NF-κB Promotes Breast Cancer Cell Migration and Metastasis by Inducing the Expression of the Chemokine Receptor CXCR4

Gregory Helbig¹, Kent W. Christopherson II§, Poornima Bhat-Nakshatri ||, Suresh Kumar¹, Hiromitsu Kishimoto¹, Kathy D. Miller¶, Hal E. Broxmeyer§¶, and Harikrishna Nakshatri ‡¥

Departments of ‡Surgery, §Microbiology and Immunology, ¶Medicine, ¥Biochemistry and Molecular Biology, ||Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana 46202; ‡Walther Cancer Institute, Indianapolis, Indiana 46208

†Contributed equally to this work

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*Corresponding Author: Harikrishna Nakshatri
R4-202 Indiana Cancer Research Institute
1044 West Walnut Street
Indianapolis, IN 46202
Phone 317 278 2238
Fax 317 274 0396
E-mail: hnakshat@iupui.edu
SUMMARY

Metastasis of cancer cells is a complex process involving multiple steps including invasion, angiogenesis, and trafficking of cancer cells through blood vessels, extravasations, organ-specific homing, and growth. While matrix metalloproteinases (MMPs), urokinase plasminogen activator (uPA) and cytokines play a major role in invasion and angiogenesis, chemokines such as SDF-1α and their receptors such as CXCR4 are thought to play a critical role in motility, homing and proliferation of cancer cells at specific metastatic sites. We and others have previously reported that the extracellular signal-activated transcription factor NF-κB upregulates the expression of MMPs, uPA and cytokines in highly metastatic breast cancer cell lines. In this report, we demonstrate that NF-κB regulates the motility of breast cancer cells by directly upregulating the expression of CXCR4. Overexpression of the inhibitor-of-kappaB (IκB) in breast cancer cells with constitutive NF-κB activity results in reduced expression of CXCR4 and a corresponding loss of SDF-1α mediated migration in vitro. Introduction of CXCR4 cDNA into IκB-expressing cells restored SDF-1α mediated migration. Electrophoretic mobility shift assays and transient transfection assays revealed that the NF-κB subunits p65 and p50 bind directly to sequences within the 66 to +7 region of the CXCR4 promoter and activate transcription. We also show that the cell surface expression of CXCR4 and the SDF-1α mediated migration is enhanced in breast cancer cells isolated from mammary fat pad xenografts compared to parental cells grown in culture. A further increase in CXCR4 cell surface expression and SDF-1α mediated migration was observed with cancer cells that metastasized to the lungs. Taken together, these results implicate NF-κB in the migration and the organ-specific homing of metastatic breast cancer cells.
INTRODUCTION:

Morbidity and mortality in cancer are mainly due to organ-specific metastasis and the failure of chemotherapeutic drugs to selectively kill cancer cells at the sites of metastasis. Metastasis is a non-random process and each cancer type has its own preferred sites of metastasis (1). For example, breast cancer cells preferentially metastasize to the regional lymph nodes, lungs, liver, and bone (1,2). Prostate cancers usually metastasize to bone. While there has been a considerable progress in identifying genes that promote the metastasis of cancer cells, little is known about the genes that enable cancer cells to seed, survive, and proliferate at sites of metastasis. Three models of organ-specific metastasis are currently under consideration: 1) Selective survival and proliferation of cancer cells in a particular organ due to local production of appropriate growth factors. 2) Organ-specific endothelial cells trap circulating tumor cells by expressing appropriate adhesion molecules on their surface. 3) Organ-specific attractant molecules helping in the homing cancer cells to specific sites (3). While data supporting the first two models are still scanty, a recent study provided evidence supporting the third model. Muller et al., (4) demonstrated that metastatic breast cancer cells overexpress the chemokine receptor CXCR4. Additionally, sites to which breast cancer cells metastasize express abundant amounts of stromal derived factor-1 (SDF-1α, recently renamed CXCL12), the ligand for CXCR4. Moreover, antibodies against CXCR4 significantly inhibited lymph node and lung metastasis in xenograft models of breast cancer. These results suggest that SDF-1α serves as a homing factor for cancer cells and the signaling pathways activated upon interaction of CXCR4 with SDF-1α play a role in the survival and proliferation of cancer cells once they are localized in a specific organ.

