12-2009

Mechanisms mediated by CXCL12 signaling through CXCR4 and CXCR7 in breast cancer.

LaSharon Denise Mosley
University of Louisville

Follow this and additional works at: https://ir.library.louisville.edu/etd

Recommended Citation

Mosley, LaSharon Denise, "Mechanisms mediated by CXCL12 signaling through CXCR4 and CXCR7 in breast cancer." (2009). *Electronic Theses and Dissertations.* Paper 1012.
https://doi.org/10.18297/etd/1012

MECHANISMS MEDIATED BY CXCL12 SIGNALING THROUGH CXCR4 AND CXCR7 IN BREAST CANCER

By

LaSharon Denise Mosley
B.S., Mississippi University For Women, 2000
M.S., University Of Louisville, 2007

A Dissertation
Submitted To The Faculty Of The
Graduate School Of The University Of Louisville
In Partial Fulfillment Of The Requirements
For The Degree Of

Doctor Of Philosophy

Department Of Pharmacology And Toxicology
University Of Louisville
Louisville, Kentucky

December 2009

MECHANISMS MEDIATED BY CXCL12 SIGNALING THROUGH CXCR4 AND CXCR7 IN BREAST CANCER

By

LaSharon Mosley
B.S., Mississippi University for Women, 2000
M.S., University of Louisville, 2007

A Dissertation Approved On

November 18th, 2009

By The Following Dissertation Committee:

_____

Dr. James W. Lillard, Jr. – Dissertation Chair

_____

Dr. Jason Chesney

_____

Dr. John Eaton

_____

Dr. Zhao-Hui Song

_____

Dr. Jill Suttles

ii

DEDICATION

This dissertation is dedicated to my parents and the memory of my grandparents

Mr. and Mrs. Joe (Mattie) Baylor

And

Mr. and Mrs. Kelly (Marie) Ford

for their love, support, guidance, and invaluable educational opportunities.

ACKNOWLEDGMENTS

ABSTRACT

MECHANISMS MEDIATED BY CXCL12 SIGNALING THROUGH CXCR4 AND CXCR7 IN BREAST CANCER

LaSharon D. Mosley

DECEMBER 2009

Interactions between chemokines and their receptors are involved in organ-specific homing and propagation of metastatic breast cancer (BrCa) cells. BrCa cells express higher levels of CXCR4 and CXCR7 mRNA and cell surface protein, than normal human mammary epithelial cells (HMECs). CXCR4 is ubiquitously expressed by a multitude of cancerous and normal cell types; while CXCR7 is differentially expressed by T helper lymphocytes, cancer cells, and normal epithelium. Importantly, recent studies show CXCR7 is highly expressed by activated endothelial cells (i.e., neovasculature), fetal liver cells, and many tumors, but not by non-transformed (or normal) human tissues. Unlike other chemokine receptors, activation of CXCR7 does not cause Ca^{2+} mobilization/flux or cell migration. However, CXCR7 activation by its ligands (CXCL12 or CXCL11) provides signals for growth, survival, and adhesion. Preliminary studies demonstrated CXCR7 expression is significantly higher in BrCa cases than compared to non-neoplastic tissues. Further, CXCR7 mRNAs are elevated in the BrCa cell line MCF-7, with modest expression in MDA-MB-231 when

compared to HMEC. Other studies revealed cell-type specific expression of CXCR7 may modulate BrCa progression. Together, these findings provided the rationale to support the **hypothesis** that *CXCR7 and its interactions with CXCR4 and CXCL12 promote BrCa cell collagenase expression, alter NF-κB and ERK1/2 localization to the nucleus, and correlates with cell cycle.* Two **Specific Aims** were used to test this hypothesis. **Aim 1** - Determined the differential and cell cycle-dependent expression of CXCR7, CXCR4, CXCL12, matrix metalloproteinase proteins MMP-1 and/or -13 in BrCa cells, which were affected by CXCL12 stimulation. **Aim 2** - Characterize some of the CXCL12-dependent mechanisms involved in BrCa progression.

