CXCL12 ACTIVATES A ROBUST TRANSCRIPTIONAL RESPONSE IN HUMAN PROSTATE EPITHELIAL CELLS

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CXCL12 is a CXC-type chemokine that plays important roles in hematopoiesis, development, and organization of the immune system, and also supports the survival or growth of a variety of normal or malignant cell types. Our laboratory recently showed that CXCL12 is secreted by aging stromal fibroblast cells and is a major paracrine factor that specifically stimulates the proliferation of prostate epithelial cells. The current study shows that this CXCL12-mediated proliferative response may be either ERK-dependent or ERK-independent. Moreover, CXCL12 initiates a previously undefined and complex global transcriptional response in prostate epithelial cells. This CXCL12-mediated transcriptional response directly stimulates the expression of genes encoding proteins that are involved in the promotion of cellular proliferation and progression through the cell cycle, tumor metastasis, and cellular motility, and directly represses the transcription of genes encoding proteins involved in cell-cell adhesion and resistance to apoptosis. Thus, CXCL12 may play a major role in the etiology of benign proliferative disease in the context of an aging tissue microenvironment.

INTRODUCTION

Many studies have shown that paracrine interactions help regulate the proliferation of both stromal and epithelial cells in the prostate and other endocrine organs (1-5). However, previous studies have not been designed to examine how aging as a risk factor contributes to potential changes in stromal/epithelial paracrine interactions permissive for cellular proliferation. This is an important concern, as the coincident development of benign proliferative disease in the prostate with increasing patient age suggests that paracrine interactions may become dysfunctional and promote cellular proliferation as a consequence of the aging process. Moreover, the gradual increase in prostate volume with patient age that is characteristic of benign prostatic enlargement (BPE) suggests that mechanisms fostering low-level, but cumulative, cellular proliferation are involved in the etiology of this disease.

Recent studies from our laboratory utilizing an in vitro model system to examine the effects of the aging microenvironment on the development of benign proliferative disease in the human prostate demonstrated that paracrine interactions between human prostate epithelial cells and stromal fibroblasts are disrupted in aging prostate tissues. Specifically, these studies showed that the CXC-type chemokine, CXCL12, is secreted by aging human prostate stromal fibroblasts at sub-nanomolar quantities that stimulate the proliferation of prostate epithelial cells (6). CXC-type chemokines have two conserved cysteines separated by one unconserved amino acid. There are several members of the CXC-type chemokine family with both conserved and diverse functions, though all CXC-type chemokines signal though membrane-bound G-protein coupled receptors (GPCRs) (7). Many studies to-date have focused on the role of CXCL12 in the acquisition and expression of an invasive, metastatic phenotype in human solid tumors, most notably, breast and prostate cancer (8-18). Taken together, these studies suggested that CXCL12 may play a role in acquisition of the proliferative phenotype in benign, and perhaps malignant, proliferative diseases of the prostate.
CXCL12-mediated signaling activates molecules known to stimulate gene transcription and cellular proliferation, including ERK and NFκB (6,19). However, the definition and extent of the CXCL12-mediated transcriptional response, and the potential impact of this transcriptional response on expression of the proliferative phenotype, has not been elucidated. To address this issue, we have now evaluated the molecular signatures of prostate epithelial cells stimulated with sub-nanomolar levels of CXCL12 similar to those secreted by aging prostate stroma and known to stimulate cellular proliferation. These studies reveal that CXCL12-mediated signaling induces a robust transcriptional response in prostate epithelial cells that includes the up-regulation of genes encoding proteins that promote cellular proliferation and resistance to apoptosis, as well as the down-regulation of genes encoding proteins that normally act to promote apoptosis, cell-cell adhesion, and cell cycle arrest. This is the first report to show that CXCL12-mediated signaling induces a robust transcriptional response that may facilitate the acquisition of a proliferative and invasive phenotype by prostate epithelial cells.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures.** N15C6 cells, developed as described previously, were used at passages 35-45 and were maintained in 5% HIE media [Ham’s F12 (Mediatech Inc. Herndon, Virginia) with 5% FBS (Life Technologies, Inc.), 5 μg/ml insulin, 10 ng/ml EGF, 1 μg/ml hydrocortisone (Sigma Aldrich), 5 μM ethanolamine (Sigma Aldrich), 10 mM HEPES (Sigma Aldrich), 5 μg/μl transferrin (Sigma Aldrich), 10 μM 3,3’,5-triiodo-L-thyronine (Sigma Aldrich), 50 μM sodium selenite (Sigma Aldrich), 0.1% BSA (JRH Biosciences Lenexa, Kansas), 0.05 mg/ml gentamycin (Gibco), and 0.5 μg/ml fungizone (Cambrex Bioscience, Walkersville, Maryland). N15C6 cells are non-transformed prostate epithelial cells and grow continuously in culture but do not form colonies in soft agar or tumors in immunocompromised mice (20, 21). LNCaP cells, a widely-used transformed prostate epithelial cell line originating from a prostate cancer lymph node metastasis, were acquired from the American Type Culture Collection (ATCC# CRL-1740), were maintained in 10% RPMI media and 0.5 μg/ml fungizone, and were used at passages 25-35(22).