The transcription factors that regulate CXCR4 expression in breast cancer cells are currently unknown. We considered the possibility that the extracellular signal-activated transcription factor NF-κB is involved in the expression of CXCR4 because NF-κB has been shown to upregulate the expression of several pro-metastatic and pro-angiogenic genes including interleukin 6 (IL-6), IL-8, urokinase plasminogen activator (uPA), matrix metalloproteinase 9 (MMP9), and vascular endothelial growth factor (5-8). In addition, inhibitors of NF-κB have been shown to reduce metastasis in xenograft
models (9). NF-κB is a heterodimeric complex of Rel family proteins that is physically confined to the cytoplasm of normal cells through its interaction with inhibitor-of-kappaB (IκB) proteins (6). A heterodimer composed of p50 and p65 subunits is the predominant form of NF-κB, although several other cell type-specific heterodimers have been identified. Upon exposure of cells to growth factors and cytokines such as epidermal growth factor (EGF), interleukin 1 (IL-1), and tumor necrosis factor alpha (TNFα), a series of signaling events target IκB for degradation, promoting the nuclear translocation of NF-κB. NF-κB binds to its response elements (5’ GGGPuNNNPyPyCC 3’) in the promoter region of target genes and activates transcription (5). CXCR4 may be one of the NF-κB target genes, as a putative NF-κB binding site (5GAGGCATTTCC3, 230 to 240) is present in the promoter region of CXCR4 (10).

Several laboratories, including ours, have demonstrated constitutive activation of NF-κB in a variety of cancers (11-17). We have shown that constitutively active NF-κB is responsible for overexpression of pro-metastatic and anti-apoptotic genes in breast cancer cells (14,18-20). In addition, we and others have shown that cancer cell-derived heregulin, IL-1α and/or overexpression of epidermal growth factor receptor (EGFR) is involved in constitutive NF-κB activation in breast cancer (21-24). The present study was initiated to determine whether NF-κB promotes organ-specific metastasis by selectively upregulating CXCR4. We demonstrate that NF-κB directly regulates the expression of CXCR4, which appears to be critical for the motility of cancer cells in response to SDF-1α in vitro. In addition, we also show that cells that express CXCR4 are clonally selected during their growth in the mammary fat pad of nude mice. A further increase in CXCR4 expression and SDF-1α-mediated migration was observed in cancer cells that metastasized to the lungs.
EXPERIMENTAL PROCEDURES:

Breast cancer cell lines. The human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from the ATCC and grown in MEM + 10% FCS. LxSN11, IxBαSR6 and IxBαSR10 cells, which are derived from MDA-MB-231 cells, have been previously described (19).

Plasmid constructs and transient transfection assays. The CXCR4 promoter was recloned by PCR using genome walker kit (Clontech). PCR amplified DNA was cloned into the pBL-CAT3 vector and sequenced. Deletion mutants were also generated by PCR. The p65, p50, Bcl-3 expression vectors were a generous gift from Dr. W. Greene (Gladstone Institute for Virology and Immunology, San Francisco, CA). The expression vectors for NIK, IKKα and IKKβ were generous gift from Dr. D. Donner (Indiana University School of Medicine). The CXCR4 expression vector was a kind gift from Dr. G. Alkhatib (Indiana University School of Medicine). MCF-7 and MDA-MB-231 cells were transfected with the CXCR4/CAT reporter and expression vectors along with a β-galactosidase expression vector (RSVβ-gal and pcH110 for MCF-7 and MDA-MB-231, respectively) by the calcium phosphate method. The β-galactosidase activity and CAT activity in an equal number of β-galactosidase units were measured as previously described (14).

Electrophoretic mobility shift assay (EMSA). COS-1 cells were transfected with 10 µg of expression vectors and whole cell extracts or nuclear extracts were prepared 48 hour after transfection as previously described (14,19). EMSA was performed with CXCR4, NF-κB and SP-1 probes as previously described (14).