The results from this study indicate that differential and cell cycle-dependent CXCR7 and CXCR4 expression plays a critical role in enhancing BrCa cell invasion and survival cell signals, which were CXCL12-dependent but often G protein independent. Further, CXCL12-induced CXCR4 mRNA expression required $G_{\beta\gamma}$ protein and/or JAK/STAT signal transduction. It was also shown that CXCL12-mediated CXCR7 mRNA expression in a pertussis toxin sensitive and no doubt required $G\alpha i$ protein signaling. In confirmation with previous studies from Wendt et al., CXCL12 expression is negatively regulated by the presence of CXCR4. Lastly, CXCL12-dependent MMP-1 and MMP-13 mRNA expression is $G\alpha$ protein-, $G\beta$ protein-, and $G\gamma$ protein-dependent presumably through CXCR4, while elevated expression of CXCR7 suppresses these effects.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Metastasis is a non-random and specific process that includes: survival

and growth of the primary tumor, detachment of tumor cells from the primary site,

invasion into the vascular or lymphatic vessels, homing and adherence to the

secondary site, survival, growth and angiogenesis of the metastasized cells at

the secondary site (1). This process is responsible for all breast cancer (BrCa)-

related deaths; therefore, therapies designed to prevent the spread of BrCa cells

are greatly needed. The current theory for malignant cancer is that metastasis is

the final stage of disease (2). Most importantly, cancer cells involved in the

progression of the disease have acquired the necessary hallmarks of malignancy

as they accumulate multiple, rate-limiting mutations that occur over an extended

period of time. The primary idea behind early cancer detection and prevention

strategies is that carcinogenesis can be suppressed during the post-initiation

stage of disease, specifically due to cancer's progressive nature and long latency

(3). However, the current concept of cancer is that disease progression is a

multi-step process that arises late from rare transformed cells with metastatic

potential (4, 5). For example, histological studies of epithelial cancers showed

progression of the disease from benign through malignant stages. Further,

metastatic tumor cells must be able to colonize at distant organs (6).

BrCa is the second leading cause of cancer-related deaths among women in the US. Similarly, the numerous anti-apoptotic mechanisms employed by BrCa cells to survive, serum-free environments or apoptosis-inducing therapies significantly contributes to the morbidity and mortality of this disease. Its progression consists of atypical ductal hyperplasia, pre-invasive ductal carcinoma *in situ* (DCIS), and invasive ductal carcinoma; however, the mechanisms responsible for BrCa metastasis and resistance to therapy are incompletely understood. Many factors have been implicated in the process of metastasis, serum-free survival and resistance to therapy, but the precise mechanisms are not entirely known. Brugge *et al.* demonstrated that a combination of oncogenes, one for constitutive proliferation along with another that inhibits apoptosis, are required for metastatic progression (7). Other studies have implicated physiological changes in the stroma or microenvironment maybe key to tumor progression or phenotypic reversion. Indeed, disrupting tumor cell interaction with the extracellular matrix (ECM) via integrin blocking antibodies resulted in a phenotypic reversion (8). Other observations in prostate and breast cancer models demonstrated that normal stromal fibroblasts are replaced by smooth muscle reactive myofibroblasts or carcinoma-associated fibroblasts to promote disease (CAFs). These CAF cells alter ECM composition, elevate cytokine production, and induce the infiltration of inflammatory cells (9, 10). Along this line, the pro-tumorigenic functions of CAFs in mammary carcinomas

2

are partially mediated by CXCL12, is expressed at increased levels by mammary carcinoma CAFs and it plays a critical role in disease progression (11).

CXCL12 directly stimulates tumor growth by binding to and signaling through CXCR4 on tumor cells. This chemokine also recruits ECs into tumors by facilitating angiogenesis (12). Local injury, inflammation, and/or tumor cells (via paracrine signaling to stromal cells) are able to induce a microenvironment that is conducive for tumor cell progression. These studies also demonstrate that pathological changes in the tumor microenvironment maybe just as important for BrCa progression as mutational-oncogenic profiles of the tumor cell. Given the importance of stromal-epithelial interactions in normal mammary gland development, tissue homeostasis, and tumor progression, chemokines and their receptors play critical roles in each process (13). The goal of this dissertation is to determine or gain a better understanding of chemokine ligand CXCL12 and its receptors, CXCR4 and CXCR7, interactions.