**Proliferation Assays.** Cellular proliferation was assessed after plating cells at 50,000 cell/well in triplicate in six well plates and counting cells after 24 and 96 hours of incubation as described previously (20). To assess the effects of exogenous CXCL12 on cellular proliferation, recombinant human SDF1 alpha/CXCL12 (R&D Systems, 350-NS) was added at the desired concentration in 1 ml SF HIE (or 1 ml SF HIE alone for control) to each well. The cells were re-fed at 48 and 72 hours growth and counted at 24 and 96 hours growth. Cell counts were normalized to 50,000 cells at 24 hours to account for any plating discrepancies. Averages and standard deviations of cell number were calculated for each time point under each media condition. Experiments utilizing U0126 were performed as above, except that cells were pre-treated for 2 hours with either .05% DMSO (vehicle) or .05% DMSO+5μM U0126 before the addition of CXCL12. Cells were maintained in media containing U0126 and/or DMSO for the entirety of the experiment. For the antibody blockade experiments, 10,000 cells per well were plated in triplicate in 24 well dishes and pre-incubated with mouse anti-human CXCR4 (BD Pharmingen 555971) and rabbit anti-human CXCR7/RDC1 (Abcam ab12870-50) or mouse anti-human CXCR2 (Biosource AHR1532X) at 1μg/ml for 1 hour prior to CXCL12 addition. Cells were maintained in media containing appropriate antibodies for the entirety of the experiment, and cells were counted at 24 and 96 hours as above.

**Affymetrix Human Genome U133 plus 2.0 Array Data Acquisition.** RNA was purified from trypsinized cultured cells by homogenization in Trizol (Invitrogen, Carlsbad, CA) and additional processing using the RNeasy (Qiagen, Valencia, CA) cleanup procedure. Ten ug of RNA was used to obtain labeled cRNA following the Affymetrix Standard
Expression intensity values for each gene were estimated using a method called Robust Multi-array Average (RMA) using tools available through Bioconductor (www.bioconductor.org). GeneChip gene expression values were normalized using a quantile normalization procedure.

Quantitative Real-Time PCR. All quantitative real-time assays were conducted as previously described with an Applied Biosystems 7900HT instrument and reagents (21). Cells were grown to 70% confluence in 60mM dishes prior to RNA purification using the Trizol reagent (Invitrogen Life Technologies). Experiments utilizing U0126 were performed as above, except that cells were treated with DMSO (vehicle) or DMSO+5uM U0126 for 2 hours, then grown in the presence or absence of CXCL12. For all experiments, one microgram of RNA was reverse transcribed by use of Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). The resulting cDNA was diluted 1:100. Real-time PCR was performed by use of Assays on Demand (Applied BioSystems, Foster City, CA) according to the manufacturer’s instructions. Reactions were performed in triplicate, including no-template controls and an endogenous control probe, RPLPO (ribosomal protein, large, PO), to assess template concentration. Cycle numbers to threshold were calculated by subtracting averaged control from averaged experimental values, and Fold Gene Expression was calculated by raising these values to the log 2. FAM conjugated, gene specific assays were Hs00152928_m1 for EGR1, Hs00193306_m1 for EGFR, Hs00170433_m1 for ERBB2, and Hs99999902_m1 for the control, RPLPO.

Western Blot and Protein Analysis. Cells were lysed, proteins resolved by electrophoresis, and electroblotting was carried out as described previously (6, 23). Proteins were detected using antibodies against phospho-ERK1/2 (Cell Signaling #9101), total ERK1/2 (Cell Signaling, #9102), CXCR4 (Abcam #ab2074), CXCR7 (aka RDC1, Abcam #ab12870), ELK-1 (Cell Signaling #9182), phospho ELK-1 (Cell Signaling #9181), and beta-actin (Santa Cruz #sc-1615), in conjunction with an ECL detection system. Secondary antibodies included goat anti-rabbit (Cell Signaling, #7074) and goat anti-mouse (Santa Cruz, #SC-2005), and both were used at a 1:5000 concentration. Immunoblots shown are representative of triplicate experiments. Densitometric quantitation of immunoblot films was accomplished by scanning the original films and converting the .tiff files to grayscale. Images were inverted, and mean band intensities were measured using ImageJ (www.nih.gov). The mean intensity of adjacent background was also measured for each band and subtracted from band intensity.

Statistical Analysis. Densitometric data from 3 experiments was averaged and standard deviations calculated for graphical depiction and statistical analysis. Normalized array-acquired transcript expression values were analyzed using a t-statistic test and by calculation of fold change between data sets. Genes that exhibited both a large t-statistic (> 10.0) and a large fold change (> 2.0) were considered to be differentially expressed. All other data was assessed by t-test or analysis of variance with p<.05 considered statistically significant.

RESULTS
ERK-dependence of CXCL12-mediated cellular proliferation.