RNase protection assay. Total RNA was prepared using RNeasy kit (Qiagen Inc). The RNase protection assay with hCR6 or hAPO-5 probes (BD PharMingen) was performed as previously described (19). The hCR6 probe set simultaneously measures the expression of CXCR1, CXCR2, CXCR3, CXCR4, BLR-1, BLR-2, and V28. The hAPO-5 probe measures XIAP, TRAF-1, TRAF-2, TRAF-3, TRAF-4, cIAP-1, cIAP-
2, and TRPM-2.

**Mouse mammary fat pad injection and isolation of cancer cells.** MDA-MB-231 cells (10^6) were injected into the mammary fat pad of 7-week-old nude mice. Mammary tumors were resected after six weeks, minced and digested for one hour at 37°C in digestion buffer (2 mg/ml collagenase type 3, 1 mg/ml hyaluronidase in PBS, 10 ml/gm of tumor). Cell pellets were digested again for 20 minutes at 37°C with protease (12.5 mg/ml in PBS). Cell pellets were washed in PBS and plated in growth media. Metastatic cells from the lungs were similarly isolated with the exception that the lungs were collected eight weeks after removal of the primary tumor. CXCR4 expression was measured periodically in cultured tumor- and metastasis-derived cancer cells. Data presented in the text were obtained from cells cultured for one month.

**Cell surface expression of CXCR4.** Cell surface expression of CXCR4 was measured by flow cytometry. MDA-MB-231 cells were stained with fluorochrome-conjugated monoclonal antibodies to either CXCR4 or an isotype control (BD PharMingen) in accordance with the manufacturer’s specifications and then analyzed by flow cytometry (25). The staining protocol used is as follows. Cells were first washed in PBS/Pen/Strep/1% BSA and resuspended in 100 µl PBS/Pen/Strep/1% BSA containing the appropriate antibody. Samples were mixed and incubated at 4°C in the dark for 40 minutes. The cells were then washed twice in PBS/Pen/Strep/1% BSA and fixed in PBS/1% paraformaldehyde. One hundred thousand events were accumulated for each analysis. Samples were analyzed in triplicate and the data was averaged for statistical analysis. Data is presented as mean ± standard error of the mean (SEM) and comparisons were made using the two-tailed student’s t-test.

**Chemotaxis Assay.** Chemotaxis assays were performed using 96-well chemotaxis chambers (NeuroProbe) in accordance with the manufacturer’s instructions as previously described with minor variations (25). Briefly, 0, 12.5, 25, 50, 100, 200, 400, or 800
ng/mL of CXCL12/SDF-1α was added to 300 µL of phenol red-free RPMI supplemented with 10% FBS in the lower chamber. Twenty-five thousand fluorescent-tagged (4 µg/mL Calcein AM, Molecular Probes, Eugene, OR) cells in 50 µL of media were added to the upper chamber, separated from the lower chamber by a membrane (5.7 mm diameter, 5 µm pore size, polycarbonate membrane). Total cell migration was obtained by measuring the fluorescence (excitation 485nm, emission 530nm) on a microplate spectrofluorometer and calculating the cell number in the lower chamber in comparison to a cell number standard curve after 2 hours of incubation at 37° C, 5% CO2. Percentage migration was calculated by dividing the number of cells in the lower chamber by the total cell input multiplied by 100 and subtracting random migration (always less than 7%) to the lower chamber in the absence of SDF-1α. Three samples were analyzed separately in triplicate, and the data was averaged for statistical analysis. Data is presented as mean ± standard error of the mean (SEM) and comparisons were made using the two-tailed students t-test.
RESULTS:

MDA-MB-231 cells overexpressing IκBα super-repressor display a lower levels of CXCR4 mRNA.