Chemokines

Chemokines are 8-10 kDa chemotactic cytokines that are classified into four groups (CXC, CC, C, and CX3C) based on the position of the first two cysteines (14). Chemokines bind receptors that belong to the G protein-coupled receptor superfamily often coupled to pertussis toxin sensitive G_i proteins (15) **(Figure 1)**. In general, chemokine receptors exhibit promiscuity, being able to bind multiple ligands; however, 6 of the 18 chemokine receptors bind a single ligand (16). In recent years, chemokines and their receptors have been implicated in several
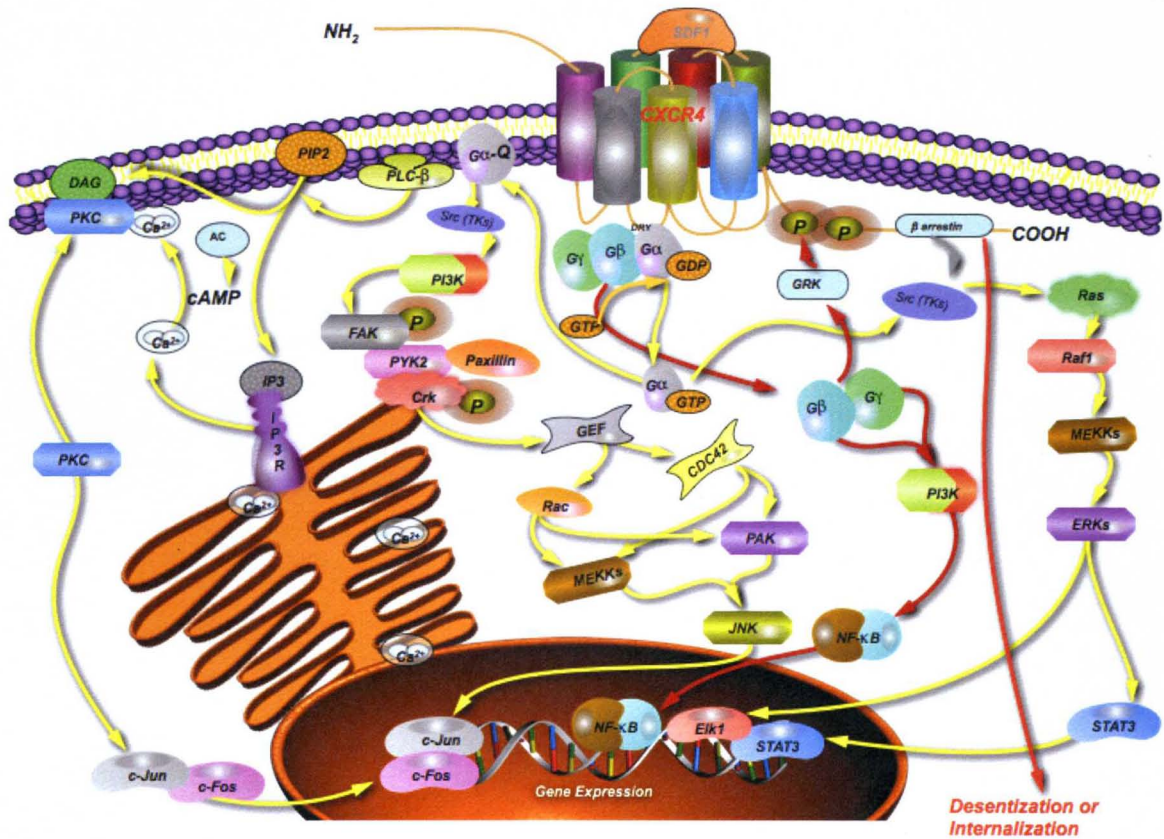
Figure 1. Previously shown CXCR4 cell-signaling pathways. The GPCR, CXCR4 binding of SDF-1 has been shown to activate G-proteins (heterotrimer $\alpha/\beta/\gamma$) mediated signaling to elevate intracellular Ca^{2+}(Calcium), activate MAPK (Mitogen Activated Protein Kinases), JNK (Jun N-terminal Kinases) and PI3K (Phosphoinositide-3 Kinase). Cell signaling through these pathways lead to cell growth, migration and polarization. CXCR4 stimulation by SDF-1 also results in increased phosphorylation of focal adhesion components *e.g.* the related adhesion focal tyrosine kinase (RAFTK/PYK2), Crk and Paxillin. Crk, which belongs to the adaptor family of proteins composed of SH2 (Src Homology 2) and SH3 domains, has a putative role in signaling. The phosphorylation and

