism of enhanced cellular invasion mediated by the *ERG* fusion proteins. *ERG* overexpression in either BPH-1 or PNTla cells enhances cellular migration [13,47]. Interestingly, co-overexpression of multiple *ERG* transcript variants increases cell proliferation rates, suggesting a potential synergism between co-expressed *ERG* variants [13]. These cell culture studies indicate that *ERG* overexpression mediated by *TMPRSS2-ERG* fusion can result in increased invasiveness, cell proliferation, and cellular migration. These effects are due to the aberrant regulation of downstream target genes of *ERG*, which in turn are able to alter normal cellular activity.

ERG mediates transcriptional regulation at specific gene loci via direct protein-protein and DNA binding interactions

In vitro studies on *ERG* protein structure and interactions using co-immunoprecipitation (Co-IP) revealed that the *ETS* domain is involved in DNA binding and protein interactions. Studies showed that wild-type *ERG* proteins physically interacted through their *ETS* domain via heterodimerization with other *ETS* transcription factor family members such as Fli-1, *ETS*-2, Er81, and Pu-1 [3,5]. Different *ERG* isoforms are also involved in the formation of a ternary complex with AP1 family members Fos and Jun, forming an *ERG*/Fos/Jun complex. In the same study, *ERG* proteins were shown to physically interact via homodimerization and homo-iso-dimerization with the same isoform or with other *ERG* isoforms [3]. Another study verified that these wild-type *ERG* protein-protein interactions influenced the regulation and expression of the MMP1 and MMP3 gene promoter regions. *ERG* interacted with Fos and Jun in order to activate MMP1 promoter activity, and in contrast, *ERG* inhibited *ETS*-2-induced activation of MMP3 promoter activity [56]. As shown by Co-IP followed by mass spectrometry analysis or western blot analysis in VCaP cells and *ERG* fusion positive human prostate cancer tissues, *ERG* physically interacts with PARP1 and DNA-PKcs in a DNA independent manner and with

Ku70 and Ku80 in a DNA dependent manner. *ERG* interacts with all four proteins through its C-terminal region, and *ERG* interacts with DNA-PKcs specifically through the *ERG-ETS* domain amino acid Y373. These protein interactions mediate DNA double stranded breaks and transcriptional regulation. In this same study, PARP1, DNA-PKcs, Ku70, and Ku80 were shown via chromatin immunoprecipitation assay to bind to *ERG* target genes, and PARP1 and DNA-PKcs were required for *ERG* transcriptional activation of the gene PLA1 [57]. These results suggest that *ERG* can regulate gene transcriptional activity in a protein interaction dependent manner, which is most likely coupled with DNA binding transcriptional regulation.

ERG is associated with the aberrant expression of many genes. Several of these genes have been identified through chromatin immunoprecipitation (ChIP) assays to be direct binding targets of *ERG*. Tomlins et al. [50] used such an assay to show that *ERG* can directly bind the proximal promoter of Urokinase Plasminogen Activator (*PLAU)* and *MMP3*. ChIP has also identified the recruitment of *ERG* to the *C-MYC* promoter upstream *Ets* element [54]. This study demonstrated that *ERG* was recruited to the *PSA* enhancer and to the *prostein* promoter upstream *Ets* element, showing direct interaction of *ERG* with *PSA* expression [54]. Additionally, *ERG* binds the *GNMT* promoter and the *SARDH* promoter [58]. A ChIP assay also found that *ERG* directly binds both *CXCR4* and *ADAMTS1* promoter regions, and the expression of these two genes is upregulated in the presence of *ERG* overexpression [47]. Additionally, *ERG* binds directly to the CXCR4 gene promoter region in response to R1881 treatment [55]. In a study by Tomlins et al. [50] ChIP identified *LAMC2*, *KNCS3*, and *PLA1A* as direct *ERG* targets. *ERG* was shown to be recruited to many gene regions including, PLAU, PLAT, MMP9, NDRG1, CUTL2, AR, KLK3 (PSA), KLK2, SLC45A1, FKBP5, EZH2, and ZBTB16 [59]. *ERG* also cooperates with AR during the recruitment of *ERG* to the TFF3 gene locus [60]. One study identified *ERG* as a modulator of prostaglandin signaling and showed that *ERG* directly bound to the core gene promoter region of the HPGD gene. In this study, *ERG* siRNA in VCaP cells resulted in an increase in HPGD expression, suggesting that *ERG* negatively regulates expression of the HPGD promoter [61]. *ERG* also promotes epithelial to mesenchymal transition, and *ERG* directly binds the ZEB1, SPINT1, and IL1R2 gene regions [62]. A study by Flajollet et al. [63] revealed that *ERG* directly bound to an *ETS* binding sequence in the promoter of the osteopontin gene (OPN), which resulted in an increase in OPN gene expression. In another study, *ERG* was shown to negatively regulate the expression of the prostate specific membrane antigen (PSMA) by direct binding to the PSMA gene locus [64]. One recent study revealed an important novel finding that wild-type *ERG* gene expression is under the control of the *TMPRSS2-ERG* fusion protein. This was verified by ChIP assay showing that *ERG* bound directly to the wild-type *ERG* locus in VCaP fusion positive prostate cancers, but not in LNCaP fusion negative cancers [65]. This finding is important as it reveals a feed-forward mechanism of *ERG* overexpression in *TMPRSS2-ERG* fusion positive PC cells, further suggesting an important role of *ERG* overexpression in the progression of PC. These ChIP assays demonstrate that many genes are direct targets of *ERG*, providing a potential explanation for their aberrant regulation.

ERG and AR co-mediate transcriptional regulation of the *ERG* and AR gene loci, as well as their target genes in prostate cancer

The role of AR in *TMPRSS2-ERG* fusion positive PC cells has also been evaluated using cell culture studies. In VCaP cells, *ERG* physically interacts with AR, as verified by Co-IP. *In vitro* studies revealed that the *ERG*-AR protein-protein interaction was mediated through the *ERG-ETS* domain in a DNA independent manner. This same study revealed that *ERG* transcriptional regulation often occurred concomitantly with AR. ChIP assays demonstrate that *ERG* and AR co-occupy many androgen regulated genes, such as KLK3 (PSA) and AR. In this study, *ERG* had an inhibitory effect on AR mRNA and protein expression, as well as an inhibitory effect on the mRNA expression of several AR regulated genes, some of which were co-occupied by *ERG* and AR. *ERG* mediates its inhibitory effects via direct DNA binding and transcriptional repression of AR target genes and by protein-protein interactions with AR [59]. Another study showed that *ERG* regulated expression of TFF3 (an androgen regulated gene) via direct binding to the TFF3 gene region in hormone-naïve PC and in CRPC [60]. Interestingly, *ERG* suppressed TFF3 expression in hormone naïve prostate cancer, and induced TFF3 expression in CRPC.

It has been shown that AR positively regulates *ERG* expression and possibly *ERG* target gene expression. As mentioned previously, a study by Cai et al. [55] showed that the synthetic androgen R1881 upregulates *ERG* and CXCR4 expression in fusion positive VCaP cells. In addition, R1881 activation of *TMPRSS2-ERG* fusions functionally activates CXCR4 expression in VCaP cells [55]. These data suggest that increased CXCR4 levels following R1881 treatment are indirectly attributed to AR activation via the R1881/AR induced upregulation of *ERG*. Consistent with these results, treatment of VCaP cells with R1881 increased *TMPRSS2-ERG* expression in several other independent studies [2,64,66,67]. However, *TMPRSS2-ERG* expression was not increased after R1881 treatment in AR negative, fusion positive NCI-H660 cells [66]. As mentioned previously, treatment of androgen sensitive VCaP cells with R1881 increased both *TMPRSS2-ERG* and wild-type *ERG* transcript expression. However, only the *TMPRSS2-ERG* transcript was directly increased by AR signaling, whereas wild-type *ERG* transcript was increased indirectly via the *TMPRSS2-ERG* fusion binding directly to the wild-type *ERG* locus [65]. Interestingly, AR signaling is implicated in a causal role in the formation of the *TMPRSS2-ERG* gene fusion rearrangements, further suggesting a strong involvement of AR with *TMPRSS2-ERG* gene fusions [68-71].

Expression of the *TMPRSS2-ERG* gene fusion persists in patients with Castration Resistant Prostate Cancer

TMPRSS2 gene expression is normally under the control of AR, so whether *TMPRSS2-ERG* fusions play a role in CRPC (hormone-refractory prostate cancer) has remained a controversial topic that is currently being investigated. A study by Hermans et al. [19] showed that although *TMPRSS2-ERG* gene fusions were present in four androgen independent prostate tumor xenografts, *ERG* fusion mRNA transcript was not detected in any of these fusion positive samples. Three of the four androgen independent prostate tumor xenografts also showed little to no expression of AR mRNA transcript, with the fourth showing only a low level of AR transcript expression. Another study showed that two AR negative, hormone-refractory xenografts LuCaP49 and LuCaP93 [72,73] were also *TMPRSS2-ERG* fusion positive, but did not express the *ERG* fusion transcript [29]. Thus, it seems that with the exception of the NCI-H660 cell line, AR negative PC cells harboring the *TMPRSS2-ERG* gene fusion generally do not express *ERG* fusion transcripts.

Despite these findings, several other studies have consistently shown that *TMPRSS2-ERG* fusion gene is present and expressed in AR positive CRPC patient tumors and xenografts [74]. Cai et al. [74] showed that *TMPRSS2-ERG* gene fusion mRNA expression was present in VCaP xenografts from castrated mice as well as in 11 of 29 (38%) of CRPC patient samples. These same fusion positive CRPC patient samples also showed increased AR mRNA expression levels relative to androgen dependent prostate cancer samples. This suggests that AR may continue to play a role in *TMPRSS2-ERG* gene expression even in a CRPC state. Additionally, *ERG* mRNA expression in VCaP mouse xenografts was decreased four days after mice underwent castration surgery. However, after approximately six weeks mice experienced tumor relapse, and *ERG* mRNA expression increased above levels seen prior to the castration surgery. These same xenograft mice showed progressively increasing AR mRNA and protein levels throughout the six week relapse period, consistent with the AR overexpression seen in the CRPC patient samples [74]. Relapse is a common occurrence among prostate cancer patients who undergo androgen deprivation therapies (ADT) in the clinic, so these findings seem to closely mimic the CRPC clinical setting. At the time of relapse, this same xenograft study found that there was an upregulation in the mRNA expression of several enzymes present in the DHT biosynthetic pathway [74]. Additionally, several studies have also shown that prostate cancer cells can synthesize androgens *de novo* [75,76]. Therefore, upregulation of DHT biosynthetic enzymes and *de novo* androgen synthesis by prostate cancer cells suggests a possible mechanism in which AR signaling and *TMPRSS2-ERG* expression may be reactivated in a CRPC state, possibly contributing to ADT resistance. These findings suggest a role for *TMPRSS2-ERG* in the progression of CRPC, although the mechanism of how CRPC cells bypass ADT and continue the AR induced expression of *TMPRSS2-ERG* remains to be fully characterized. It has been suggested that *TMPRSS2-ERG* expression may eventually become androgen independent in CRPC, but no data have yet confirmed this hypothesis; further studies will be needed in order to investigate this possibility [77].