We previously reported the generation of MDA-MB-231 breast cancer cells overexpressing the IκBα super-repressor (19). MDA-MB-231 cells contain constitutively active NF-κB and express a number of pro-metastatic (uPA, IL-6 and IL-8) and anti-apoptotic genes (cIAP-2 and TRAF-1) in an NF-κB dependent manner (18-20). Constitutive NF-κB DNA binding activity and cIAP-2 expression were lower in IκBα super-repressor expressing cells (IκBαSR6 and IκBαSR10) compared to cells transduced with retrovirus vector alone (LxSN11) (19). To determine whether NF-κB similarly regulates the expression of CXCR4 in these cells, we performed an RNAse protection assay with RNA from LxSN11, IκBαSR6 and IκBαSR10 cells. CXCR4 transcripts could be detected in LxSN11 cells but not in IκBαSR6 and IκBαSR10 cells (Fig. 1A). To further confirm the involvement of NF-κB in CXCR4 expression, an RNAse protection assay was performed with RNA from untreated and parthenolide-treated MDA-MB-231 cells. Parthenolide is a relatively specific inhibitor of NF-κB and has been used by a number of investigators for in vitro studies (26). Parthenolide reduced the expression of CXCR4 in MDA-MB-231 cells (Fig. 1B). To determine whether IL-1α, a potent inducer of NF-κB, alters CXCR4 expression, we performed RNAse protection assay with RNA from MCF-7 breast cancer cells containing the empty vector (pcDNA3) or cells that stably overproduce IL-1α. These cell lines have been described elsewhere (Kumar et al., submitted). Consistent with the role of NF-κB in CXCR4 expression, IL-1α overproducing cells displayed elevated CXCR4 compared to cells with the pcDNA3 vector (Fig. 1C).

To ensure that there is a correlation between CXCR4 mRNA and cell surface expression of the CXCR4 protein, we performed flow cytometric analysis with an antibody that specifically recognizes CXCR4 on the cell surface. Cell surface expression of CXCR4 was observed in MCF-7 cells that overexpress IL-1α but not in MCF-7-pcDNA3 cells (Fig. 2). Taken together, these results indicate that CXCR4 expression in breast cancer cells is regulated by NF-κB, as well as the cytokines that induce NF-κB.
NF-κB directly regulates the CXCR4 promoter.

To determine whether NF-κB directly regulates CXCR4 promoter activity, we performed a transient transfection assays in MCF-7 breast cancer cells with a CXCR4/CAT reporter. Two reporters, one with the 897 to +7 region [CXCR4(-897)/CAT] and the other with the 209 to +7 [CXCR4(-209)/CAT] region of the CXCR4 promoter were used. Reporter activity was measured in the absence or presence of the NF-κB inducers TNFα, IL-1α or TPA. IL-1α and TPA induced CXCR4/CAT reporter activity (Fig. 3A). Constitutive CXCR4 promoter activity in MDA-MB-231 cells was reduced by IκBαSR (Fig. 3B). Induction of CXCR4(-209)/CAT by IL-1α and TPA suggests that induction of CXCR4 by IL-1α and TPA is an indirect effect of NF-κB activation or that the NF-κB binding site in the CXCR4 promoter is distinct from the putative site predicted from the TFSEARCH computer program. To distinguish between these two possibilities, we generated additional deletion mutants (CXCR4(-121)/CAT and CXCR4(-66)/CAT) and tested them for activity in the presence of various subunits of NF-κB. The p65 but not the p50 subunit of NF-κB increased the activity of both reporters (Fig. 3C). Among the upstream kinases involved in NF-κB activation, NIK activated the CXCR4 promoter. IKKα but not IKKβ activated CXCR4(-897)/CAT. Although the basal activity of CXCR4(-66)/CAT was five times lower than CXCR4(-121)/CAT, the p65 subunit still activated this reporter. These results suggest that the NF-κB response element is located within the 66 to +7 sequence of the CXCR4 promoter.

