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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.

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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 × 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°C, and centrifuged at 10,000 × g; supernatant containing nuclear proteins was collected. For EMSA, 2μg of protein was incubated with IR Dye™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 5×10^5 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. 5×10^6 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°C. Subsequently, cells were treated with 200 ng/ml CXCL12 as shown in figure. 6×10^4 cells were seeded on plates and incubated for 1 hour at 37°C 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 4°C 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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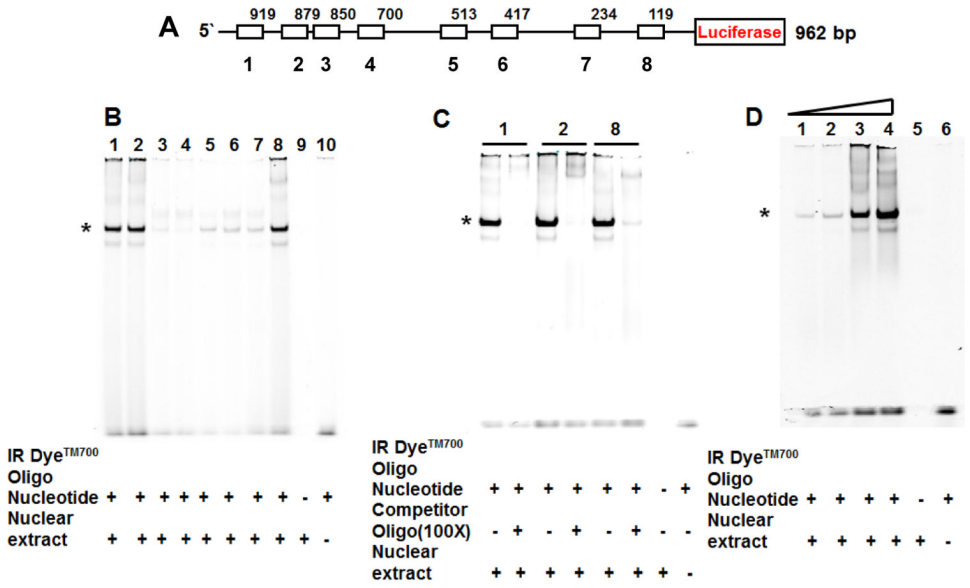


Figure 1. Electrophoretic mobility shift assay of CXCR4 promoter elements with VCaP cell nuclear extracts

(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).

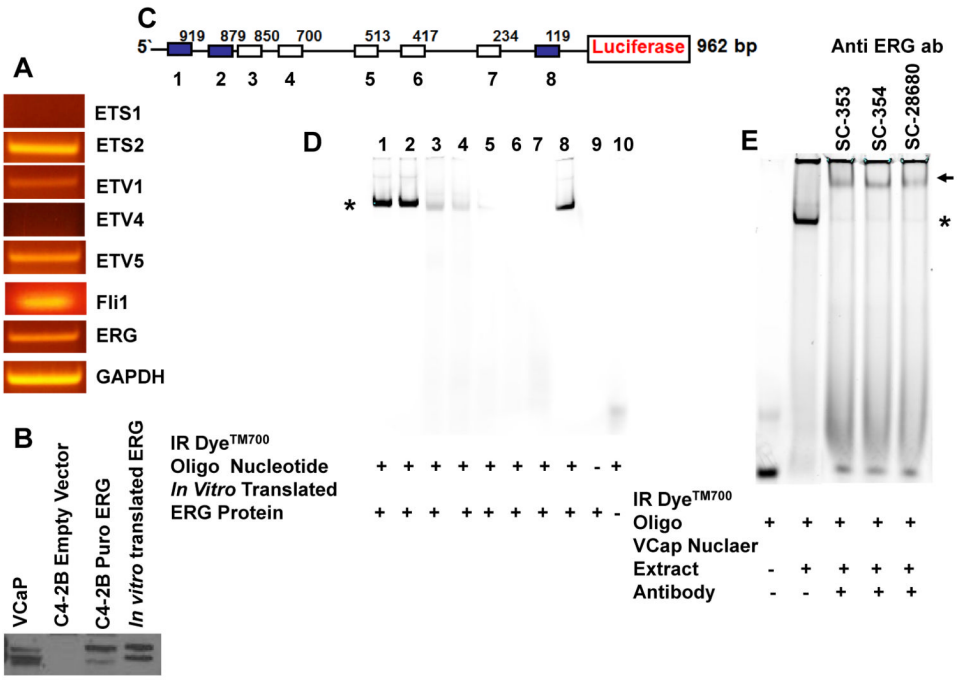


Figure 2. *In vitro* expressed ERG binds with CXCR4 promoter elements

(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.