4

translocation of NF-kB, c-jun/c-fos, and ERK1/2 are also critical for activating

genes and transcriptional factors involved in many cellular events including:

tumorigenesis, differentation, migration, growth and survival. JNK, which is

moderately activated by v-Crk as well as Rac and Cdc42 (Cell Division Cycle 42),

can activate genes needed for cell proliferation and survival. All of these

signaling pathways are key for the role of CXCR4 in BrCa progression.

diseases, including cancer. They are secreted cytokine-like proteins that induce the direction of metastasis and survival of neoplastic cells through G-protein-coupled receptors, cytoskeleton rearrangement and adhesion of neoplastic cells to leukocytes and endothelial cells (14, 17, 18). These secreted factors act in a coordinated fashion with cell-surface proteins to direct the homing of various subsets of haematopoietic cells to specific organ sites (19). Studies have shown that secondary sites of BrCa metastasis, *e.g.,* lung, liver, and bone are abundant sources of these ligands (19). Studies within our laboratory and others **(Table 1)** have shown chemokine involvement in cancer development and progression, including metastasis. This association with metastasis is not unusual since it is not a random process of cell migration. Metastasis has many features in common with normal cell migration; however, key differences lie in abnormal chemokine receptor expression, regulation or use (20). The role of chemokines and their receptors with regard to cancer has been divided into three broad categories. First, they must provide directional cues for tumor cells to migrate or metastasize at secondary sites. Second, they can favorably shape the tumor microenvironment for the "metastasized" cells. Third, they provide survival and growth signals for cancer cells (21, 22). Although chemokines and their receptors' involvement in each of these categories has been well established, their exact mechanism of action are not well understood and the underlying complexity of chemokine networks makes it difficult to characterize them definitively. However, CXCL12 and its receptor CXCR4 have been implicated in 23 different types of cancers (23).

Chemokine Receptor	Chemokine Ligand (s)	Disease Investigated & Pub
CXCR3	CXCL9, 10, & 11	Cystitis, 2008 & 2003
CCR5	CCL5	Chlamydia, 2008
CXCR3	CXCL10 & 11	Colitis, 2008
CXCR3	CXCL9, 10, & 11	IRB, 2007
CCR5	CCL3, 4 & 5	Pneumonia, 2006
CCR1, 3, & 5	RANTES	Malaria, 2005
CCR9	CCL25	Prostate Cancer, 2004
CCR3, 8	I-309, vMIP-11, & TCA-3	Mucosal Immunity, 2004 & 2001
CXCR4	CXCL12	Prostate Cancer, 2004
CXCR5	CXCL13	Prostate Cancer, 2008
CXCR1 and 2, IL-8Rβ	GCP-2 & IL-8	Adaptive Immunity, 2004 & 2003
CCR1	RANTES	Mucosal Immunity, 2001

Table 1. Chemokines and Disease: Chemokine ligands and their receptors expression in various diseases.