We next determined direct binding of the NF-κB subunits to the 66 to +7 region of the CXCR4 promoter by electrophoretic mobility shift assays (EMSA) using extracts from COS-1 cells transfected with various subunits of NF-κB and the 66 to +7 region of CXCR4 as a probe. The p50 subunit, either alone or in combination with the p65 subunit bound to the probe (Fig. 4, lanes 1-4). Similar results were obtained when nuclear extracts instead of whole cell extracts were used (Fig. 4, lanes 5-7). Neither c-Rel nor Bcl-3 bound to this region (data not shown). Unlabelled oligonucleotide with a classical NF-κB binding site from the immunoglobulin promoter but not the SP-1 binding site reduced the CXCR4 probe:protein complex formation (Fig. 4, lanes 8-10). The CXCR4 DNA probe:protein complex could be disrupted by an antibody against p65 and
supershifted partially by an antibody against p50 (lanes 11-14). A non-specific antibody (against the p110 subunit of PI3 kinase) had no effect on the CXCR4 probe:protein complex (lane 14). These results indicate that the p50 and p65 subunits of NF-κB directly bind to the CXCR4 promoter. Because this region of the promoter lacks a classical NF-κB response element, it appears that the p50 and p65 subunits bind to a non-classical response element. The exact p50 and p65 binding site sequence is yet to be determined because results of a DNAse I foot-printing assay were inconclusive (data not shown). Also, we did not detect the binding of NF-κB subunits using EMSA when the probe contained only the 66 to 33 or the 32 to +1 regions of the CXCR4 promoter (data not shown). Thus, it appears that NF-κB DNA binding requires the entire 66 to +1 region.

**NF-κB regulates SDF-1α-mediated migration of MDA-MB-231 cells through CXCR4.**

To determine the consequence of NF-κB inhibition on SDF-1α mediated migration of MDA-MB-231 cells, we performed a chemotaxis assay with LXSN11, IκBαSR6 and IκBαSR10 cells with increasing concentrations of SDF-1α. SDF-1α induced motility was observed with LxSN11 cells, but not with IκBαSR6 and IκBαSR10 cells (Fig. 5A). We confirmed the requirement of NF-κB for the SDF-1α-induced migration of MDA-MB-231 cells by performing chemotaxis assay with cells pre-treated with parthenolide. Parthenolide at 5 µM completely inhibits constitutive NF-κB DNA binding activity in these cells (19). Inhibition of SDF-1α dependent migration was observed with cells pre-treated with parthenolide (Fig. 5B).

Recent studies indicated that NF-κB regulates migration of MDA-MB-231 cells through upregulation of uPA (27). In that case, the failure of IκBαSR cells and parthenolide pre-treated cells to migrate in response to SDF-1α could be due to reduced uPA instead of CXCR4 expression. To directly prove that reduced SDF-1α dependent migration of IκBαSR cells is due to lower levels of CXCR4 in these cells, we reintroduced CXCR4 by transient transfection and performed a chemotaxis assay. SDF-1α dependent migration was restored in IκBαSR6 and IκBα10 cells upon reintroduction of CXCR4 cDNA (Fig. 5C). These results confirm that NF-κB is directly involved in SDF-
α mediated migration of breast cancer cells.

**MDA-MB-231 cells selected after growth in nude mice express higher levels of cell surface CXCR4.**

Although a major function of SDF-1α is to transiently upregulate the expression of integrins involved in the binding of CXCR4-expressing cells to the endothelium and egress from the circulation, SDF-1α is also known to activate other growth-promoting signaling pathways (28,29). If that is the case, unlike in cell culture models, cancer cells that express CXCR4 should grow well in a xenograft model, as these cells can take advantage of circulating SDF-1α. Because flow cytometry with an antibody against CXCR4 revealed that less than 10% of MDA-MB-231 cells grown in culture express CXCR4 on their surface, our hypothesis was that only those cells that express higher levels of CXCR4 should grow in nude mice. Furthermore, cells expressing CXCR4 should metastasize to organs that express SDF-1α, such as the lungs. To test this possibility, we implanted MDA-MB-231 cells into the mammary fat pad of nude mice, resected the tumors after six weeks and allowed the cancer cells isolated from the tumor to grow in culture. After an additional eight weeks, cells from the lungs of these mice were cultured. MDA-MB-231 cells isolated after their growth in the mammary fat pad (named TMD231 hereafter) or those that metastasized to the lungs (hereafter called LMD231) expressed very high levels of CXCR4 compared to parental cells grown in culture (Fig. 6A-C). In fact, the highest CXCR4 expression was seen in lung metastatic cells. Increased cell surface expression of CXCR4 in tumor- or lung-derived cells were maintained even after three months of growth in culture, which suggests that there is clonal selection of CXCR4-expressing cancer cells in the mammary fat pad. TMD231 and LMD231 cells are free of contaminating mouse cells as indicated by cell surface expression of the epithelial cell surface antigen (ESA) (Fig. 6D-F). Both TMD231 and LMD231 cells showed enhanced SDF-1α mediated migration compared to cells grown in culture (Fig. 6G). CXCR4 mRNA levels were increased in TMD231 and LMD231 cells compared to MDA-MB-231 cells, as determined by an RNase protection assay using a human-specific CXCR4 probe (Fig. 7A). All three cell types expressed similar levels of
XIAP, TRAF-3 and TRAF-4 transcripts, which suggests that there is no global increase in transcription in LMD231 and TMD231 cells compared to parental cells (Fig. 7B). Thus, it appears that cancer cells that express CXCR4 on their surface are selected during growth in the mammary fat pad, which could help cancer cells to respond to SDF-1α mediated growth as well as migration signals. In addition, these CXCR4-expressing cells have a higher potential to metastasize to the lungs.