Mehra et al. [35] found that *TMPRSS2-ERG* mRNA expression was present in 10 of 27 (37%) of hormone-refractory metastatic prostate cancer patient samples. Of these 10 patients, 100% of metastatic sites contained a *TMPRSS2-ERG* fusion formed through interstitial deletion (Edel). In accordance with these findings, all metastatic tumor sites in one individual harbored the same fusion subtype, and this same fusion subtype was also found in the primary tumor site of the prostate. This suggests a mechanism of clonal selection and clonal expansion of the *TMPRSS2-ERG* fusions in metastatic fusion-positive CRPC. Attard et al. [67] found similar results, with 9 of 15 (60%) CRPC tumor samples expressing the *TMPRSS2-ERG* transcript, and circulating tumor cells (CTCs) collected from 11 CRPC patients showing the same *ERG* gene status as prostate tumor tissues from the same individual.

Many studies have revealed the presence of *TMPRSS2-ERG* transcript in CRPC; Rickman et al. [60] revealed a unique biological role of the *TMPRSS2-ERG* fusions in CRPC. In this study, 14 of 19 (73%) *TMPRSS2-ERG* fusion positive CRPC samples were found to express the *ERG* fusion transcript. Importantly, this study found that *ERG* fusions induced expression of TFF3 in CRPC, but inhibited TFF3 expression in hormone-naïve PC samples. Additionally, *ERG* fusion regulation of TFF3 was dependent on AR signaling, and overexpression of TFF3 in fusion positive CRPC resulted in an increase in cellular invasiveness. This study revealed a novel functional role of *TMPRSS2-ERG* fusion proteins in the progression of CRPC, and that the function of *ERG* fusions can change depending on the selective pressures and progressive state of the disease.

Concluding Remarks

Overall, the studies discussed here support a model (Figure 1) in which circulating androgens in PC patients activate *TMPRSS2-ERG* fusions, with the resulting *ERG* protein regulating genes whose expression and function facilitate PC progression. As mentioned above, this can increase invasiveness, cellular motility, and disease aggressiveness. In addition, it has been shown to cause the upregulation of oncogenes, as well as the downregulation of prostate differentiation genes. This, as well as the other evidence outlined above, indicates the importance of *TMPRSS2-ERG* fusions in PC. Considering the prevalence of *TMPRSS2-ERG* fusion in PC, and the large number of men who develop PC each year, the further study of these fusions is vital to the understanding of PC biology. Once better elucidated, the associations between this fusion and prognostic indicators and disease aggressiveness could give clinicians a more informed way of classifying PC for prognostic and treatment purposes. The furthered understanding of *ERG*'s biological roles in PC will augment this and potentially provide novel target pathways for future therapies, potentially through inhibition of *ERG* itself. Continued research of gene fusions, in particular *TMPRSS2-ERG*, is needed to better understand their biological roles and relationship to PC development and progression.

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Transcriptional regulation of CXCR4 in prostate tumor cells: Significance of TMPRSS2-ERG fusions. ¶

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Abstract: CXCR4 is a chemokine receptor that mediates 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 the ERG transcription factor regulates CXCR4 expression in prostate cancer cells. We further showed that androgens regulate CXCR4 expression via increasing ERG transcription factor expression. Herein, we investigated molecular mechanisms of ERG-mediated CXCR4 promoter activation, phosphorylation of ERG by intracellular kinases and subsequent CXCR4 expression, as well as expression of ERG and CXCR4 in human prostate tumor tissues. Using multiple molecular strategies, we demonstrate that: (a) ERG expressed in TMPRSS2-ERG fusion positive VCaP cells selectively binds with specific ERG/Ets bindings sites in the CXCR4 promoter; (b) distal binding sites mediate promoter activation; (c) exogenously expressed ERG promotes CXCR4 expression; (d) ERG is phosphorylated at Serine 81 and 215, both IKK and Akt kinases induce serine phosphorylation, and Akt mediates CXCR4 expression; (e) ERG-induced CXCR4 drives CXCL12-dependent adhesion to fibronectin; (f) ERG and CXCR4 were co-expressed in human prostate tumor tissues, consistent with ERG-mediated transcriptional activation of CXCR4. These data demonstrates that ERG factor activates CXCR4 expression by binding to the specific ERG/Ets responsive elements and intracellular kinases phosphorylate at ERG at serine residues to induce CXCR4 expression. These findings may provide a mechanistic link between TMPRSS2-ERG translocations and intracellular kinase mediated phosphorylation of ERG on enhanced metastasis of tumor cells via CXCR4 expression and function in prostate cancer cells.

Introduction:

TMPRSS2-ETS gene fusions are highly prevalent in prostate cancer (PC) patients, where the androgen responsive *TMPRSS2* gene promoter is fused with *ETS* transcription factor coding sequences (1). Approximately 50% of prostate cancers harbor *TMPRSS2-ETS* fusions, of which greater than 90% involve ERG factor (2). Presence of TMPRSS2-ERG fusions associate with high grade disease (3) and different subsets of rearrangements, including 2+Edel, T2-E4 and presence of 72 bp insert in ERG gene, are associated with aggressive disease characteristics (4-7).

Tumor biology studies show that oncogenic ERG overexpression along with tumor suppressor PTEN loss contributes to invasive PC development (8, 9). Clinical studies also further validate that *TMPRSS2-ERG* fusions are significantly enriched for loss of the tumor suppressor *PTEN (8)*. Several studies demonstrate that TMPRSS2-ERG fusions promote invasive phenotype of prostate cancer cells via the expression of several protease family members (6, 10) and prometastatic genes (8, 11, 12), but the underlying mechanisms related to how these genes were transcriptionally regulated are only beginning to be investigated (12). ERG has been shown to interact with other transcription factors via dimerization (13-15), and a recent study demonstrates that ERG interacts with PARP1 and DNA-PKcs to mediate target gene expression (16). ERG has been shown to regulate gene expression both positively and negatively (11, 12, 15, 17, 18); thus, understanding the molecular mechanisms of gene regulation can link ERG oncogenic transcription factor function with specific pathological functions in tumor cells. Furthermore, there has been considerable progress in mapping the ERG transcriptome in TMPRSS2-ERG fusion positive tumors, but little is known about functional aspects of ERG regulated genes in prostate cancer progression. Towards this end, recent studies have demonstrated that ERG expression regulates the expression of CXCR4, a prometastatic chemokine receptor (8, 11), which contributes to cancer progression.

CXCR4 function has been implicated as a major contributor to the cross-talk between tumor cells and the microenvironment. At the cellular level, CXCL12 with its receptor CXCR4 functions to increase tumor aggressiveness by enhancing adhesion of tumor cells to extracellular matrix components and endothelial cells (19). Tumor microenvironment interactions further activate the CXCL12/CXCR4 pathway in tumor cells and promote invasion by expression and subsequent function of several types of proteases (20-24). In PC, CXCR4 expression increases during progression; localized prostate carcinoma and bone metastasis tissue express significantly higher levels than benign prostate tissue (25, 26). Higher expression of CXCR4 was documented in prostate tumor tissues from African Americans, who often have more aggressive disease (27). CXCR4 expression in PC is also associated with poor survival (28). The CXCL12/CXCR4 axis has been shown to play an important role in PC cell proliferation, migration, and invasion (19-21, 23, 25, 29-33). We showed that CXCL12/CXCR4 signals through the PI3 kinase/Akt pathway to induce matrix metalloproteinase (MMP) expression and secretion, ultimately leading to migration and invasion of PC cells(22).

Transcriptional regulation of the CXCR4 gene is a key determinant of net cell surface expression of the CXCR4 and its subsequent metastatic function in cancer cells. Several factors and organ microenvironments have been shown to regulate CXCR4 expression in tumor cells (31, 34-41). Previous studies demonstrate that ERG factor regulates CXCR4 gene expression via androgen-induced activation of TMPRSS2-ERG fusions in PC cells (11). Herein, we show that ERG binding and activation of upstream elements in the CXCR4 promoter mediate functional CXCR4 expression.

Materials and Methods

Cell Culture and Reagents

VCaP, LNCaP, and HEK293T cells were obtained from American Type Culture Collection (Manassas, VA). VCaP cells were cultured in DMEM medium (American Type Culture Collection, Manassas, VA) with 10% regular fetal bovine serum (FBS) and 1% penicillin and streptomycin. LNCaP cells were cultured in RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA), and HEK293T cells were cultured in DMEM medium, supplemented with 10% heat inactivated FBS and 1% penicillin and streptomycin. C4-2B cells were obtained from Dr. Leland Chung (42) and maintained in T-media supplemented with 10% FBS and 1% penicillin and streptomycin. All cell lines were tested for Mycoplasma contamination before use in the experiments with VenorGeM Mycoplasma detection kit from Sigma Biochemicals (St. Louis, MO). PD 325901 (cat #P-9618), LY294002 (cat #L-7962) were obtained from LC Laboratories (Woburn, MA), BMS34551 (cat #B9935) was obtained from Sigma Aldrich (St. Louis, MO), Akt Inhibitor IV (cat #50-230-3383) was obtained from Fisher Scientific (Pittsburgh, PA), and CXCL12 (cat #300-28A) was obtained from Peprotech (Rockyhill, NJ).

CXCR4 Promoter Cloning and Luciferase Reporter Transfections

Human genomic DNA (Roche Diagnostics, Indianapolis, IN) was used for cloning 962bp CXCR4 promoter in pGL3 basic vector (Promega, Madison, WI). Forward primer, 5'-TACCCATCTCTCCGGGCTTATTTG-'3, and reverse primer, 5'-TACCCCGCAGCCAACAAACTGA-'3, were used in PCR amplification and cloned at KpnI site in pGL3 basic vector. 899bp promoter was obtained from Dr. Nakshatri, Indiana University (43) and sub-cloned into pGL3 basic vector. 231bp CXCR4promoter fragment was PCR

amplified and sub-cloned into PGL3 basic vector. HEK293T cells were transfected with either pGL3-CXCR4 plasmid containing 962bp, 899bp, and 231bp promoters or pGL3 basic vector along with either pIRES-puro (Clontech, Palo Alto, CA) or pERG-IRES-puro plasmids. Renilla luciferase vector pRL-NULL (Promega, Madison, WI) or pRL-CMV was cotransfected to serve as an internal control for normalizing transfection efficiency. Cell lysates were assayed for luciferase and Renilla luciferase activities.

ARR2-pb-ERG-Luc cloning

EF2-IRES-Luc plasmid was obtained from Dr. Alexander Kazansky, Baylor College of Medicine and used in cloning ARR2-Pb to generated ARR2-Pb-Luc plasmids. ARR2-Pb was obtained from Dr. Robert J. Matusik`s laboratory Vanderbilt Univeristy (44). ERG gene was cloned in-between ARR2-Pb promoter and IRES sequence to generate ARR2-pb-ERG-Luc.