Previous studies have shown that both transformed PC3 and LNCaP, as well as non-transformed N15C6 and BPH-1 prostate epithelial cells, express CXCR4, one of the receptors for CXCL12 (6, 19, 24). We now show that these cells also robustly express CXCR7, a recently-identified second receptor that recognizes CXCL12 (FIGURE 1A) (25). Our laboratory has previously shown that the stimulation of non-transformed N15C6 and transformed LNCaP cells with CXCL12 at sub-nanomolar levels similar to those secreted by aging prostate stromal fibroblasts induces these cells to proliferate (6). As seen in FIGURE 1B, N15C6 cells stimulated with 1pM or 10pM CXCL12 proliferate to levels significantly higher than those achieved by unstimulated cells (p<.001). This proliferation is maintained even after the cells are pre-treated with an antibody...
against an unrelated chemokine receptor, 
CXCR2 (FIGURE 1B). However, pre-
treatment with antibodies against the CXCL12-
specific receptor, CXCR4, or against both 
CXCR4 and CXCR7, significantly (p<.001) and 
equivalently ablated the ability of CXCL12 to 
stimulate proliferation. Thus, CXCL12-
stimulated cellular proliferation is mediated 
through interactions with receptors that 
specifically recognize this chemokine.

The Ras-mediated mitogen-activated 
kinase pathway is known to stimulate cellular 
proliferation. In this pathway, mitogen-activated 
kinase 1 (MEK1) phosphorylates extracellular 
regulated kinase (ERK), which in turn 
phosphorylates and activates other molecules, 
including transcription factors, involved in 
cellular proliferation. Treatment of prostate 
epithelial cells with nanomolar levels of 
CXCL12 has been shown to activate the 
MEK/ERK pathway and promote cytokine 
secretion, angiogenesis, and transendothelial 
cellular migration (19, 26). Our laboratory has 
also shown that both sub-nanomolar and 
nanomolar levels of CXCL12 stimulate 
MEK/ERK pathway activation in prostate 
epithelial cells (FIGURE 1C, D and E, and ref. 
6). However, LNCaP cells treated with 
CXCL12 at the sub-nanomolar levels that 
stimulate a proliferative response, e.g., 50 pM, 
demonstrate only a modest activation of ERK 
(FIGURE 1C). In contrast, N15C6 cells treated 
with CXCL12 at the sub-nanomolar levels that 
stimulate a proliferative response, e.g., 1 pM or 
10 pM, demonstrated rapid, transient ERK 
phosphorylation and activation (FIGURE 1D 
and E) (6). The observed differential extent of 
ERK activation in N15C6 and LNCaP cells 
treated with levels of CXCL12 known to 
stimulate proliferation in each cell line 
suggested that ERK activation might not be 
equivalently required for CXCL12-mediated 
proliferation in both cell lines. To test this, the 
the mitogen-activated protein kinase kinase 1 
(MEK1) inhibitor, U0126, was utilized to inhibit 
the ability of MEK1 to phosphorylate ERK, thus 
preventing ERK activation. As seen in FIGURE 
1D, treatment of N15C6 cells with 1pM, 10pM 
or 1000pM CXCL12 results in rapid and 
transient MEK1-mediated ERK 
phosphorylation. However, co-treatment of the 
cells with CXCL12 and 1.0 uM or 5.0 uM 
U0126 greatly diminishes ERK phosphorylation. 
Densitometric analysis of the immunoblot 
confirms these observations and shows that cells 
pre-treated with either concentration of inhibitor 
fail to phosphorylate ERK in response to 
treatment with CXCL12 (FIGURE 1E).

Moreover, the proliferative response of N15C6 
cells to CXCL12 is nearly ablated when the cells 
are co-treated with CXCL12 and 5.0 uM U0126 
in .05% DMSO compared to co-treatment with 
CXCL12 without inhibitor in .05% DMSO 
(FIGURE 1F). In contrast, the proliferative 
response of LNCaP cells to CXCL12 was not 
affected by co-treatment with U0126 (FIGURE 
1F).

CXCL12-mediated signaling activates ELK1 
and promotes EGR1 gene transcription.

Both N15C6 and LNCaP cells were 
extreamed for CXCL12-mediated activation 
of ELK-1, an Ets domain-type transcription 
factor which is itself activated through 
phosphorylation by both MAPK-dependent and 
independent mechanisms (27, 28). N15C6 and 
LNCaP prostate epithelial cells stimulated with 
CXCL12 demonstrated rapid and transient 
phosphorylation of ELK-1 as visualized by 
immoblot and quantitated by densitometry 
(FIGURE 2). ELK-1 is a transcriptional 
activator of the Early Growth Response 1 
(EGR1) gene, which encodes a C2H2-type zinc-
finger protein that is induced by mitogenic 
stimulation and has been shown to stimulate 
tumor cell growth, play a role in tumor 
progression, and stimulate angiogenesis and 
improved survival of tumor cells (29).