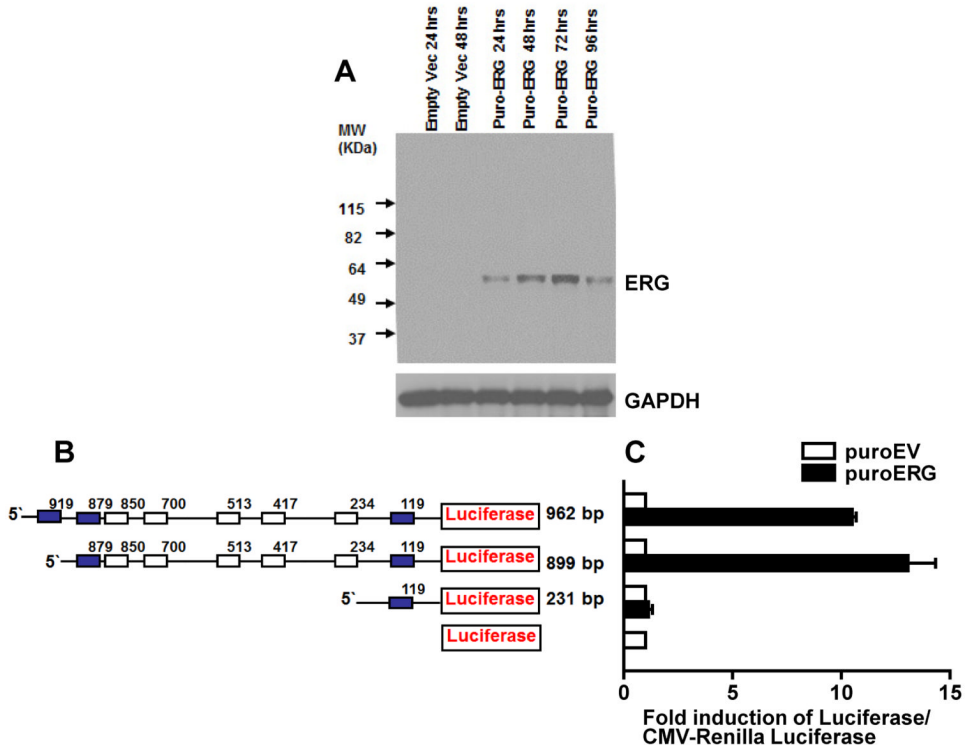


Figure 3. ERG regulates CXCR4 promoter activation

(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.

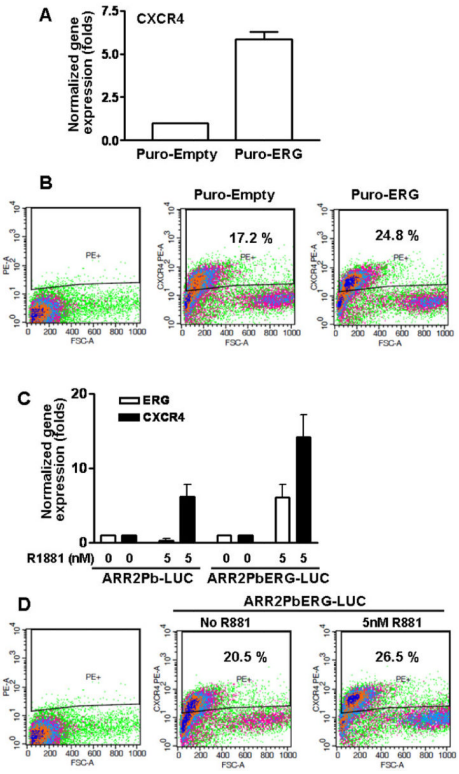


Figure 4. Androgen-induced ERG factor regulated CXCR4 cell surface expression

(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).

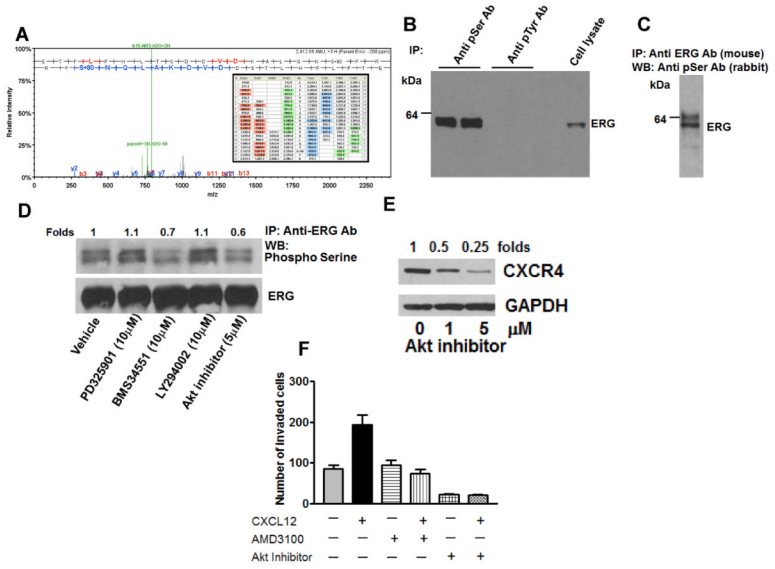


Figure 5. ERG is a phospho-Serine protein in VCaP cells, IKK and Akt kinases phosphorylate ERG, and Akt kinase regulates CXCR4 expression

(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.