***In Vitro* Translation of ERG**

PCR cloning method was used to clone full length ERG in pT7CFE1-CHis vector. ERG was *in vitro* transcribed and translated per manufacturer's instructions (Pierce Biotechnology, Rockford, IL). *In vitro* translated ERG protein was resolved by 9% SDS gel and immunoblotted with anti-ERG antibody (sc-28680, Santa Cruz Biotechnology Inc, Santa Cruz, CA).

ERG shRNA lentivirus infection

Six different ERG shRNA plasmids were purchased from OpenBioSystems and tested in transient transfections with VCaP cells. ERG6515 plasmid consistently downregulated ERG in two independent transfections. ERG6515 plasmid was used in preparation of lentivirus using Trans-Lentiviral ORF packaging kit (part number TLP5918) form Fisher Scientific (Pittsburgh, PA). HEK293T cells were transfected with ERG6515 shRNA plasmid and scrambled shRNA

plasmid along with virus packaging constructs as per manufacturers recommendations. Forty-eight hours post-transfection, supernatant containing viral particles were collected and used to infect VCaP cells. Forty-eight hours post-infection, VCaP cells expressing scrambled and ERG shRNA were selected with 0.25 μl/ml puromycin.

Immunoprecipitation and Western Blot Analysis

Total cellular proteins were extracted with buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 1 mM PMSF, and 1X protease inhibitor cocktail (Roche, Indianapolis, IN); for IP studies, cellular proteins were extracted in 1X RIPA buffer. Protein content was quantified with a BCA protein assay (Pierce, Rockford, IL). For immunoprecipitation, 500 μg of protein were incubated with anti-ERG antibodies (sc-28680) and protein-G agarose beads for overnight, washed with 1X RIPA buffer and resolved in 9 % SDS PAGE. For Western blot, equal amounts of protein were resolved by 9% SDS PAGE. Immunoblot was performed with antibodies against ERG (sc-28680), pTyr and pSer (9419S and 9646S, Cell Signaling Technology, Boston, MA), anti-CXCR4 antibody (Millipore, Billerica, MA) and V5 fusion antibody (P/N - 46-0708, Invitrogen, Carlsbad, CA).

Electrophoretic Mobility Shift Assay (EMSA)

VCaP cells were treated with buffer A (10mM Tris –PH: 7.8, 5mM $MgCl_2$ and 0.05% Triton X-100) for 30 min on ice, homogenized by dounce homogenizer for 20-40 strokes, and centrifuged for 20 min at 10,000 x g. The pellet containing nuclear proteins was suspended in buffer B (10mM Tris –PH: 7.8, 5mM $MgCl_2$ and 500mM NaCl), vortexed, mixed in rotary for 20 min at 4^0C, and centrifuged at 10,000 x g; supernatant containing nuclear proteins was collected. For EMSA, 2μg of protein was incubated with IR Dye^{TM}700 labeled CXCR4 promoter oligo nucleotides and binding mix (LICOR, Lincoln, Nebraska, USA). Samples were loaded on 6%

gel. EMSA competitor was performed using 100x excess oligo nucleotide in the reaction mix. For super shift assay, anti-ERG antibodies were included in the binding reaction (Cat # Sc-353, Sc-354, Sc-28680, Santa Cruz Biotechnology Inc, Santa Cruz, CA).

Quantitative Polymerase Chain Reaction

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 μg of total RNA with an oligo (dT) primer and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). 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 as described previously (11).

Fluorescence-Activated Cell Sorting Analysis (FACS)

A total of $5x10^5$ cells were suspended in phosphate-buffered saline supplemented with 5% FBS and incubated with either phycoerythrin (PE)-conjugated anti-CXCR4 antibody (BD Pharmingen, San Diego, CA) or isotype-matched IgG2a (BD Pharmingen, San Diego, CA) for 30 minutes on ice. Antibody-bound 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.

Cell Adhesion

96-well plates were coated with 5 μg/ml fibronectin, and control wells were coated with 2% BSA to determine nonspecific adhesion. $5x10^6$ PC-3 cells overexpressing Neo and CXCR4 and VCaP cells were loaded with 5 μl of Calcein AM (Molecular Probes, Inc., Eugene, OR) in a one ml volume and incubated for 30 min at 37^0C. Subsequently, cells were treated with 200 ng/ml CXCL12 as shown in figure. $6x10^4$ cells were seeded on plates and incubated for 1 hour at 37^0C

in a cell culture incubator. Non-adherent cells were removed from the plate under static condition using a static cell adhesion wash chamber (Glycotech, Rockwille, MA). Subsequently, wells were washed with HEPESCaMg buffer (50 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) under static condition five times, and cellular fluorescence was measured at 494 nm excitation and 517 nm emissions maximum.

***In vitro* migration and invasion assay**

In vitro migration and invasion assays were performed as previously described with minor modifications (21, 22). Briefly, for invasion studies scrambled and ERG shRNA infected VCaP cells were seeded on matrigel coated transwell inserts. For migration studies cells were seed on empty transwell inserts.

LC/MS/MS and Data Analysis.

ERG protein was immunoprecipitated from VCaP cells, resolved in SDS-PAGE, eluted from gel, and trypsinized to generate peptides. Peptides were either used for MS analysis or enriched with TiO_2 beads for phosphopeptides. Peptides were separated by reverse phase chromatography before introduction into a linear ion trap mass spectrometer (LTQ-XL, Thermo Scientific). A data-dependent neutral loss method was employed. For MS2, the top seven peaks from MS1 were selected for fragmentation by collision-induced dissociation with dynamic exclusion turned on (one repeat within 5 sec, then excluded for 20 sec; mass list = 200). An MS3 fragmentation event was triggered if a neutral loss of 24.5, 32.7 or 49.0 was found within the top three fragments of the MS2 spectrum. For protein identification, Proteome Discoverer (ver 1.3; Thermo Scientific) was used to prepare peak lists from MS2 and MS3 spectra that were sent to the Mascot search engine (ver 2.3; Matrix Science). Data were simultaneously searched against human sequences in the UniProtKB database and a decoy database. Mascot scores were then

imported into Scaffold (ver 3.3; Proteome Software), which incorporates the X! Tandem search engine and the PeptideProphet and ProteinProphet algorithms for probability assignment.

Immunohistochemical Analysis of CXCR4 and ERG.

Slides of 29 formalin-fixed, paraffin-embedded human prostate carcinoma specimens were obtained from the Wayne State University Pathology Research Services facility. Tissue slides were deparaffinized, and antigen retrieval was performed by steaming for 20 min in a sodium citrate buffer (BioGenex, Freemont, CA). Slides were incubated overnight at 4ºC in a humidified chamber with either anti-CXCR4 Ab (R&D Systems MAB170, 1:750 dilution), or anti-ERG Ab (Epitomics, 2805-1, 1:100). Sections were then washed twice with PBS and incubated with VECTASTAIN ABC Kit according to manufacturer's protocol, followed by incubation with 3,3'-diaminobenzadine tetrahydrochloride (DAB, Vector Labs), counterstained with Mayer's hematoxylin, dehydrated, and mounted with Permount (Fisher Scientific, Pittsburgh, PA).

Statistical Methods.

The association of ERG and CXCR4 expression in IHC samples was examined using the Mantel-Haenszel test statistic of association (Q_{cs}), which is sensitive to the ordinal categorical feature of the two coded gene variables. To measure the strength of linear association of ERG presence/absence and CXCR4 presence/absence, we calculated the Spearman rank correlation coefficient (rho), and its 90% confidence interval (CI). The 90% confidence level is appropriate for a preliminary investigation with a modest sample size (N=29). Exact statistical inference methods were used to produce exact (not asymptotic) p-values for testing the null hypothesis $Q_{cs} = 0$, and for testing the null hypothesis rho = 0. Statistical analyses and calculations were performed using the Frequency procedure in SAS 9.3 software (45). For tumor gene expression

analysis, ERG and CXCR4 expression values were analyzed in Graphpad prizm software (ver 3.0), and Pearson r values were calculated.

Results:

Identification of specific ERG binding sites in CXCR4 promoter: We and others have shown that the ERG factor regulates CXCR4 expression in prostate cancer cells (8, 11), and we have further demonstrated that androgen-mediated activation of TMPRSS2-ERG fusions enhances CXCR4 expression in fusion positive VCaP cells (11). Analysis of the CXCR4 promoter reveals eight putative Ets/ERG binding sites spanning between -919 to -119 upstream of transcription start site (Figure 1A) (11). To determine whether ERG directly regulates CXCR4 gene via the binding and activation of these putative Ets/ERG binding sites, we performed electrophoretic mobility shift assay (EMSA) with IR Dye labeled oligos with each individual Ets/ERG binding site. EMSA data show that VCaP cell nuclear proteins bind with -919, -879 and -119 Ets/ERG binding sites (Figure 1B). Specificity of this binding was tested with competition binding assay; where 100 fold excess unlabeled oligos in assay abrogated VCaP nuclear protein binding to these sites (Figure 1C). Further, higher amounts of nuclear proteins in EMSA showed enhanced binding (Figure 1D).

Previous data demonstrate that VCaP cell nuclear extracts are active in binding to Ets/ERG factor binding sites in CXCR4 promoter oligos. To determine the expression of other Ets family members in VCaP cells, RT-PCR analysis was performed. Analysis of other Ets members' expression in VCaP cells show that Ets2, ETV1, ETV5 and Fli-1 are expressed in addition to ERG (Figure 2A). To determine whether ERG binds to CXCR4 promoter oligos, ERG was sub-cloned into an *in vitro* translation vector, and ERG protein was prepared using an *in vitro* translation system (Figure 2B). EMSA analysis of *in vitro* translated ERG with CXCR4 promoter oligos show that -919, -879, and -119 Ets/ERG binding elements in CXCR4 promoter are active in binding (Figure 2C and D). To determine whether VCaP cell expressed ERG binds to these elements, EMSA assay was performed using anti-ERG antibodies with VCaP nuclear

extracts. The results show a supershift of ERG with CXCR4 promoter oligos in the presence of anti-ERG antibodies (Figure 2E). Further, the reciprocal experiment was performed, where -919 and -879 sites were mutated (Supplementary figure 1A). The results showed that mutation at these sites abrogated the VCaP cell nuclear protein binding to oligos (Supplementary Figure 1B). The data suggest that these three Ets/ERG binding sites (-919, -879, and -119) are required for ERG binding to CXCR4 promoter using VCaP nuclear extracts. ChIP seq analysis of immunoprecipitated ERG in VCaP cells (46) show that ERG interacts with CXCR4 promoter (Supplementary Figure 2)

ERG activates CXCR4 promoter: To determine the significance of ERG binding to -919, -879 and -119 Ets/ERG binding elements in CXCR4 promoter, promoter luciferase activation experiments were utilized. The 962bp CXCR4 promoter was cloned from human genomic DNA into pGL3 basic vector containing luciferase reporter gene. Additionally, deletion constructs lacking one or more elements of Ets/ERG binding sites were sub-cloned, generating 899 and 231bp CXCR4 promoter fragments. Transfection of ERG into HEK293 cells resulted in expression of ERG protein (Figure 3A). To determine the role of ERG mediated transactivation of CXCR4 promoter, ERG and each individual CXCR4 promoter luciferase constructs were co-transfected into HEK293 cells. ERG activated 962 and 896 bp CXCR4 promoter luciferase constructs by 10-15 fold, with a greater effect on the 896 bp construct. Deletion of both upstream Ets/ERG binding sites abrogated ERG-induced CXCR4 promoter activation (Figure 3B and C). These results suggest that -919 and -879 Ets/ERG binding elements are sufficient for ERG mediated CXCR4 promoter activation.