Human Stromal Cell-Derived Factor-1, also known as CXCL12, has two known isoforms (SDF-1α (89 aa) and SDF-1β (93 aa)) that binds the 352 aa rhodopsin-like GPCR, CXCR4 with comparable affinity (K_d of 7.5 and 13.7nM respectively) (24, 25). The CXCL12/CXCR4 axis is one of the best studied, primarily due to their roles in HIV entry (26) as well as their ability to mediate the metastasis of a variety of cancers (27). Studies have shown that CXCL12 affects growth and spread of cancer cells through interactions with CXCR4 (19). CXCR4 has been shown to play a major role in the progression of multiple types of carcinomas and this chemokine receptor is widely expressed by astrocytes, glial cells, and neurons of the adult nervous system as well as normal epithelial cells. Of particular interest, CXCR4 is constitutively expressed by cortical and hippocampal neurons as well as epidermal cells.

Other factors have also been shown to enhance CXCR4 expression specifically during cancer progression. Further, CXCR4 is expressed by many cancer cells, cell lines and endothelial cells around tumor lesions (28). Other studies have shown that CXCR4 may influence migration in the peritoneum, a major route for BrCa cell spread to lymph nodes (19, 29). Increased cell surface expression of CXCR4 maybe the result of altered regulation, independent of effects on transcription or translation. Ubiquiination of CXCR4 is a post-translational modification regulating the expression of this receptor (30). Interestingly, BrCa cells that are HER2/neu positive have increased expression of CXCR4 as a result of reduced CXCR4 ubiquitination. This provides a potential link between HER2 positive BrCa and the attenuated degradation of CXCR4

(31). To this end, neutralizing CXCR4 significantly impaired BrCa cell metastasis to regional lymph nodes and lung (19).

Several clinical studies have noted that the progression of BrCa and tumor burden correlates with inflammatory and acute phase protein levels in sera. For example, patients with metastatic BrCa express significantly more serum IL-1β, IL-6, and TNF-α when compared to healthy donors or patients without neoplasms or with benign or localized breast tumor (32). Stimulation of fibroblast or endothelial cells with IL-1β, IL-6, and TNF-α, which are elevated during BrCa burden, results in the production of several factors including CXCL12 (33) and CXCL11 (34). Elevation of these factors often correlates with poorer disease outcomes, tumor size, and metastasis. To explain, the bone marrow responds to infection (and tumor burden) by producing more mature leukocytes from a small pool of committed progenitor cells under the influence of colony-stimulating factors (CSF) (35). Similarly, factors produced during this innate response are involved in the expression of chemokines and cell adhesion molecules by human bone marrow endothelial (HMBE) cells and osteoblasts (36, 37). Of importance, osteoblasts have been demonstrated to express and respond to CXCL12 (38).

Mutation of CXCL12 leads to a defect in the guided migration of the lateral line of zebrafish. This defect caused by CXCL12 mutation is surprisingly stronger than those caused by CXCR4 deficiency (39). Perhaps tumor cell invasion, like tissue development, is a collective phenotype of several signals and cells. Perhaps, extrinsic cues and interactions influence a small number of **"leader"** as well as **"trailing"** cells. Recent studies have shown that CXCR4 is

required at the leading edge, while another chemokine receptor that also binds CXCL12, CXCR7, is expressed by trailing regions to presumably support migration, differentiation, and/or adhesion (i.e., arrest). Knockdown of CXCR7 affected trailing cells and caused stretching and incomplete or "stalled" tissue formation in zebrafish (40, 41). Other studies demonstrated that CXCR4 and CXCR7 act independently to regulate cell migration and this relationship is believed to be synergistic; however, activation of both receptors is controlled directly by CXCL12 (34). While there are several mechanisms that are common among tissue development or wound healing and tumor metastasis, further studies are required to determine how this elegant interaction between CXCR7 (CXCL11 and CXCL12) and CXCR4 (CXCL12) may determine breast tumor development. My dissertation addressed and dissected some of these cellular and molecular mechanisms.