We next examined whether inhibitors of NF-κB can reduce CXCR4 expression in LMD231 cells. Cells were incubated with either parthenolide (5 µM) or MG132 (10 µM), a proteosomal inhibitor that inhibits NF-κB activation by reducing IκBα, for four or eight hours. Parthenolide inhibited CXCR4 expression by ~50% whereas MG132 reduced CXCR4 expression by ~80% after eight hours treatment (Fig. 7C). Thus, NF-κB inhibitors have the potential to reduce growth and survival of cancer cells at metastatic sites through inhibition of CXCR4 expression.
DISCUSSION:

In this report, we show that the extracellular signal-activated transcription factor NF-κB regulates the expression of the chemokine receptor CXCR4, which has recently been implicated in organ-specific metastasis of breast cancer (4). NF-κB-dependent expression of CXCR4 appears to require a non-classical response element present within the 66 to +7 sequence of the CXCR4 promoter. This response element binds to either the p50 homodimer or p65:p50 heterodimer in vitro. Because the NF-κB:DNA complex generated with the CXCR4 promoter fragment and a classical response element displayed a similar mobility pattern in EMSA, it is less likely that interaction of NF-κB subunits to the CXCR4 promoter is facilitated by additional transcription factors. Moreover, the reporter gene containing only the 66 to +7 region of the CXCR4 promoter was responsive to NF-κB, which implies that the transactivation by NF-κB subunits is direct. A number of known activators of NF-κB, including TPA and CD30, have previously been shown to induce CXCR4 (10,30). Because of a lack of the classical NF-κB-related binding sites in the promoter, it was suggested that transcription factors such as AP-1, SP-1 and NRF-1 are responsible for constitutive and inducible expression of CXCR4. This study provides more compelling evidence for the direct involvement of NF-κB in the regulation of CXCR4 expression. Mapping of the precise NF-κB binding site within the 66 to +7 region may require additional studies such as chromatin immunoprecipitation assays.

Vascular endothelial growth factor (VEGF) has been shown to induce CXCR4 in breast cancer cells (31). Because VEGF is a NF-κB inducible gene (32), it is possible that the regulation of CXCR4 by NF-κB may be indirect in some cell types. However, VEGF may not be responsible for the increased expression of CXCR4 in IL-1α overexpressing MCF-7 cells as both parental and IL-1α overexpressing cells contained similar levels of VEGF transcripts (data not shown). Because both MCF-7 and MDA-MB-231 cells do not express IL-8 receptors CXCR1/2, IL-8 is less likely involved in CXCR4 expression (data not shown, also the riboprobe used in Fig. 7 measures CXCR1/2). Taken together, our results as well as published results by others suggest both direct and indirect regulation of CXCR4 expression by NF-κB in breast cancer cells.

Most of the current work on breast cancer metastasis focuses on the role MMPs...
and uPA/uPAR/plasminogen network (33,34). It is believed that the uPA/uPAR/plasminogen network activates pro-MMP-1, MMP-3, MMP-9 and MMP-13 produced by stromal cells. Activated MMPs breakdown the physical barriers of metastasis, thus promoting invasion, intravasation and extravasation of cancer cells (33,34). In addition, MMPs promote the growth of cancer cells at both primary and metastatic sites. We and others have shown that the expression of uPA and MMPs in cancer and stromal cells is regulated by NF-κB (8,18). The present report adds CXCR4 to the list of pro-metastatic genes under the control of NF-κB. Thus, inhibitors of NF-κB should reduce breast cancer metastasis by reducing the expression of a number of pro-metastatic genes. NF-κB inhibitors should also reduce metastasis of other cancers, as several cancers including melanoma, ovarian, prostate, brain and pancreatic cancers are dependent on CXCR4 for migration, survival and/or metastasis (3,35-38). This also provides an explanation for the therapeutic benefits observed in patients treated with PS341, a proteosome inhibitor with an anti-NF-κB properties (39).