TMPRSS2-ERG fusions transcripts undergo alternate splicing and produce different isoforms. Major isoforms expressed in patient tumors produce either full length ERG or a form that is lacking the N-terminal 39 amino acids; the latter form is predominantly expressed in

tumor tissues. A C-terminal truncation lacking the DNA binding domain has been also reported to be expressed patient tumor tissues (47). We determined the effect of C- and N-terminus truncations of ERG in regulating CXCR4 promoter activation. Full length ERG as a native or C-terminus V5 fusion, and N-terminus and C-terminus truncations as V5 fusions were cloned and expressed in HEK293 cells (Supplementary Figure 3A). Transfection of 962 and 896 promoter constructs with different forms of ERG factor show that full length and N-terminus mutants activated CXCR4 promoter constructs to similar levels. The C-terminus ERG truncation resulted in loss of CXCR4 promoter transactivation (Supplementary Figure 3B and C). Both native and V5 fusions activated the CXCR4 promoter to similar levels, suggesting that the addition of the V5 tag did not alter the ERG function in transactivating the CXCR4 promoter.

ERG regulates CXCR4 gene expression: To determine whether the ERG binding and activation of CXCR4 promoter can induce CXCR4 expression, we transfected the ERG expression vector into LNCaP cells, which lack TMPRSS2-ERG fusions, and measured CXCR4 gene expression. Q-RT-PCR analysis showed that ERG induced expression of CXCR4 mRNA (Figure 4A), and FACS analysis showed ERG transfection into LNCaP cells enhanced cell surface expression of CXCR4 (Figure 4B). Our previous report demonstrates that synthetic androgens induce CXCR4 gene expression in TMPRSS-ERG fusion positive cells in an ERG dependent manner (11). To determine if androgen-induced ERG can regulate cell surface CXCR4 expression, the ERG gene was cloned in ARR2-Pb-luc promoter; LNCaP cells were transfected with both ARR2-Pb-Luc and ARR2-Pb-ERG-Luc constructs. Q-RT-PCR analysis showed that synthetic androgen R1881 induced both ERG and CXCR4 only in ARR2Pb-ERG-Luc transfected cells. R1881 also induced CXCR4 expression in ARR2-Pb-Luc transfected cells, but its expression was higher in ARR2-Pb-ERG-Luc transfected cells (Figure 4C). Analysis of another CXCL12 receptor, CXCR7, showed that its expression was inhibited by

R1881 treatment (Supplemental Figure 4), suggesting the CXCR4 gene may be a target for R1881-induced ERG factor. FACS analysis of ARR2-Pb-ERG-Luc transfected cells revealed that R1881 induced expression of CXCR4 expression at the cell surface (Figure 4D).

ERG is a serine phosphorylated protein in TMPRSS2-ERG fusion positive cells:

Transcription factor activity of Ets family members is modulated by post-translational phosphorylation at serine/threonine and tyrosine (48). To determine whether ERG is phosphorylated at any serine, threonine, or tyrosine, ERG was immunoprecipitated from VCaP cells, and the protein was subjected to MS analysis. MS/MS analysis of peptides revealed that Ser 215 was phosphorylated with 94% certainty (Figure 5A). To confirm this phosphorylation, ERG was immunoprecipitated from VCaP cells and subjected to tryptic digestion, then peptides were captured on titanium dioxide column and analyzed on MS. Ser 215 and Ser 81 phosphorylation (Supplementary Figure 5) was detected in ERG protein (Gene accession #NM_182918). To confirm serine phosphorylation, VCaP cell lysates were isolated on pSerine agarose and pTyrosine agarose beads and subjected to Western blot analysis with anti-ERG (Figure 5B). ERG was detected in pSerine agarose beads and absent in pTyrosine agarose beads. Reverse immunoprecipitation studies with anti-ERG IP followed by Western blot analysis with anti-pSer antibodies confirmed ERG phosphorylation at serine (Figure 5C). To determine the upstream kinase(s) phosphorylating ERG, cells were treated with MEK inhibitor (PD0325901), IKK inhibitor (BMS34551), PI3K inhibitor (LY294002) and Akt inhibitor (Akt Inhibitor IV); ERG was immunoprecipitated and immunoblotted with anti-pSer antibody. Both IKK and Akt inhibitors reduced serine phosphorylation to 0.3 and 0.4 folds respectively (Figure 5D). To further determine if Akt inhibitor-induced reduction in ERG phosphorylation regulates CXCR4 expression, cells were treated with 1 and 5 μM Akt inhibitor followed by Western blot analysis. Akt inhibitor reduced CXCR4 expression in cells in a dose dependent manner (Figure 5E).

These biochemical studies suggest that the TMPRSS2-ERG fusion expressed ERG protein is post-translationally modified by phosphorylation at Ser 81 and 215 in VCaP cells, both IKK and Akt pathways mediate the ERG phosphorylation, and Akt-induced ERG phosphorylation regulates CXCR4 expression.

CXCL12/CXCR4 axis induces adhesion of prostate cancer cells, and ERG-induced CXCR4 expression mediates CXCL12 dependent adhesion, invasion, and migration of TMPRSS2-ERG fusion positive tumor cells: We have previously shown that overexpression of CXCR4 in PC-3 cells promotes intraosseous tumor growth in SCID-human prostate cancer model (22). The initial growth of bone tumors could be due to enhanced adhesion of tumor cells to extracellular matrix proteins. We tested CXCR4 overexpressing cells for the adhesion to fibronectin matrix. CXCR4 overexpression enhanced adhesion to fibronectin, and CXCL12 activation further enhanced binding to fibronectin by PC-3 cells (Figure 6A). To verify that ERG-regulated CXCR4 expression mediates adhesion to fibronectin as well as *in vitro* migration and invasion, ERG expression was stably knocked down by ERG shRNA lentiviral infection. ERG shRNA lentiviral infection downregulated both ERG and CXCR4 RNA (Figure 6B) and protein (Figure 6C) expression in VCaP cells compared to scrambled shRNA infection. CXCL12 treatment enhanced VCaP cell binding to fibronectin and matrigel invasion in scrambled shRNA infected cells. ERG knockdown reduced VCaP cell binding to fibronectin and matrigel invasion, and CXCL12 treatment did not promote adhesion and invasion (Figure 6D), while cell migration was not affected by the ERG knockdown. However, CXCL12 induced cell migration only in cells transfected with scrambled shRNA. Taken together, these data demonstrate that ERG-induced CXCR4 expression is functionally active in CXCL12-expressing cells and induced adhesion to extracellular matrix fibronectin, matrigel invasion, and migration.

ERG and CXCR4 co-localized to tumor cells in human prostate tumors: To determine whether ERG and CXCR4 are co-expressed in human prostate tumor tissues, we performed immunohistochemistry on human prostate tumors. From a total of 29 prostate cancer patient tissues, 16 tumor samples stained positive for ERG expression, and 18 samples stained positive for CXCR4 expression. Histological studies show that there is a tendency for co-expression of ERG and CXCR4 in tumor tissues (Figure 7A).

The two-way frequency distribution of the 29 prostate cancer patients by ERG presence or absence versus CXCR4 presence or absence is shown in Figure 7B. The Mantel-Haenszel test statistic ($Q_{cs} = 5.3853$) provided evidence of a statistically significant positive association ($p = 0.0266$). The Spearman rank correlation coefficient was rho = 0.44, with 90% confidence interval (0.17 – 0.71). The magnitude of the statistic rho = 0.44 suggests a modest linear correlation and is statistically significantly different from zero ($p = 0.0266$).

Secondary analyses of expression data sets were performed to determine correlation between ERG and CXCR4 expression in prostate tumor tissues (Figure 7C). Two data sets were analyzed for correlation (49, 50). The data sets show a Pearson r value of 0.4238 ($p=0.0002$) and 0.4633 ($p<0.0001$), suggesting a statistically significant moderate correlation between ERG and CXCR4 expression. Together, these data show that ERG factor may regulate CXCR4 expression in prostate tumor tissues.

Discussion:

Previous studies demonstrated that androgens act through TMPRSS2-ERG fusion to increase the ERG expression. We and others have shown that ERG enhances the expression of prometastatic gene, CXCR4 (8, 11). In fusion positive VCaP cells, androgens can induce functional CXCR4 expression via the expression of ERG (11). The CXCR4 promoter contains eight ERG/Ets factor binding sites within the 1 kb of promoter (Figure 1A). We showed that ERG factor regulates CXCR4 expression in TMPRSS2-ERG fusion positive cells via two distal promoter elements. This is the first study to molecularly characterize ERG-mediated CXCR4 expression in prostate cancer cells.

ERG regulation of downstream gene expression is complex, with its transactivation potential depending on the protein interactions with other heterodimerizing partners as well as structural features of ERG factor (13-15). In addition, different domains on ERG also have both inhibitory and activating function (46, 51) that can influence target gene expression. Due to this fact, ERG can activate or repress gene expression depending on the context of heterodimerizing partners and structural changes in ERG. Published reports are consistent with ERG's dual role on transcriptional regulation, showing that PLAU (6), CXCR4 (8, 11), MMPs (6) and osteopontin (12) are upregulated, and TFF3 (18) and PSMA (17) are downregulated upon ERG transcriptional activation. A recent study demonstrates that ERG function as a negative regulator of androgen receptor activity (46). This dual regulation also depends on the nature of ETS binding sites, as some binding sites have an inhibitory function as opposed to the activating function on transcription because of occupancy by heterodimeric partners (13-15). The CXCR4 gene contains eight binding sites for ERG/Ets factors (Figure 1A), and our studies in VCaP cells showed that these cells express several other Ets-family transcription factors (Figure 2A). To