Quantitative RT-PCR assays showed that both 
N15C6 and LNCaP cells stimulated with low, 
picomolar levels of CXCL12 similar to those 
observed to promote N15C6 and LNCaP cellular 
proliferation rapidly and robustly accumulate 
EGR1 gene transcript (FIGURE 3A).

ERK-dependence of CXCL12-mediated 
EGR1 gene transcription.

The specificity of the EGR1 gene 
transcriptional response to CXCL12-mediated 
ERK signaling was next examined using the
mitogen-activated protein kinase kinase 1 (MEK1) inhibitor, U0126. For these experiments, cells plated in triplicate at 50,000 cells/well were pre-treated with .05% DMSO or .05% DMSO and 5 uM U0126 (Cell Signaling) for 2 hours, then stimulated with CXCL12 at concentrations shown previously to induce EGR1 gene transcription, e.g., 10 pM CXCL12 for N15C6 cells and 50 pM CXCL12 for LNCaP cells. As seen in FIGURE 3A, pre-treatment with the MEK1 inhibitor inhibited the EGR1 gene transcriptional response in both cell lines, though more profoundly in N15C6 than LNCaP cells. These results suggest that CXCL12-mediated EGR1 gene transcription is more ERK-dependent in N15C6 than LNCaP cells.

**CXCL12 stimulates a global transcriptional response in prostate epithelial cells that is partially ERK-dependent.**

We had previously reported that both non-transformed N15C6 and transformed LNCaP prostate epithelial cells respond proliferatively to low, picomolar levels of CXCL12, similar to those secreted by aging human prostate stromal fibroblasts (6). The observation that these same levels of CXCL12 stimulated ELK1 activation and EGR1 gene transcription raised the possibility that other genes may also be transcribed in response to CXCL12 stimulation. To explore this possibility, N15C6 and LNCaP cells were treated in replicate with CXCL12 and the MEK1 inhibitor, U0126 in .05% DMSO; CXCL12 and .05% DMSO; or .05% DMSO alone, for 30 minutes. The cells were immediately lysed and the purified RNA was subjected to gene expression profiling using Affymetrix Human Genome U133 Plus 2.0 Arrays. The results of these experiments showed that N15C6 cells demonstrated up-regulation of 370 genes and down-regulation of 162 genes at significance levels of p<.05 and fold levels >1.5 consequent to stimulation with 10 pM CXCL12. LNCaP cells demonstrated up-regulation of 185 genes and down-regulation of 413 genes consequent to stimulation with 50 pM CXCL12 (TABLE I). Thus, the transcriptional response of N15C6 cells to CXCL12 stimulation was largely positive and resulted in the up-regulation of the majority (370/532, or 70%) of genes differentially transcribed. In contrast, the transcriptional response of LNCaP cells to CXCL12 stimulation was largely negative and resulted in the transcriptional repression of the majority (413/598, or 69%) of genes differentially transcribed.

Although the gene transcription profiles induced by CXCL12 were largely different between N15C6 and LNCaP cells, 85 genes were transcribed in common (TABLE II). The use of both the Gene Ontology and Information Hyperlinked over Proteins (iHOP) databases permitted a limited functional assessment of proteins encoded by genes commonly transcriptionally down- or up-regulated consequent to CXCL12 stimulation (30). This assessment showed that the predicted functional consequences of loss of expression for many of the proteins encoded by the 52 commonly down-regulated genes and 33 up-regulated genes was consistent with promoting cellular proliferation or tumor progression. Quantitative real-time PCR of RNA purified from N15C6 or LNCaP cells treated for 30 minutes with CXCL12 demonstrates a robust and significant accumulation of transcript from the EGFR gene and a significant decrease in transcription of the ERBB2 gene compared to treated cells at 0 minutes, parallel to RNA profiling results obtained using Affymetrix GeneChips (FIGURE 3B and TABLE II).

**Differential ERK-dependence of the CXCL12-stimulated transcriptional response.**

Co-treatment of N15C6 or LNCaP cells with CXCL12 and 5uM U0126 reduced the number of differentially expressed genes in both cell lines compared to treatment with CXCL12 alone. The proportion of differentially expressed genes that were down-regulated consequent to treatment with CXCL12+U0126 was 91% (296/325 genes) for N15C6 cells and 45% (13/39) for LNCaP cells (TABLE III). Seven of the 39 genes affected transcriptionally by the treatment of LNCaP cells with both CXCL12 and U0126 were similarly affected in N15C6 cells, including ITGB4 (up-regulated) and CD44, EGR1, IL8, MAFF, TXNIP and ARRD4C4 (down-regulated). Reports in the literature suggested that the majority of these
genes are transcriptionally regulated by ERK 1/2 (ITGB4, CD44, EGR1, IL8) or inflammatory cytokines (MAFF) (30).