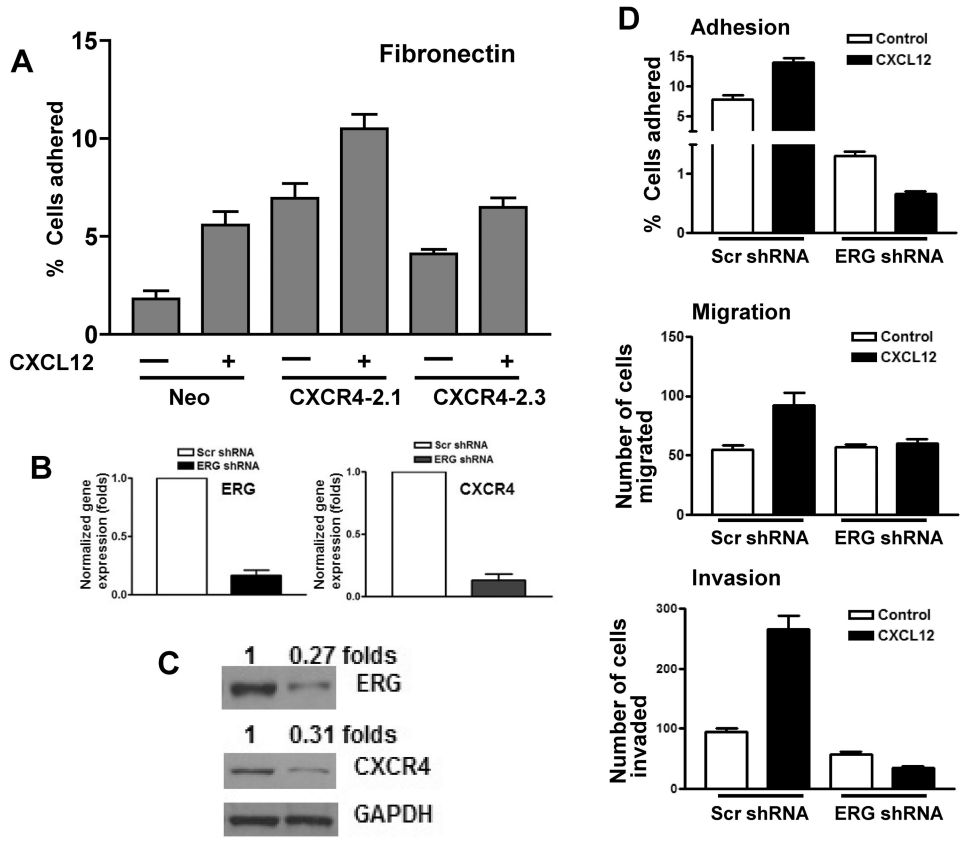
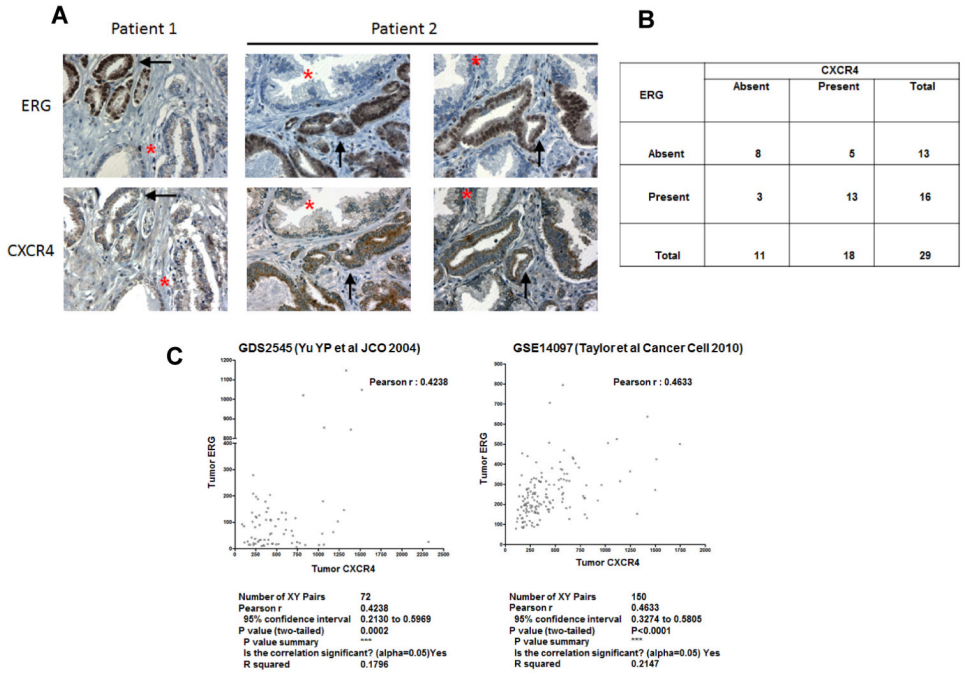


Figure 6. ERG-induced CXCR4 is functionally active in cellular adhesion

(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.



ERG	CXCR4		
	Absent	Present	Total
Absent	8	5	13
Present	3	13	16
Total	11	18	29

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.