molecularly characterize ERG mediated transcriptional regulation of the CXCR4 gene; we performed EMSA studies, which showed that VCaP nuclear proteins selectively bind certain elements in CXCR4 gene. As VCaP cells express multiple Ets factor family members, we performed EMSA studies with *in vitro* translated ERG, and these studies confirmed the binding data from VCaP cells. Furthermore, to determine the nature of ERG/Ets binding sites, we mutated these sites in the CXCR4 promoter, and our data confirmed that specific ERG binding sites localize to -919, -879, and -119 in CXCR4 promoter. Previous studies show that ERG can differentially regulate promoter activities of downstream genes (14, 15). To determine the ERG binding sites in CXCR4 promoter elements in promoter activation, we made promoter deletion constructs and tested the promoter activation in ERG overexpression system. These studies confirmed that ERG binding activates the promoter, and this activation is confined to upstream promoter binding elements in CXCR4 promoter (Figure 4). Taken together, transcriptional regulation of CXCR4 gene is under transcriptional control of ERG genes. Blast analysis of -919 and -879 oligo primer sequences (Supplementary Table 1) do not give a complete homology to any other sequences in human genomic plus transcript database, but Ets core sequence flanked by four nucleotides at the 5' and 3' ends in both primers identified several homologous sequences in the human genome. Therefore, it appears that ERG binding is confined to -919 and -879 sequences in the CXCR4 gene. To determine the nature of structural requirements of ERG protein, we utilized full length and N-terminus truncated form that corresponds to T1-E4 form. Our data show that both forms similarly activated CXCR4 promoter activation, and deletion of C-terminus results in loss of CXCR4 promoter transactivation. ERG transfection in LNCaP cells that are TMPRSS2-ERG fusion negative induced cell surface expression compared to empty vector transfected cells. In addition, androgen-induced expression of ERG in LNCaP cells also induced cell surface CXCR4 expression. Two previous studies show that androgens regulate CXCR4 gene expression in LNCaP cells (11, 52). This regulation appears to be indirect and

requires the expression of a transcription factors. Consistent with these published AR ChIP seq data do not identified a AR binding site in CXCR4 gene (46). Collectively, these data demonstrate that androgens do not directly regulate CXCR4 gene expression, but androgen-induced ERG factor regulates CXCR4 expression. Recent studies demonstrate that ERG in fusion positive cancer cells extinguishes AR signaling in a negative feedback manner in repressing AR differentiation process. In TMPRSS2-ERG fusion positive cells ERG also overexpress EZH2 gene and mediate epigenetic repressive program. In addition to activating epigenetic silencing, EZH2 has novel function as a AR co-activator (53), suggesting that the AR/ERG/EZH2 axis works in concert to promote AR mediated cancer progression. ERG activation also induces invasive signaling in fusion positive cancer by inducing protease expression (6, 10), and our data with pharmacological inhibition of Akt and CXCR4 suggest that the AR/ERG/CXCR4 axis promotes CXCL12-dependent cancer cell invasion. Altogether, these studies convincingly demonstrate that ERG transcriptionally regulates CXCR4 in TMPRSS2-ERG fusion positive cells.

Transcription factor function has been shown to be regulated by post-translational phosphorylation. This phosphorylation facilitates nuclear transport and interaction with co-activators and subsequent DNA binding. Ets family factors have been shown to be either activated or repressed by the phosphorylation. For ERG mediated transcriptional regulation, a key question that needs to be addressed is whether deregulated ERG expression via TMPRSS2-ERG fusion activation is sufficient for transcriptional regulation of responsive genes or whether overexpressed ERG requires activating signals through phosphorylation. Towards understanding this key question, we investigated the phosphorylation status of ERG in VCaP cells through MS analysis. Our data identified two serine phosphorylation sites in ERG factor, suggesting that at the basal level ERG is phosphorylated specifically at Ser 81 and 215 positions in TMPRSS2-

ERG fusion positive cells. To our knowledge, this is the first report presenting phosphorylation status of ERG in fusion positive prostate cancer cells. Based on the collaboration of ERG with alternative signaling pathways such as alterations in PI3K signaling via PTEN loss and androgen receptor in driving adenocarcinoma (8, 9, 54) and our current data suggesting phosphorylation of ERG in VCaP cells (Figure 5), it could be postulated that these altered signaling pathways drive pathological progression via phosphorylation mediated activation of ERG factor. Recent studies show that ERG in fusion positive tumors interacts with poly ADP-ribose polymerase 1 (PARP) and DNA protein kinase (DNA-PK) to activate gene programs including invasion (16, 55), suggesting that phosphorylated ERG may interact with these proteins to promote downstream transcriptional program leading to cellular invasion. Our data with Akt inhibitor-mediated inhibition of ERG phosphorylation, subsequent CXCR4 expression and CXCL12-induced invasion further support this notion that Akt kinase activation is a an upstream signal for CXCR4 expression. These studies also further suggest that Akt/ERG/CXCR4 axis as molecular mediators in previously identified co-operation between PTEN and ERG in driving adenocarcinoma development.

The potential biological relevance of the CXCL12/CXCR4 axis has been shown to be mediated by selective adhesion to extracellular matrix components (19) and to enhance migration and invasion by increasing protease expression (21, 23). CXCR4 overexpression enhanced binding to fibronectin in PC-3 cells, suggesting that CXCL12/CXCR4 mediated adhesion is a key event in tumor metastasis. Moreover, in ERG-knocked down cells, CXCL12 is unable to enhance adhesion, migration, and invasion due to lower CXCR4 expression, implying ERG-induced CXCR4 is functionally involved in tumor cell adhesion, migration, and invasion. Based on the critical role of ERG in tumor cell invasion (6), our data for the first time assign ERG-induced gene expression in initial phases of invasion, i.e. adhesion of tumor cells to extra cellular

matrix proteins. CXCL12/CXCR4 signaling also induces protease expression mediating tumor cell invasion (21, 23); thus, ERG regulation of CXCR4 contributes to multiple steps of tumor metastasis. The clinical relevance of the ERG/CXCR4 axis is well supported by data from human tumor tissue studies. ERG has been shown to be expressed in human tumor tissues as a fusion gene with androgen responsive TMPRSS2 promoter in approximately 50% of patients. Its expression in tumors is multifocal, and expression is strongly associated with prostate cancer and persistent in metastatic prostate cancer. Similarly, CXCR4 expression is enhanced during prostate cancer progression (25). CXCR4 expression is also associated with aggressive phenotypes of prostate cancer (27). Our data are the first to determine expression of both genes in human prostate tumor cells and analysis of multiple tumor specimens reveals a statistically significant positive association between the expressions of both genes in human prostate tumor tissues. These data are consistent with secondary analysis of tumor microarray gene expression, where both ERG and CXCR4 were co-expressed in prostate tumors (11) (Figure 7C). Based on these studies, targeting TMPRSS2-ERG fusion positive cancers with CXCR4 inhibitors may have therapeutic benefit for prostate cancer patients.

In summary, we show that ERG factor specifically binds to upstream ERG/Ets sites and activates CXCR4 promoter. In TMPRSS2-ERG fusion positive cells, ERG is expressed as a phosphoprotein, suggesting the presence of post-translational modification of ERG protein. ERG factor induced CXCR4 is functionally active in adhesion of tumor cells to extracellular matrix protein. These data suggest that CXCR4 is a relevant target for androgen-mediated activation of TMPRSS2-ERG fusion in prostate tumor cells.

Legends:

Figure 1. Electrophoretic mobility shift assay of CXCR4 promoter elements with VCaP cell nuclear extracts. (A) Depicted is the 962bp CXCR4 promoter containing eight (number 1 to 8) ERG putative binding sequences (refer to Supplementary Table 1 for sequences) that are represented by rectangular boxes. (B) Electrophoretic mobility shift assay (EMSA) of IR Dye TM^{700} labeled CXCR4 promoter sequences with VCaP nuclear extracts (lane 1-8), control experiment lacking either oligo (lane 9) or nuclear extract (lane 10) in assay. (C) Specificity of oligos 1, 2, and 8 binding to nuclear protein were shown with inclusion of 100 fold excess of unlabeled competitor oligo in the assay and last two lanes are controls either lacking oligo or nuclear extract in the assay. (D) 0.01, 0.1, 1, and 2 mg of VCaP nuclear extracts were incubated with oligos in EMSA assay (lane 1-4) control assay lacking either oligo (lane 5) or nuclear extract (lane 6).

Figure 2. *In vitro* expressed ERG binds with CXCR4 promoter elements. (A) RT-PCR analysis of VCaP cell mRNA show expression of other Ets family transcription factors ETS1, ETS2, ETV1, ETV4, ETV5, Fli-1, ERG, and GAPDH. (B) Western blot with *in vitro* translated ERG protein, VCaP extracts, and C4-2B cells transfected with empty vector and puro-ERG were shown as positive controls. (C) 962 bp CXCR4 promoter showing 1-8 putative ERG binding sites. Blocked boxes represent positive for elements binding to VCaP cell nuclear extracts as shown in Figure 1. (D) *In vitro* translated ERG with 1-8 oligos in EMSA assay (lanes 1 to 8) and controls lacking either oligo (lane 9) or *in vitro* translated ERG (lane 10). (E) Supershift assay with three different ERG antibodies (SC-353, SC-354 and SC-28680 antibodies) in EMSA assay. * Represents shifted band in EMSA, and arrow represents antibody mediated super shift of ERG and labeled oligo.

Figure 3. ERG regulates CXCR4 promoter activation. (A) Empty vector and puro-ERG plasmids were transfected to HEK293 cells, and western blot analysis was performed with anti-

ERG and GAPDH antibodies as a loading control. (B) Different CXCR4 promoter (962, 899, and 231 bps) luciferase constructs used in the transfection experiment. (C) Different CXCR4 promoter luciferase reporter plasmids, either puro-empty vector or puro-ERG plus CMV Renilla luciferase plasmids were co-transfected into HEK293 cells and luciferase and Renilla luciferase activities were measured. Fold induction of luciferase activities in puro-ERG transfected cells over empty vector transfected cells were determined. Data shown are from triplicate transfections in three independent experiments.

Figure 4. Androgen-induced ERG factor regulated CXCR4 cell surface expression. (A) LNCaP cells were transfected with vector control and ERG expression plasmid, and CXCR4 gene expression was quantitated. (B) FACS analysis of cell surface CXCR4 expression was determined in puro-empty and puro-ERG transfected cells (middle and right panel). Isotype IgG PE was used as control for background signal (left panel). (C) LNCaP cells were transfected with ARR2Pb-LUC as a vector control and ARR2Pb-ERG-LUC plasmids and treated with R1881. Relative gene expression of CXCR4 and ERG were determined. (D) Cell surface expression of CXCR4 was analyzed by FACS in ARR2Pb-LUC and ARR2PbERG-LUC transfected cells (middle and left panel). Isotype IgG PE was used as a control for background staining (left panel).