A principal components analysis (PCA) of the gene expression profiling data shows that N15C6 or LNCaP cells treated with .05% DMSO or CXCL12 and .05% DMSO are widely separated along the first two principal components, consistent with the observed robust CXCL12-stimulated transcriptional response exhibited by both cell lines (FIGURE 3C). Although the gene expression profiles of CXCL12 + DMSO-treated compared to CXCL12 + U0126 + DMSO-treated N15C6 cells are also distinctly separated in the PCA plot, those of similarly treated LNCaP cells demonstrate considerable overlap along the first principal component (PC1) and some overlap along the second principal component (PC2) (FIGURE 3C). This analysis is consistent with the observation that CXCL12-stimulated transcriptional responses in N15C6 cells is more ERK-dependent than that observed for LNCaP cells.

DISCUSSION

CXCL12 is a CXC-type chemokine that activates downstream signaling through binding the G-protein coupled receptors, CXCR4 or CXCR7. CXCL12 has been shown to play important roles in hematopoiesis, development, and organization of the immune system (31). Recent studies demonstrated that CXCL12 also supports the survival or growth of a variety of normal or malignant cell types. For example, Orimo et al. recently showed that carcinoma-associated fibroblasts (CAFs) extracted from human breast tumors promote the growth of breast carcinoma cells significantly more than those of normal fibroblasts, and that this effect was at least partially mediated by stromally-derived CXCL12 (32). We have previously reported that CXCL12 is secreted at sub-nanomolar levels by aging human prostate stromal fibroblasts, and that these levels of CXCL12 stimulate prostate epithelial cell proliferation (6). The current study shows that sub-nanomolar levels of CXCL12 similar to those secreted by aging fibroblasts stimulate a proliferative and robust transcriptional response in both pre-malignant and malignant prostate epithelial cells.

Consistent with data shown here, the MEK/ERK pathway can be activated in prostate epithelial cells by nanomolar levels of CXCL12. Activation of the MEK/ERK pathway at these levels of CXCL12 has been shown to promote cytokine secretion and transendothelial cellular migration by prostate epithelial cells (19, 26). Experiments reported here also show that prostate epithelial cells stimulated with sub-nanomolar levels of CXCL12, comparable to those secreted by aging prostate stroma, demonstrated differential ERK activation. Non-transformed N15C6 cells transiently but robustly activate ERK, whereas transformed LNCaP cells minimally activate ERK, in response to sub-nanomolar levels of CXCL12. Moreover, experiments utilizing the mitogen-activated protein kinase kinase 1 (MEK1) inhibitor, U0126, showed that ERK activation was required for CXCL12-mediated cellular proliferation in non-transformed N15C6, but not transformed LNCaP, prostate epithelial cells.

In addition to stimulating cellular proliferation, activated ERK can translocate from the cytoplasm to the nucleus and, in turn, phosphorylate and activate several downstream effectors, including the transcription factor, ELK-1 (33). ELK-1, in turn, can activate the promoters of multiple genes containing serum response elements (e.g., EGR1) or ternary complex factor (TCF) binding sites (34, 35). Although ERK phosphorylation in response to CXCL12 was clearly more robust in N15C6 compared to LNCaP cells, both cell lines demonstrated ELK-1 phosphorylation consequent to CXCL12 stimulation. Several studies have shown that ELK-1 can be phosphorylated and activated by multiple MAP kinases, including ERK, JNK and p38 (27, 28). Taken together, these results suggest that the common CXCL12-mediated proliferative and transcriptional responses observed for prostate epithelial cells may, in fact, be governed by multiple signaling pathways.
In addition to stimulating EGR1 gene transcription, the studies reported here show, for the first time, that CXCL12 stimulates a robust, global transcriptional response in both non-transformed N15C6 and transformed LNCaP prostate epithelial cells. Although these responses were largely contrary, that is, CXCL12-stimulated differential gene transcriptional was up-regulatory in N15C6 but repressive in LNCaP cells, they also resulted in the transcriptional regulation of a common set of genes that encoded proteins consistent with the promotion of cellular proliferation. In particular, the transcriptional down-regulation of several genes encoding proteins that normally promote cell cycle arrest, including TP53, MAFK, CUGBP1, CDK2 and CDK9 or resistance to apoptosis, e.g., HIPK3, MAPK8IP2 and CANX, by CXCL12 might functionally promote cellular proliferation. Similarly, the observed transcriptional down-regulation of genes encoding proteins involved in cell-cell adhesion, including CDH1, CTNNB1, CPSF1, EXOSC6, ITGB4, LOXL2, and SORBS3, or of genes involved in cytoskeleton organization, including MAPRE3, DOCK9, ARPC4, and MARCKS, could facilitate cellular motility, a trait associated with tumor progression and metastasis. Conversely, several genes that encode proteins that are over-expressed in many tumor types, some of which are functionally involved in cellular proliferation and tumor metastasis, including EGFR, CD44, ANKR D12, JMJD1C, and STRAP, were up-regulated consequent to CXCL12 stimulation. Moreover, two of these genes – CD44 and EGFR – are known to be transcriptionally induced by EGR1, which is itself encoded by a gene transcriptionally up-regulated by CXCL12. Thus, CXCL12 stimulates a complex transcriptional response which includes the common regulation of a set of genes encoding proteins involved in cellular proliferation.