A major observation in our present study is that cancer cells expressing CXCR4 are clonally selected during growth in the mammary fat pad of nude mice and that there is a further increase in CXCR4 expression in cancer cells that metastasize to the lungs. Because elevated CXCR4 expression was maintained in TMD231 and LMD231 cells even after three months in culture, it is less likely that the tumor microenvironment played any role in the transcriptional upregulation of CXCR4. Thus, we believe that there is a clonal selection of CXCR4-expressing cells, which needs to be further verified by immunohistochemistry within the context of the primary tumor and the metastatic sites. However, we feel that that is beyond the scope of the current investigation because of the technical difficulties involved in such analysis. For example, the tumor microenvironment may contribute to the transient expression of CXCR4 in cells that are not clonally selected for metastasis.

Why CXCR4 expression is advantageous to cancer cells remains to be determined. The SDF-1α:CXCR4 activated signaling pathways may provide a growth advantage to cancer cells at both the primary and metastatic sites. SDF-1α has been shown to enhance tyrosine phosphorylation and association of components of the focal

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adhesion complex (28). In addition, it induces PI3 kinase, p44/42 MAP kinases but not stress induced kinases such as p38 kinase and c-Jun amino-terminal kinase (29,40). The AKT/PKB pathway, activated by the PI3 kinase, protects a variety of cell types against cytokine-, stress- and chemotherapy-induced apoptosis (41). Thus, it is possible that cancer cells with cell surface CXCR4 are better equipped to protect themselves from host cytokine- as well as chemotherapy-induced apoptosis. The MAP kinase pathway may provide proliferation signals for the cancer cells that express CXCR4 to grow out in the tumor microenvironment. SDF-1α:CXCR4 ligation induced PI3 kinase along with uPA may promote the migration of these cells from the primary site. Taken together, our results reveal multiple functions of NF-κB in the growth, migration and organ specific metastasis of breast cancer cells, which in part appears to be mediated through the induction of CXCR4.
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ABBREVIATIONS:

NF-κB, Nuclear Factor-kappaB
IκBαSR, Inhibitor-of-kappaB alpha Supper Repressor
IL-8, Interleukin 8
EMSA, Electrophoretic Mobility Shift Assay
UPA, Urokinase Plasminogen Activator
SDF-1, Stromal Derived Factor 1
MMP, Matrix Metalloproteinase
REFERENCES:
FIGURE LEGEND:

**Fig. 1: Regulation of CXCR4 expression by NF-κB.** A) IκBαSR reduces CXCR4 expression in MDA-MB-231 cells. CXCR4 expression in MDA-MB-231 cells with retrovirus vector alone (LxSN11) or IκBαSR (IκBαSR6 and 10) was measured by the RNAse protection assay. B) Parthenolide reduces CXCR4 expression in MDA-MB-231 cells. Cells were treated with parthenolide (5 µM) for 4 hours. C) IL-1α overexpression leads to increased CXCR4 expression in MCF-7 cells.

**Fig. 2: Cell surface expression of CXCR4 is enhanced in IL-1α expressing MCF-7 cells compared to parental cells.** CXCR4 expression (black line), shown superimposed on the isotype control (gray shaded), was measured by flow cytometry. The control cells containing the empty vector (TMCF-7pcDNA3) show no significant expression of CXCR4. An increase in the percentage of cells expressing CXCR4 (20%) is observed in cells transfected with IL-1α (TMCF-7IL-1α(1) and TMCF-7IL-1α(2)).