Figure 5. ERG is a phospho-Serine protein in VCaP cells, IKK and Akt kinases phosphorylate ERG, and Akt kinase regulates CXCR4 expression. (A) MS3 spectrum of trypsin-digested ERG from VCaP cells. Inset table shows the masses of B and Y ions in collision-induced fragmentation of MS2 peptide and shows Serine 81 has an additional mass of 80, suggesting phosphorylation. (B) VCaP cell lysates were affinity purified with anti-phospho-Serine and anti-phospho-Tyrosine agarose beads and Western immunoblotted with anti-ERG antibody. (C) VCaP cell lysates were imumoprecipitated with anti-ERG antibodies and immunoblotted with anti-phospho-Serine antibodies. (D) VCaP cells were treated with 10 μM of

PD32901, 10 μM of BMS34551, 10 μM of LY294002, and 5 μM of Akt Inhibitor IV overnight; cell lysates were immunoprecipitated with anti-ERG antibody and immunoblotted with anti-phospho-Serine and anti-ERG antibodies. Fold changes in serine phosphorylation in ERG were determined by densitometric scanning and quantitation of pSerine and ERG expression with ImageJ software and normalized for ERG expression. (E) VCaP cells were treated with 0, 1, and 5 μM of Akt Inhibitor IV overnight, and cell lysates were immunoblotted with anti-CXCR4 and anti-GAPDH antibodies. Fold changes in CXCR4 expression were determined by densitometric scanning and quantitation of CXCR4 and GAPDH expression and normalized for GAPDH expression. (F) VCaP cells were treated with Akt Inhibitor IV overnight and AMD3100 for two hours. Untreated and inhibitor-treated cells were seeded on the upper chamber in transwell inserts. Either serum free media or CXCL12 (200ng/ml) were added to the bottom chamber. Number of invaded cells were scored after 24 hour invasion.

Figure 6. ERG-induced CXCR4 is functionally active in cellular adhesion. (A) PC-3 Neo, CXCR4-2.1 and CXCR4-2.3 cells were treated with vehicle or 200 ng/ml CXCL12 and seeded in fibronectin-coated 96 well plates. After one hour plates were washed and adhered cells were measured for fluorescence. Based on standard curve, fluorescence values were converted to number of cells, and percent adhered cells were determined. (B) ERG was knocked down with shERG lentivirus in VCaP cells; ERG, CXCR4, and GAPDH mRNA expressions were determined. ERG and CXCR4 expressions were normalized for GAPDH expression, and in (C) ERG, CXCR4 and GAPDH protein expression were determined. Fold expression of ERG and CXCR4 were determined by densitometric scanning of bands and normalized for GAPDH expression. (D) VCaP scrambled and shERG lentivirus infected cells were treated with vehicle or 200ng/ml CXCL12 followed by adhesion, migration, and invasion assays.

Figure 7. Expression of ERG and CXCR4 in human prostate tumors. (A) Immunohistochemical analysis of ERG and CXCR4 in human prostate tumor tissue specimens.

* indicates no positivity of tumor cells for ERG and CXCR4; arrow represents tumor tissues showing positivity for both genes. (B) Distribution of 29 patients by presence or absence of CXCR4 and ERG in prostate tumor tissues. (C) Expression array data for ERG and CXCR4 were obtained from GDS2545 and GSE 14097 (CXCR4 ID 7934 and ERG ID 23711) record from Gene Expression Omnibus database. Correlation analysis was performed between ERG and CXCR4 expression data to determine Pearson r value.

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PTEN loss mediated Akt activation promotes prostate tumor growth and metastasis via CXCL12/CXCR4 signaling.[¶]

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Abstract

Introduction: The chemokine CXCL12, also known as SDF-1, and its receptor, CXCR4, are overexpressed in prostate cancers and in animal models of prostate-specific PTEN deletion, but their regulation is poorly understood. Loss of the tumor suppressor PTEN (phosphatase and tensin homolog) is frequently observed in cancer, resulting in the deregulation of cell survival, growth, and proliferation. We hypothesize that loss of PTEN and subsequent activation of Akt, frequent occurrences in prostate cancer, regulate the CXCL12/CXCR4 signaling axis in tumor growth and bone metastasis.

Methods: Murine prostate epithelial cells from $PTEN^{+/+}$, $PTEN^{+/-}$, and $PTEN^{-/-}$ (prostate specific knockdown) mice as well as human prostate cancer cell lines C4-2B, PC3, and DU145 were used in gene expression and invasion studies with Akt inhibition. Additionally, HA-tagged Akt1 was overexpressed in DU145, and tumor growth in subcutaneous and intra-tibia bone metastasis models were analyzed.

Results: Loss of PTEN resulted in increased expression of CXCR4 and CXCL12 and Akt inhibition reversed expression and cellular invasion. These results suggest that loss of PTEN may play a key role in the regulation of this chemokine activity in prostate cancer. Overexpression of Akt1 in DU145 resulted in increased CXCR4 expression, as well as increased proliferation and cell cycle progression. Subcutaneous injection of these cells also resulted in increased tumor growth as compared to neo controls. Akt1 overexpression reversed the osteosclerotic phenotype associated with DU145 cells to an osteolytic phenotype and enhanced intra-osseous tumor growth.

Conclusions: These results suggest the basis for activation of CXCL12 signaling through CXCR4 in prostate cancer driven by the loss of PTEN and subsequent activation of Akt. Akt1-associated CXCL12/CXCR4 signaling promotes tumor growth, suggesting that Akt inhibitors may potentially be employed as anticancer agents to target expansion of PC bone metastases.

Introduction

Chemokines are a superfamily of cytokines known to regulate the migration of cells and play a key role in the regulation of metastasis. The chemokine CXCL12, also known as stromal-derived factor-1 (SDF-1), is a potent chemoattractant for hematopoetic cells[1] and activate signaling events through its two distinct receptors, CXCR4 and CXCR7. CXCR4 has been shown to be a key receptor in mediating the metastasis of multiple types of tumors. Binding of CXCL12 to CXCR4 induces trimeric G protein signaling leading to activation of the Src, PI3K/Akt, ERK, and JNK pathways, contributing to protease production and cellular migration and invasion. In addition, we recently found that epidermal growth factor receptor family members are activated downstream of CXCL12/CXCR4 signaling, providing proliferative signals in bone tumor growth. CXCL12 and its receptors have been strongly linked to prostate cancer bone metastasis and are markers for poor prognosis[2-5].

The tumor suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN, also known as MMAC1/TEP1) is a lipid and protein phosphatase that serves as a negative regulator of the phosphatidylinositol-3 kinase (PI3K) pathway [6]. PTEN dephosphorylates phosphatidylinositol-3,4,5-trisphosphate (PIP3), thus serving as an inhibitor of the PI3K signaling pathway. Through its attenuation of the PI3K pathway, PTEN is a critical regulator of growth factor signaling and is able to regulate key cellular processes such as cell proliferation, motility, protein synthesis, glucose metabolism, genomic stability, and survival. Mutations of PTEN are associated with several diseases, including Cowden disease, Lhermitte-Duclos disease, and Bannayan-Zonana syndrome [7, 8]. In addition, loss of PTEN has been shown to be associated with many types of cancer, such as glioblastoma, endometrial carcinoma, and breast cancer [9-11]. PTEN expression is frequently altered in cancer; PTEN is lost or mutated in 50-80% of primary PC, and complete loss of PTEN is associated with aggressive and metastatic cancer [5, 12]. In mouse models of PC, loss of PTEN is critical for tumor initiation, and the level

of PTEN expression is inversely associated with prostate tumorigenesis [13, 14]. As shown by microarray analysis and immunohistochemistry, murine epithelial cells from these PTEN-deficient prostate tumors display increased expression of CXCL12 and CXCR4 as compared to the normal prostate glands of $PTEN^{+/+}$ and $PTEN^{+/-}$ mice [15]. However, it is not known whether Akt activation downstream of PTEN loss regulates CXCL12/CXCR4 expression and function in tumor cells.

In this study, by using cell lines derived from $PTEN^{+/+}$, $PTEN^{+/-}$, and $PTEN^{-/-}$ mice, we demonstrate that loss of PTEN results in increased expression of CXCL12 and CXCR4. Using inhibitor assays, we demonstrate that regulation of the PI3K/Akt pathway by PTEN in turn regulates expression of both CXCL12 and CXCR4 in mouse and human prostate cancer cells. Akt overexpression in PTEN wild type DU145 cells induced cell proliferation, tumor growth and bone metastasis. Taken together, these data define a relationship between PTEN loss and CXCL12/CXCR4 signaling in prostate cancer progression.

Materials and Methods

Cell culture. Cell lines were cultured in a humidified incubator with 5% CO2 at 37°C. All media were supplemented with 2mM glutamine, 100units/ml penicillin, and 100mg/ml streptomycin (Life Technologies Inc., Carlsbad, CA). Murine cell lines were maintained in Advanced DMEM supplemented with 5% fetal bovine serum. The benign human prostate cell line BPH-1 and human PC cell line PC3 were maintained in RPMI-1640 supplemented with 10% fetal bovine serum. Human PC cell line C4-2B and DU145 were maintained in T-Medium supplemented with 10% fetal bovine serum and DMEM supplemented with 10% fetal bovine serum, respectively.

Establishment of $PTEN^{+/+}$, $PTEN^{+/-}$, and $PTEN^{-/-}$ mouse prostate epithelial cell lines, DU145-neo and DU145-HA-Akt1 stable cell lines. Murine cell lines were established as described previously [16, 17]. Briefly, exon 5 of PTEN was deleted specifically in the murine prostate. $PTEN^{+/+}$, $PTEN^{+/-}$, and $PTEN^{-/-}$ prostate epithelial cells were isolated from prostates of corresponding mice at 8 weeks of age, and cell lines were established by serial dilution method and subsequent clonal selection. DU145 cells were transfected with PLNCX-neo and PLNCX-Hemagglutinin-tagged Akt1 constructs using lipofectamine 2000; 48 hours post-transfection, cells were exposed to Neomycin, and stable clones were selected.

Western Blot Analysis. Cells were washed with PBS, and total cellular proteins were extracted with buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 1mM PMSF, and 1X 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 against PTEN, phosphorylated Akt (S473), and total Akt (Cell Signaling Technology, Boston, MA), CXCR4 (Chemicon, Billerica, MA) and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). The band intensities

were determined by quantitation of pixel intensities using ImageJ software (version 10.2; National Institutes of Health, Bethesda, MD).

Quantitative RT-PCR. mRNA was purified from cells using the RNeasy kit (Qiagen, Valencia, CA), and cDNA synthesis was performed with iScript Select cDNA Synthesis Kit (Biorad, Hercules, CA). Real time RT-PCR was performed using SYBR Green mix plus ROX (Fisher Scientific) and the Eppendorf Mastercycler ep realplex^2 qPCR System (Hauppauge, NY) according to the manufacturer's protocol. Relative values of gene expression were normalized to GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = (\Delta Ct_{\text{target gene}} - \Delta Ct_{GAPDH})_{\text{sample}} - (\Delta Ct_{\text{target gene}} - \Delta Ct_{GAPDH})_{\text{control}}$. The fold change in relative expression was then determined by calculating $2^{-\Delta\Delta Ct}$. Forward and reverse murine specific forward and reverse primers used are as follows: CXCR4: 5'-TCAGTGGCTGACCTCCTCTT-3', 5'-TTTCAGCCAGCAGTTTCCTT-3'; CXCL12: 5'-CTTCATCCCCATTCTCCTCA-3', 5'-GACTCTGCTCTGGTGGAAGG-3'. Forward and reverse human specific forward and reverse primers used are as follows: CXCR4: 5'-GGTGGTCTATGTTGGCGTCT-3', 5'-TGGAGTGTGACAGCTTGGAG-3'; CXCL12: 5'-ATGAACGCCAAGGTCGTG-3', 5'-CTTCGGGTCAATGCACACTT-3'. Forward and reverse primers recognizing both murine and human GAPDH were 5'-ATCACCATCTTCCAGGAGCGA-3' and 5'-GCCAGTGAGCTTCCCGTTCA-3', respectively.