The studies reported here were initiated to further understand how observed changes in stromal/epithelial paracrine interactions consequent to aging are permissive for cellular proliferation in the human prostate and may promote the development of benign prostatic enlargement (BPE). Previous studies have shown that BPE comprises a gradual increase in prostatic volume that occurs over decades of life. BPE is characterized by low-level, but cumulative, cellular proliferation that increases post-pubertal prostatic volume by approximately 0.8-1.6%, equivalent to only 0.2-0.4 ml, per year (36, 37). Our previous studies, as well as those shown here, demonstrate that CXCL12 secreted at sub-nanomolar levels by aging prostate stromal fibroblasts stimulates a significant, low-level proliferative response in prostate epithelial cells (6). Taken together, these findings suggest that the modest rate of cellular proliferation stimulated by CXCL12 in vitro essentially mimics the gradual rate of cellular proliferation characteristic of prostatic enlargement in vivo.

In summary, these studies show that, in addition to the activation of signaling pathways that trigger phenotypic responses typically associated with malignant transformation, e.g., cellular proliferation and motility/invasion, CXCL12 initiates a robust and complex transcriptional response in prostate epithelial cells. The potential net result of this transcriptional response is the promotion of these same phenotypic responses, including cellular proliferation and progression through the cell cycle, tumor metastasis, and cellular motility; impaired cell-cell adhesion, and resistance to apoptosis. Importantly, these effects are stimulated at low, sub-nanomolar levels of CXCL12 similar to those secreted by aging prostate stromal fibroblasts. Together, these results suggest that CXCL12 may be an important paracrine factor that plays a central role in the promotion of proliferative disease in the aging prostate. Future studies should elucidate how the proteins encoded by genes that are transcriptionally activated or repressed consequent to CXCL12-mediated activities contribute to the development of such disease.
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Figure 1. Receptor-specificity and ERK-dependence of CXCL12-mediated cellular proliferation.

A. Immunoblot demonstrating that PC3 (lane 1), LNCaP (lane 2), N15C6 (lane 3) and BPH-1 (lane 4) prostate epithelial cells abundantly express CXCR7 (aka RDC1), a receptor that binds CXCL12. The primary antibody concentrations used were 1:1000 for CXCR7 and 1:5000 for actin (as loading control).

B. N15C6 prostate epithelial cells grown for 96 hours in serum-free HIE media supplemented with 1pM CXCL12 (white bars) or 10pM CXCL12 (gray bars) proliferated to significantly higher levels than cells grown in serum-free HIE media alone (p<.001, indicated by *). Cellular growth following pre-incubation with an antibody against an unrelated chemokine receptor, CXCR2, followed by supplementation with CXCL12 and maintenance of growth in CXCL12+anti-CXCR2-containing media was similar to that observed for non-pre-treated cells grown in CXCL12-supplemented media and was significantly higher than that in serum-free HIE media alone (p<.001, indicated by *). Pre-incubation of cells for one hour with 1ug/ml antibody against CXCR4, a receptor for CXCL12, followed by supplementation with CXCL12 and maintenance of growth in CXCL12+anti-CXCR4-containing media significantly ablated the proliferative response (p<.001, indicated by #). Pre-incubation of cells for one hour with 1ug/ml antibodies against both known receptor for CXCL12, CXCR4 and CXCR7, followed by supplementation with CXCL12 and maintenance of growth in CXCL12+anti-CXCR4/CXCR7-containing media also significantly ablated the proliferative response (p<.001, indicated by #) though not significantly more so than that obtained upon pre-treatment with anti-CXCR4 alone. All data is shown normalized to growth in un-supplemented SF HIE media, which was set at 1-fold.

C. LNCaP cells demonstrate minimal ERK phosphorylation (pERK) upon treatment with 100 ng/ml epidermal growth factor (EGF) or 50pM CXCL12. Primary antibody concentrations used were 1:500 for phosphoERK and 1:2000 for total ERK. Phosphorylation of ERK relative to total ERK quantitated from the immunoblot is shown in the densitometry plot as pERK/tERK.

D. N15C6 cells grown in SF HIE media, pre-treated for 2 hours with the solvent control .05% DMSO, then supplemented with 1, 10, or 1000 pM CXCL12 demonstrate rapid and transient ERK phosphorylation (pERK) at 5 and 10 minutes post-stimulation compared to cells at the initiation of the experiment (time 0). Pre-treatment of the cells the MEK1 inhibitor, U0126 (dissolved in .05% DMSO), either reduces (1uM) or ablates (5uM) ERK phosphorylation. Primary antibody concentrations used were as described in B.