**Fig. 3: Induction of CXCR4 promoter activity by TPA, IL-1α, and NF-κB subunits.** A) TPA and IL-1α induce CXCR4/CAT activity. MCF-7 breast cancer cells were transfected with the indicated CXCR4/CAT reporters (5 µg) with RSVβ-gal (2 µg) as an internal control. TPA (125 nM) or IL-1α (5 ng/ml) was added 24 hours after transfection. CAT activity in an equal number of β-galactosidase units was measured 36 hours after transfection. B) IκBαSR reduces CXCR4/CAT activity in MDA-MB-231 cells. C) Induction of CXCR4/CAT activity by p65, IKKα and NIK. MCF-7 cells were transfected with the indicated CXCR4/CAT reporter constructs and expression vectors (0.5 µg) or the control vector pcDNA3. CAT activity was measured as described above.

**Fig. 4: Binding of NF-κB subunits to CXCR4 promoter.** COS-1 cells were transfected with the indicated NF-κB subunits and whole cell extracts were prepared 48 hours after transfection. Radiolabelled CXCR4 promoter region (-66 to +1) was incubated with whole cell extracts and subjected to an electrophoretic mobility shift assay. As controls,
DNA binding of the general transcription factor SP-1 and binding of NF-κB to the classical response element was also measured (lanes 1-4). Nuclear extracts instead of whole cell extracts were used in EMSAs in lanes 5-7. In oligonucleotide competition assays (lanes 8-10), cell extract was incubated with a 25-fold excess of unlabelled oligonucleotides prior to the addition of labeled CXCR4 probe. Lanes 11-14 show antibody supershift assays with nuclear extracts of cells transfected with p65 and p50. 

**Fig. 5: IκBαSR reduces SDF-1α-dependent chemotaxis of MDA-MB-231 cells.** A) Cells transfected with IκBαSR, IκBαSR-6 (square) and IκBαSR-10 (triangle), exhibit a loss of SDF-1α induced migration when compared to empty vector, LXSN11 (diamond) (p≤0.05 at 100, 200, 400, and 800 ng/ml, n=6). B) Loss of SDF-1α induced migration was observed following 13 hrs of parthenolide treatment (p≤0.05 at 100, 200, 400 and 800 ng/ml n=7). C) Loss of migration induced by IκBαSR was partially rescued by transfection with CXCR4, IκBαSR-6-CXCR4 (open square) and IκBαSR-10-CXCR4 (open triangle), compared to empty vector controls, IκBαSR-6-pcDNA3 (filled square) and IκBαSR-10-pcDNA3 (filled triangle) (p≤0.05 at 400 and 800 ng/ml, n=6).

**Fig. 6: CXCR4 expression and SDF-1α dependent migration of parental, tumor-derived (TMD231) and lung-metastasis-derived (LMD231) MDA-MB-231 cells.** A-C) CXCR4 expression. Cell surface expression in parental (A), TMD231 (B) and LMD231 (C) was determined by flow cytometry (black line). Flow cytometry analysis of cells stained with isotype control antibody (gray shaded) is also shown. D-F) Cells stained with epithelial cell specific antigen (ESA). Parental (D), TMD231 (E) and LMD231 (F) showed similar levels of ESA expression. G) Migration of parental (diamond), TMD231 (square) and LMD231 (triangle) cells in response to SDF-1α.

**Fig.7: CXCR4 mRNA is increased in TMD231 and LMD231 cells compared to parental cells.** A) CXCR4 expression was measured by an RNAse protection assay as described in
Fig. 1. B) MD231, TMD231 and LMD231 cells express similar levels of XIAP, TRAF-3 and TRAF-4 transcripts. RNase protection assay was performed with the hAPO-5 probe. C) Parthenolide or MG132 inhibits CXCR4 expression in LMD231 cells. Cells were incubated with parthenolide (5 µM) or MG132 (10 µM) for indicated time and subjected to an RNase protection assay as described in Fig. 1.
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NF-κB promotes breast cancer cell migration and metastasis by inducing the expression of the chemokine receptor CXCR4

Gregory Helbig, Kent W. Christopherson II, Poornima Bhat-Nakshatri, Suresh Kumar, Hiromitsu Kishimoto, Kathy D. Miller, Hal E. Broxmeyer and Harikrishna Nakshatri

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