Inhibition of Akt signaling pathway. Cells were cultured with growth media supplemented with 1% FBS and treated with indicated concentrations of Akt Inhibitor IV (Fisher Scientific, Pittsburgh, PA) or vehicle control for 18 hours. Subsequently, mRNA and protein were collected from cells and subjected to quantitative RT-PCR analyses or western blot analysis, respectively.

Invasion assay. Cells were cultured with complete growth media and treated with 10μM Akt Inhibitor IV or vehicle control; after five hours, media was replaced with growth media supplemented with 1% FBS containing 10μM Akt Inhibitor IV or vehicle control. After overnight culture, cells were trypsinized and plated on the upper chamber of matrigel-coated transwell filters ($2x10^5$ cells/filter) in growth media supplemented with 1% FBS containing 10μM Akt Inhibitor IV or vehicle control, with 200ng/mL CXCL12 added to bottom chamber. After 24 hours, cotton swabs were used to remove unmigrated cells from the upper chamber, and inserts were stained with 0.9% crystal violet. Total number of migrated cells was counted under 10X magnification. Assay was performed in triplicate.

***In vivo* studies and tumor tissue analyses.** Both subcutaneous and intratibial tumor inoculation studies were performed as described previously[18]. Briefly, five-week-old male C.B.-17 severe combined immunodeficient (SCID) mice (Taconic Farms, Germantown, NY) were used in the study. For subcutaneous tumor cell implantation, $5x10^5$ cells of both DU145-Neo and DU145-Hemagglutinin-tagged AKT transfectants were mixed in 50% matrigel in a volume of 100 μl and implanted in flanks of mice. For each cell line, eight mice were used in the experiment. For intratibial implantation $1x10^5$ cells were injected per bone; for each cell line, 8-10 mice were used. Histomorphometric analyses were performed to determine tumor burden and trabecular bone area in both DU145 transfectants (Neo and HA-Akt1) as previously described[18].

Immunohistochemistry. Formalin-fixed, paraffin-embedded serial tissue sections from DU145-Neo and DU145-HA-Akt1 tumors were deparaffinized with xylene and rehydrated in graded EtOH. Endogenous peroxidase activity was blocked by incubating in 3% H_2O_2 for 20 min. For subcutaneous tumor sections, antigen retrieval was performed with Antigen Retrieval Citra Plus Solution (BioGenex, Freemont, CA) in a steamer. For bone sections, antigen retrieval was

performed with proteinase K (Sigma-Aldrich, St. Louis, MO). Slides were then blocked with Blocking Serum from ABC Vectastain Kit (Vector Labs, Burlingame, CA). Slides were incubated at 4°C overnight in a humidified chamber with antibodies directed against Ki67 (BD Biosciences, San Jose, CA), phosphorylated Akt (S473) (Cell Signaling Technology), or CXCR4 (R&D Systems, Minneapolis, MN). After washing, sections were incubated with ABC Vectastain Kit, according to manufacturer's protocol, followed by incubation with 3,3-diaminobenzidine tetrahydrochloride (Vector Labs). Nuclei were counterstained with Mayer's hematoxylin (Sigma-Aldrich). Sections were then dehydrated with graded EtOH, washed with xylene, and mounted with Permount (Sigma-Aldrich).

Statistical analyses: Data were analyzed using Microsoft Excel 2008. All data are presented as mean ± SD. Data were analyzed using Student's t-test; a p-value < 0.05 was considered statistically significant.

Results

Progressive loss of PTEN results in increased expression of CXCL12 and CXCR4 in murine prostate epithelial cells. In an effort to study the regulation of CXCL12 and its receptor, a murine model was utilized. In this model system, prostate epithelial cell lines were generated from anterior prostates of $Pten^{+/+}$, $Pten^{+/-}$, and $Pten^{-/-}$ mice, using the method previously described [15] [16] [19]. Loss of PTEN was verified at the protein level (Fig. 1A). As shown by qPCR, $PTEN^{-/-}$ cells exhibit significantly increased mRNA levels of CXCL12 and CXCR4 (Fig. 1B). These data are consistent with results from Berquin et al, where microarray and immunohistochemistry demonstrated increased expression of both CXCL12 and CXCR4 in $PTEN^{-/-}$ mice [15]. When $PTEN^{-/-}$ cells were treated with increasing concentrations of Akt Inhibitor IV, expression of both CXCR4 and CXCL12 decreased (Fig. 1C, D). As expected, Akt Inhibitor IV inhibited Serine 473 phoshporylation on Akt without changing Akt1 levels in cells. As low as 1 μM Akt Inhibitor IV reduced Serine 473 phosphorylation. At this concentration, Akt Inhibitor IV abrogated basal as well as CXCL12-induced cell invasion of $PTEN^{-/-}$ cells through Matrigel coated inserts (Fig. 1E). Notably, there was no significant difference in cell invasion when 200ng/mL CXCL12 was added to the bottom chamber, likely due to the high basal levels of CXCL12 expressed by $PTEN^{-/-}$ cells.

Akt regulates CXCR4 expression in PTEN-null human prostate cancer cells. To examine the role of PTEN in the regulation of CXCR4 in human prostate cancer, the cell lines BPH-1, C4-2B, and PC3 were utilized. As shown in Figure 2A, BPH-1 expresses PTEN, while C4-2B and PC3 are PTEN-null. Treatment with 1 and 10μM Akt Inhibitor IV resulted in decreased expression of CXCR4 in C4-2B and PC3 cell lines (Fig. 2B). As low as 1 μM Akt Inhibitor IV reduced CXCR4 expression in PC-3 cells, whereas in C4-2B cells 10 μM Akt inhibitor IV inhibited CXCR4 expression. Additionally, CXCL12-mediated invasion through a matrigel-

coated transwell insert was abrogated by treatment with 1μM Akt Inhibitor IV in both C4-2B and PC3 (Fig. 2C).

Overexpression of Akt results in increased phosphorylation of Akt, CXCR4 expression, proliferation and invasion. Multiple cell surface receptors have been shown to activate Akt kinase and induce downstream signaling events leading to cell survival. Among Akt family members Akt1 is predominantly expressed in prostate cancer cells[20]. Even though PTEN lipid phosphatase activity has been shown to regulate the PI3K-Akt pathway, several studies document PI3K-Akt-independent functions of PTEN [21-23]. PTEN loss deregulates both lipid and protein phosphatase activity [24]. Figures 1 and 2 demonstrate that Akt activation regulates CXCR4 expression. To determine Akt1 function in tumor growth and metastasis without disturbing other functions of PTEN, a novel model consisting of Akt1 overexpression in PTEN-intact DU145 cells was generated. Studies have been performed previously using a constitutively active Akt via artificially tagging membrane localization myristoylation signal to study downstream functions of activated Akt; however, in these studies, the transfected Akt must be phosphorylated in the cell to induce downstream effects similar to endogenous Akt protein. DU145 cells transfected with HA-Akt1 exhibit increased levels of pAkt Ser473, p90rskSer380 and pFKHR Ser256 in serum free media, suggesting that transfected Akt1 and its effector signaling is activated in cells (Fig. 3A). In addition, Akt1 overexpression induced a 1.29 fold increase in CXCR4 protein expression (Fig. 3A). Culture of the cells with 10% serum resulted in a further increase of phosphorylated Akt (Fig. 3B). When cells were cultured in complete serum conditions, HA-Akt1 expression resulted in an increase in proliferation compared to Neo-transfected cells (Fig. 3C). Additionally, cell cycle analysis revealed that expression of HA-Akt1 resulted in a decrease in the G_0G_1 population and an increase in the S phase population (Fig. 3D), suggesting cell cycle progression. To demonstrate that CXCR4 is a key element of Akt-induced

effects, an invasion assay was performed utilizing AMD3100, a pharmacological inhibitor of CXCR4. DU145-HA-Akt1 cells exhibited increased invasion through Matrigel coated inserts, as compared to DU145-Neo cells (Fig. 3E). Treatment of DU145-Neo cells with AMD3100 did not affect invasion. In DU145-HA-Akt1 cells, however, invasion was inhibited by this treatment, suggesting that the increased invasion of Akt1-transfected cells as compared to control cells is driven at least in part by CXCL12/CXCR4 signaling. These studies together demonstrate Akt1 activity in DU145 cells, and that this activity induces CXCR4 expression and function.

Overexpression of Akt results in increased subcutaneous tumor growth. To determine the biological importance for Akt in tumor growth, mice were injected subcutaneously with DU145-Neo or DU145-HA-Akt1 cells. As shown in Fig. 4A, HA-Akt1 expression resulted in increased tumor volume after 60 days of inoculation; the growth rate was significantly faster compared to DU145-neo cells. As shown by immunohistochemistry, tumors also exhibited increased expression of both Serine 473 phosphorylated Akt and CXCR4, suggesting that activated Akt mediates downstream gene expression, resulting CXCR4 overexpression (Fig. 4B). Furthermore, Ki67 staining revealed increased proliferation in DU145-HA-Akt1 tumors as compared to neo controls (Fig. 4C). These data demonstrate that overexpressed Akt is active in tumors and mediate tumor growth by enhancing CXCR4 signaling.

Overexpression of Akt results in increased intratibial tumor growth. Prostate cancer frequently metastasizes to the bone, and previous studies implicate key role for CXCL12/CXCR4 signaling in bone metastasis. To examine the effects of Akt in the bone environment, DU145-HA-Akt1 cells were cultured with bone conditioned media, resulting in increased Serine 473 phosphorylation. This increase in phosphorylation was not detected in DU145-Neo control cells (Fig. 5A). Further, co-culture of DU145 transfectants with human fetal

bone stromal cells show that in HA-Akt1 transfected cells Akt is phosphorylated at Serine 473, suggesting that Akt signaling in cancer cells is induced by bone stromal interactions in both a paracrine manner and in direct contact. Next, mice were injected intratibially with DU145-Neo or DU145-HA-Akt1 cells. Previous studies show that DU145 cells in intratibial model induce an osteosclerotic phenotype, as evidenced by enhanced trabecular bone formation[18]. DU145-Neo cells induced a similar osteosclerotic reaction in bone, while DU145-HA-Akt1 cells resulted in increased osteolysis at eight weeks (Fig. 5B, C). Histomorphometric analysis reveal that DU145-HA-Akt1 tumors exhibited a decreased ratio of trabecular bone area to tissue area, as well as an increase in overall tumor burden in bone tumors (Fig. 5C). Furthermore, DU145-HA-Akt1 tumors expressed higher levels of phosphorylated Akt and CXCR4 (Fig. 5E).