E. Densitometry plot of the immunoblots shown in C demonstrates phosphorylation of ERK relative to total ERK (pERK/tERK). All values are normalized to those obtained for the 0 time point, which was considered 1-fold for comparative purposes. Cells pre-treated with .05% DMSO then treated with 1, 10, or 1000 pM CXCL12 for 5 or 10 minutes demonstrated significantly higher levels of ERK phosphorylation than those at time 0 (p<.001, indicated by *). Cells pretreated with 1uM or 5uM U0126 in .05% DMSO then treated with 1, 10, or 1000 pM CXCL12 for 5 or 10 minutes demonstrated levels of ERK phosphorylation at or below those obtained at time 0 (p<.001, indicated by #)

F. N15C6 cells (black triangles) grown in SF HIE with .05% DMSO + 10pM CXCL12 (+) proliferated significantly better than those grown in SF HIE with .05% DMSO (-) (p<.001, indicated by *). Proliferation was significantly reduced in N15C6 cells (white triangles) grown under the same conditions but with the addition of 5uM U0126 (p<.001, indicated by #). LNCaP cells treated in a similar manner but supplemented with 50pM CXCL12 did not demonstrate significant differences in growth between cells treated with the solvent control (black squares) or inhibitor (white squares).
FIGURE 2. CXCL12 mediates the activation of ELK-1.

A. Immunoblot analysis shows that N15C6 cells demonstrate rapid and transient phosphorylation of the ELK-1 transcription factor (pELK-1) after exposure to 10pM or 1000pM CXCL12. Primary antibody concentrations used were 1:1000 for pELK-1 and 1:1000 for total ELK-1.

B. Densitometry plot of the immunoblots shown in A demonstrate phosphorylation of ELK-1 relative to total ELK-1 (p/ELK-1/ELK-1). All values are normalized to those obtained for the 0 time point, which was set at 1-fold for comparative purposes. Phosphorylation levels significantly higher than that those observed at time 0 are indicated by * (p<.05).

C. Immunoblot analysis shows that LNCaP cells demonstrate rapid and transient phosphorylation of the ELK-1 transcription factor (pELK-1) after exposure to 5pM, 50pM, or 500pM CXCL12. Primary antibody concentrations used were as in A.

D. Densitometry plot of the immunoblots shown in C demonstrate phosphorylation of ELK-1 relative to total ELK-1 (p/ELK-1/ELK-1). All values are normalized to those obtained for the 0 time point, which was set at 1-fold for comparative purposes. Phosphorylation levels significantly higher than that those observed at time 0 are indicated by * (p<.05).

FIGURE 3. CXCL12 mediates a robust transcriptional response.

A. Quantitative real-time PCR of RNA purified from N15C6 cells pre-treated with .05% DMSO then treated with 10pM CXCL12 (black triangles) or LNCaP cells pre-treated with .05% DMSO then treated with 50pM CXCL12 (black squares) demonstrates rapid and robust transcription of the EGR1 gene (p<.001, indicated by *). This response is significantly dampened when the cells are first pretreated for 2 hours with 5uM U0126 in .05% DMSO (N15C6 cells, white triangles; LNCaP cells, white squares) (p<.001, indicated by #).

B. Quantitative real-time PCR of RNA purified from N15C6 cells treated for 30 minutes with 10pM CXCL12 or LNCaP cells treated with 50pM CXCL12 (black bars) demonstrates a robust and significant accumulation of transcript from the EGFR gene (p<.001, indicated by *) and a significant decrease in transcription of the ERBB2 gene (p<.05, indicated by #) compared to treated cells at 0 minutes (grey bars), parallel to RNA profiling results obtained using Affymetrix GeneChips.

C. Principal Components analysis of the CXCL12-stimulated transcriptional response is shown for N15C6 and LNCaP cells in SF HIE media after a 2 hour pre-treatment with .05% DMSO (white dots, dotted ovals), the same but pre-treated with .05% DMSO and stimulated with 10pM (N15C6) or 50pM (LNCaP) CXCL12 (black dots, black ovals) or pre-treated with .05% DMSO + 5uM U0126 and stimulated with 10pM (N15C6) or 50pM (LNCaP) CXCL12 (grey dots, grey ovals). Each experiment was performed twice, and both sets of data are shown. The significant separation along both components (PC1 and PC2) between the transcriptional responses resulting from DMSO- and DMSO+CXCL12 treatments demonstrates that CXCL12 induces a robust transcriptional response in both cell lines. The separation between the transcriptional responses resulting from DMSO+CXCL12 and DMSO+CXCL12+U0126 treatments is greater for N15C6 than for LNCaP cells, suggesting that the CXCL12-mediated transcriptional response is more ERK-dependent for N15C6 than LNCaP cells.
TABLE I