CXCR7

My preliminary histological studies show that BrCa tissue also express higher levels of CXCR7 **(Figure 3)** to presumably dictate cell adhesion, invasion and survival in response to chemokine ligand CXCL12. CXCR7, formerly called RDC1, is a recently de-orphanized G-protein coupled receptor that binds with high affinity to two chemokines - CXCL11 (interferon-inducible T cell chemoattractant; I-TAC) and CXCL12 (stromal cell-derived factor-1; SDF-1). Receptor-binding studies revealed that CXCL12 has a higher binding affinity for CXCR7 than compared to CXCL11 (~3-fold more efficiency) (34). Originally,

10

cloned from a canine cDNA library as a putative G-protein coupled receptor for vasoactive intestinal peptide hormone (VIP), a connection between RDC1 and chemokines was proposed based on sequence similarity (43%) and identity (32%) with CXCR2, CXCR1 and CXCR4 on human chromosome 2. Despite its phylogenetic relation and ligand-binding properties, CXCR7 has not yet found general consent as a typical chemokine receptor (42). For example, chemokine receptors often signal via pertussis toxin sensitive Gαi-proteins and contain a conserved **DRYLAIV** motif at the N-terminus of the second intracellular loop that is sufficient for coupling to Gαi-proteins. CXCR7 coupling to G-proteins has not been demonstrated and it has an altered **DRYLSIT** motif, which may disallow it to optimally interaction with these signal transducing proteins (42). Most importantly, the third intracellular loop, which is generally less conserved between GPCRs, confers specificity for G-protein interactions **(Figure 2)**, but the function of this site remains in question, because CXCR7 activation is atypical when compared to signals induced by other **CXC** chemokine receptors (43).

Activation of CXCR7, whether by CXCL11 or CXCL12, does not cause Ca^{2+} mobilization or cell migration; however, expression of CXCR7 provides cells with increased growth and survival properties (44). In studies involving CXCR4^{+} HEK293 cells, expression of CXCR7 showed enhanced cell responsiveness to CXCL12 (44). Other studies showed CXCR7 is critical for CXCL12 mediated survival, tumor growth and aggressiveness (42). Due to these studies, it could be hypothesized that CXCR7 may signal independently of its G-proteins.
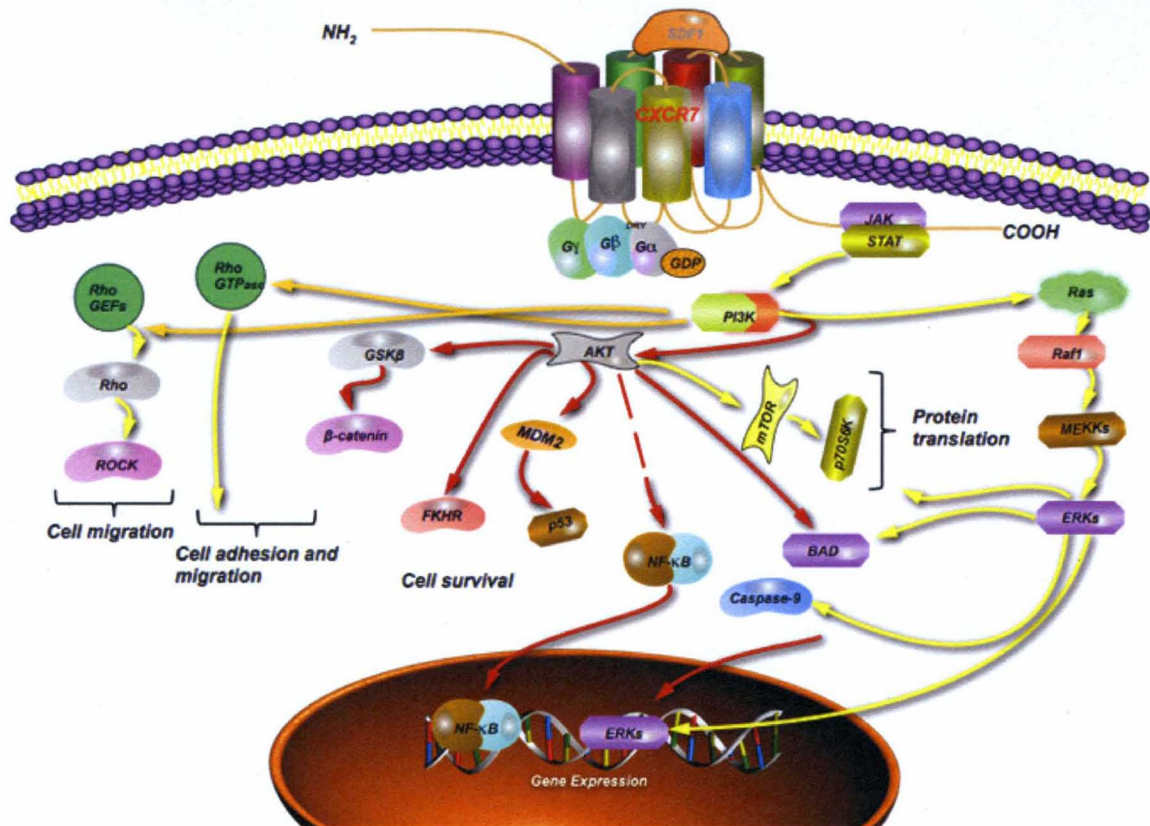
Figure 2. Hypothetical CXCR7 (G protein-independent) cell-signaling pathways. The GPCR, CXCR7 is hypothesized to signal via a pathway independent of its G-proteins (heterotrimer subunit $\alpha\beta\gamma$) but involves crosstalk with CXCR4. Interaction with JAK/STAT with the C-terminal tail of the receptor is key for the activation of PI3K that induces downstream cascade components MAPK, AKT, Rho GTPases, and Rho Gefs. These signaling pathways for CXCR7 enhances protein translation, cell adhesion, migration, and survival in BrCa.