Discussion

Chemokines and their receptors play key roles in hematopoietic cell trafficking. CXCL12/CXCR4 has also been shown to play a key role in the regulation of metastasis, and its expression has been shown to be elevated in localized and metastatic cancer, including bone metastatic prostate tumors[2],[3]. Among PC patients, higher expression of CXCR4 was documented in prostate tumor tissues from African American patients, suggesting CXCR4 expression is associated with aggressive disease phenotypes in these patients[25]. Human prostate tumor expression of CXCR4 is also associate with poor survival[4], as its expression is significantly associated with local recurrence after therapy and formation of distant metastases[5]. Taken together, these studies emphasize the clinical significance of CXCL12/CXCR4 expression in prostate tumor progression.

Tumor cells expressing CXCR4 metastasize to target organs that express high levels of CXCL12[26, 27]. Colonization of tumor cells in the bone microenvironment is thought to occur through PC cell encroachment of the hematopoietic stem cell niche by mimicking the stem cell interactions with bone resident stromal cells[28]. This concept is validated by studies showing that targeting CXCR4 function through neutralizing antibodies inhibited prostate cancer bone metastasis[29], and overexpression of CXCR4 in prostate cancer cells enhanced bone metastasis[30]. Additionally, inhibition of CXCR4 with CTCE-9908 resulted in decreased tumor growth, angiogenesis, and lymphangiogenesis, as well as increased apoptosis in a xenograft PC model [31] . Thus, these studies show that the CXCL12/CXCR4 axis in tumor cells usurps stem cell homing mechanisms to get into bone[28, 29] and subsequent colonization and growth through activation of growth factor receptor signaling[30]. Studies with colorectal cancers show that CXCR4 signaling is also involved in outgrowth of metastasis[32]. Taken together, these studies demonstrate that CXCL12/CXCR4 signaling is a critical event mediating homing of

tumor cells and subsequent expansion of metastases.

Our previous studies show that PTEN knockout mouse model epithelial tumor cells gain expression of an "osteogenic signature," thus predisposing these cells for metastasis [15]. We found that both CXCL12 and CXCR4 are overexpressed in epithelial tumor cells in PTEN knockout mice. Herein, using cultured cells from PTEN intact, heterozygous, and knockout cells, we show that both CXCR4 and CXCL12 expression is higher in PTEN knockout cells, thus confirming the immunohistochemical tumor expression findings (Figure 1). Specific inhibition of Akt resulted in downregulation of both CXCL12/CXCR4 expression, and an established PI3 kinase inhibitor also downregulated both CXCL12 and CXCR4 gene expression (data not shown). Similarly, Akt-mediated regulation of CXCR4 was observed in human prostate cancer cells with loss or mutation of PTEN (Figure 2). In addition, in both mouse and human tumor cells with either loss or mutation of PTEN, Akt Inhibitor IV treatment inhibited basal as well as CXCL12-induced invasion. PTEN loss-induced PI3K/Akt has been shown to mediate migration and invasion of prostate cancer cells in response to CXCL12/CXCR4 [20, 33, 34], suggesting that Akt can function both upstream (as an inducer of CXCR4 expression) and downstream (as a signaling kinase for induction of proteases and invasion) of CXCR4. Current studies with Akt inhibitors implicate Akt as a key member in this pathway contributing to CXCR4 expression in prostate cancer cells.

Tumor suppressive functions of PTEN independent of phosphoinositide lipid phosphatase activity play a key role in cell cycle regulation, maintaining genomic instability, and controlling DNA repair mechanisms that safeguard cells from accumulation of genetic mutations and uncontrolled proliferation [24]. PTEN loss dysregulates these genome safeguard mechanisms and also leads to Akt activation, promoting tumorigeneisis. To determine the function of Akt in

tumor growth and metastasis via regulation of CXCR4 function independent of other effects induced by PTEN loss, DU145 cells were transfected with an HA-tagged Akt1, which is not constitutively active and must be activated within the cell (Figure 3). Akt1 overexpression resulted in increased proliferation and cell cycle progression, suggesting that transfected Akt1 mimicked PTEN loss-associated hyperactivated Akt signaling. In an effort to determine the biological significance of Akt/CXCR4 axis in tumor growth, we subcutaneously implanted both low Akt1 (Neo) and high Akt1 (HA-Akt1) expressing cells in SCID mice (Figure 4). Akt1 overexpression significantly enhanced tumor growth starting from day 60. Immunohistochemical analysis further revealed that HA-Akt1 tumors proliferated at a faster rate, as shown by increased staining for Ki-67 proliferation marker. As expected, HA-Akt1 tumors have stronger Akt Serine 473 phosphorylation and CXCR4 expression, suggesting that Akt1 induced CXCR4 expression, contributing to the primary tumor growth. Our previous data demonstrate that CXCR4 overexpression in the PTEN-null cell line PC-3 enhanced bone tumor growth in a SCID-human model[20]; these tumors have activated Akt signaling, demonstrating the Akt signaling downstream of CXCR4 contributing to bone tumor growth. Herein, we utilized DU145-HA-Akt1 overexpressing cells to specifically determine the contribution of Akt1 without perturbing the lipid/protein phosphatase functions of PTEN in CXCR4 expression and downstream signaling in bone tumor growth. Activation of Akt1 in these cells was enhanced by bone factors and/or bone stromal cell interactions. In intratibial models, DU145 cells produce osteosclerotic reactions, as evidenced by enhanced bone formation measured by X-rays and histomorphometry. DU145-Neo cells produced similar bone reactions in our model, whereas overexpression of Akt1 completely reversed this phenotype to an osteolytic phenotype similar to PC-3 cells in this model, as evidenced by enhanced tumor growth and destruction of trabecular bone (Figure 5). Akt1 activation, as measured by Serine 473 phosphorylation, is higher in DU145-HA-Akt1 cells and is localized to the bone tumor interface, suggesting that transfected Akt1 is activated by bone

remodeling-released and/or stromal-expressed factors. CXCR4 expression is also enhanced in bone tumors (Fig. 5E), suggesting that CXCL12/CXCR4 signaling contributing to bone tumor growth in Akt1 transfected DU145 cells.

In summary, these data showed that PTEN loss-induced PI3K/Akt pathway induces CXCL12/CXCR4 expression, and this expression is particularly mediated by Akt kinase in both murine and human prostate cancer cells. Akt1-induced CXCR4 expression is active in CXCL12-induced cellular invasion, tumor growth, and intraosseous tumor growth in murine model systems. Given the frequency of PTEN gene alterations in advanced human prostate tumors and expression of CXCR4 in these patients, Akt1 signaling may be a therapeutic target for advanced prostate cancer patients.

Authors` contribution:

M. Katie Conley-LaComb participated in study design, performing experiments, data analysis and drafting of manuscript.

Allen Saliganan and Pridvi Kandagatla performed in vivo experiments and data analysis.

Yong Q. Chen participated in study design and PTEN knockout cell generation.

Michael L. Cher participated in study design and manuscript editing.

Sreenivasa R. Chinni is involved in conception and design of study, data preparation and analysis, manuscript drafting and revisions.

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Figure Legends

Figure 1. Loss of PTEN results in increased expression of CXCL12 and CXCR4 in murine prostate epithelial cells. A) Cell lysates were collected from $PTEN^{+/+}$, $PTEN^{+/-}$, and $PTEN^{-/-}$ cells and analyzed by Western blot for PTEN. **B)** mRNA expression levels of CXCL12 and its receptor, CXCR4, in $PTEN^{+/+}$, $PTEN^{+/-}$, and $PTEN^{-/-}$ cells were analyzed by quantitative RT-PCR**. C, D)** $PTEN^{-/-}$ cells were treated for 18 hours with increasing concentrations of Akt Inhibitor IV. Protein and mRNA expression levels were analyzed by Western blot (C) and quantitative RT-PCR (D), respectively. **E)** $PTEN^{-/-}$ cells were pretreated with or without 10μM Akt Inhibitor IV; $2x10^5$ cells were then plated on Matrigel coated inserts, allowed to invade for 24 hours, and stained with Crystal Violet. Total number of migrated cells was counted under 10X magnification in five fields. Assay was performed in triplicate. *: $p<0.005$.

Figure 2. Akt regulates CXCR4 expression in PTEN-null human prostate cells. A) Cell lysate was collected from BPH-1, C4-2B, and PC3 cells. Protein levels of PTEN and β-actin were analyzed by Western blot. **B)** BPH-1, C4-2B, and PC3 cells were treated for 18 hours with increasing concentrations of Akt Inhibitor IV. Protein levels were analyzed by Western blot. **C)** C4-2B *(left)* and PC3 *(right)* cells were pretreated with or without 1μM Akt Inhibitor IV; $2x10^5$ cells were then plated on Matrigel coated inserts, allowed to invade for 24 hours, and stained with Crystal Violet. Total number of migrated cells was counted under 10X magnification in five fields. Assay was performed in triplicate. *: $p<0.05$; **: $p<0.015$.

Figure 3. Overexpression of Akt results in increased phosphorylation of Akt, CXCR4 expression, and proliferation. A) DU145 cells were stably transfected with HA-tagged Akt1. Lysate was collected from serum-starved cells, and protein levels were analyzed by Western blot. **B)** Cells were cultured in the presence or absence of serum, lysate was collected, and

protein levels were analyzed by Western blot. **C)** Proliferation of DU145-Neo and DU145-HA-Akt1 cells was determined over three days. **D)** Cell cycle analysis of DU145-Neo and DU145-HA-Akt1 cells was performed. **E)** DU145-Neo or DU145-HA-Akt1 cells treated with 5 μM AMD3100 for two hours were plated on Matrigel coated inserts, allowed to invade for 24 hours, and stained with Crystal Violet. Total number of migrated cells was counted under 10X magnification in five fields. *: $p<0.01$

Figure 4. Overexpression of Akt results in increased subcutaneous tumor growth. DU145-Neo and DU145-HA-Akt1 cells were injected into mice subcutaneously. **A)** Tumor volume was measured over 80 days. **B)** Tissue sections from DU145-Neo and DU145-HA-Akt1 subcutaneous tumors were immunostained with antibodies directed against pAkt(S473) or CXCR4, or with negative control. Images were taken at 20X. Bar represents 50μM. **C)** Proliferation of DU145-Neo and DU145-HA-Akt1 tumors was analyzed by Ki67 staining; average number of Ki67+ cells of five 20X fields was determined. Bar represents 50μM.

Figure 5. Overexpression of Akt results in increased intratibial tumor growth. A) Cells were cultured in presence or absence of bone conditioned medium then analyzed by Western blot for pAkt(S473). **B)** Cells were injected into the tibiae of mice intratibially. After harvesting at 8 weeks, bones were analyzed by x-ray. **C)** Trabecular bone and tumor growth was analyzed at 8 weeks. **D)** H&E was performed on tissue sections from DU145-Neo and DU145-HA-Akt1 intratibial tumors. Images were taken at 5X and digitally merged. **E)** Tissue sections from DU145-Neo and DU145-HA-Akt1 intratibial tumors were immunostained with antibodies directed against pAkt(S473) or CXCR4. Images were taken at 20X and 40X (insert). Bar represents 50μM.

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