Summary of CXCL12-Regulated Transcriptional Response in N15C6 and LNCaP Cells

|                                | UP-REGULATED | DOWN-REGULATED |
|--------------------------------|--------------|----------------|
| N15C6 Response to CXCL12      | 370          | 162            |
| N15C6 Response to CXCL12 + U0126 | 29           | 296            |
| LNCaP Response to CXCL12      | 185          | 413            |
| LNCaP Response to CXCL12 + U0126 | 13           | 26             |
| Down-Regulated Genes | Promoting Effect on Cellular Proliferation/Tumor Metastasis | Down-Regulated Genes | Promoting Effect on Cellular Proliferation/Tumor Metastasis | Up-Regulated Genes | Promoting Effect on Cellular Proliferation/Tumor Metastasis |
|----------------------|----------------------------------------------------------|----------------------|----------------------------------------------------------|-------------------|----------------------------------------------------------|
| CDH1                 | impairs cell-cell adhesion                               | TPS3                 | promotes progression through cell cycle                  | EGFR              | potentially upregulates cell proliferation               |
| CTNNB1               | impairs cell-cell adhesion                               | MAPK                 | promotes progression through cell cycle                  | CDK4              | promotes resistance to apoptosis                         |
| CSP8F1               | impairs cell-cell adhesion                               | CU08P1               | promotes progression through cell cycle                  | ANKR123           | metastasis repressor in prostate cancer                 |
| EXOC6                | impairs cell-cell adhesion                               | CDK2                 | promotes progression through cell cycle                  | SSBP1             | amplified in some retinoblastomas                        |
| ITGB4                | impairs cell-cell adhesion                               | CDK9                 | promotes progression through cell cycle                  | CNT1              | associated with transcriptional up-regulation            |
| LOXL2                | impairs cell-cell adhesion                               | HPK3                 | promotes resistance to apoptosis                           | JMD1C             | controls HIV transcript elongation (with CDK5)            |
| SORBS3               | impairs cell-cell adhesion                               | MAPK8P2              | promotes resistance to apoptosis                           | HNRPO             | family members amplified in some cancers                |
| GSR                  | impairs response to oxidative stress                     | CANX                 | promotes resistance to apoptosis                           | GOPC              | involved in post-transcriptional regulation of early response genes |
| RANOGAP1             | impairs formation of mitotic spindle, disrupts normal cell division | NR2F6                | impairs transcriptional repression                         | STRAP             | may upregulate Rho GTPase activity                      |
| NUMA1                | impairs formation of mitotic spindle, disrupts normal cell division |                      |                                                          | UNCLEAR           | overexpression activates mitogen-activated protein kinase, promotes anchorage-independent growth of the cells, frequently overexpressed in human breast tumors |
| RBM14                | impairs DNA repair, facilitates mutation?                |                      |                                                          |                   | transcrititionally regulated by Elk-1                    |
| SMH1                 | impairs Basement membrane assembly                       |                      |                                                          |                   |                                                        |
| SERBP2               | Expression is associated with apoptosis in prostate and colon tumors |                      |                                                          |                   |                                                        |
| MAPRE3               | facilitates cell motility                                |                      |                                                          |                   |                                                        |
| DOCK9                | facilitates (?) cell motility                            |                      |                                                          |                   |                                                        |
| ARP4                 | facilitates (?) cell motility                            |                      |                                                          |                   |                                                        |
| MARCKS               | facilitates (?) cell motility                            |                      |                                                          |                   |                                                        |
| Up-Regulated Genes | Function | Transcriptional Regulation | Down-Regulated Genes | Function | Transcriptional Regulation |
|-------------------|----------|-----------------------------|-----------------------|----------|----------------------------|
| ITGB4             | MULTIPLE: cell communication, cell adhesion, signaling | via Ets- and NFκB-binding site motifs in promoter | CD44     | cell-cell adhesion         | EGR1 via activated ERK 1/2 |
|                   |          |                             | EGR1     | transcription              | activated ERK 1/2           |
|                   |          |                             | IL8      | MULTIPLE: angiogenesis, cell motility, chemotaxis, 1/2, NFκB, p38, cell cycle arrest, signaling, inflammatory/immune response | transcription factors |
|                   |          |                             | MAF      | transcription             | inflammatory cytokines      |
|                   |          |                             | TXNIP    | keratinocyte differentiation | via E-box motifs in promoter |
|                   |          |                             | ARRDG4   | UNKNOWN                   | UNKNOWN                     |
**A.**

- CXCR7
- actin

**B.**

Fold Proliferation Relative to SF HIE

| Condition         | CXCR2 | CXCR4 | CXCR4+CXCR7 |
|-------------------|-------|-------|-------------|
| No Pre-Treatment  |       |       |             |
| 1pM CXCL12        |       |       |             |
| 10pM CXCL12       |       |       |             |

**C.**

pERK/ERK

| Treatment Time (min) | 0  | 5  | 10 | 15 | 20 |
|----------------------|----|----|----|----|----|
| pERK/ERK             | 1  | 1  | 1  | 1  | 1  |

**D.**

Fold Proliferation

- DMSO
- 1uM U0126
- 5uM U0126

**E.**

pERK/ERK

| Condition         | 5 min | 10 min |
|-------------------|-------|--------|
| DMSO, 1uM U0126   |       |        |
| DMSO, 5uM U0126   |       |        |
| DMSO, 10 min U0126|       |        |
| DMSO, 10 min U0126|       |        |

**F.**

Fold Proliferation

- LNCaP+CXCL12
- LNCaP+CXCL12+U0126
- N15C8+CXCL12
- N15C8+CXCL12+U0126

- CXCL12