Although CXCR4 was once believed to be a receptor unique for CXCL12, my preliminary studies and those of others suggest that CXCR7 acts either as a "**scavenger receptor**" with limited function or a receptor that **amplifies** or fine-tunes CXCL12 cues for cell migration, adhesion, invasion and/or survival. When both CXCR4 and CXCR7 are expressed, studies show that these chemokine receptors efficiently form heterodimers, which could have consequences on CXCL12-mediated signals (45). Moreover, the relative CXCR4 or CXCR7 expression levels contribute to the occurrence of these heterodimers. Using bioluminescence resonance energy transfer (BRET) analyses, Levoye *et al.* demonstrated that CXCR7 expression alters CXCR4-mediated $G\alpha i$ protein complexes and impaired CXCR4-promoted $G\alpha i$ protein activation and Ca^{2+} responses. In the same study, RNA interference targeting CXCR7 and blockade of CXCL12/CXCR7 interactions contributed to reduced chemotaxis toward CXCL12 in primary T cells. This study along with others, identified CXCR4/CXCR7 heterodimers as distinct functional units with novel properties that can contribute to the activity of CXCL12. Lastly, no cellular signaling pathway(s) , *e.g.,* extracellular signal-related kinase (ERK1/2), Akt/PKB, phosphoinositide 3-kinase (PI3K), and Src, have been determined for CXCR7. Studies within this dissertation will help to elucidate some of these events.

Chemokine-Induced Integrin Activation and Firm Adhesion

Primary leukocyte adhesion to endothelium, namely, tethering and rolling, are mediated by chemokines as well as integrins. Transendothelial lymphocyte motility during inflammation is a complex process that involves cell rolling along the endothelium of extracellular matrixes (ECM). This rolling continues until a high concentration of a given chemokine is detected which results in integrin-dependent firm adhesion and cell arrest at the site of secretion. Hence, chemokines aggregate not only their respective receptors but also bring together and activate integrins to increase the binding avidity for firm cell adherence that can resist the shear force exerted by blood flow (46). Several chemokines have been shown to modulate lymphocyte adhesion to endothelium or endothelial ligands (47-51). Elucidating the mechanisms of integrin activation by chemokines at confined leukocyte–endothelium contact zones under shear flow is crucial for understanding how chemokines regulate BrCa cell metastasis. Chemokine-triggered G-protein signaling coupled to integrin clustering takes place within sub-seconds. Endothelium expressed chemokines, e.g., CXCL11 and CXCL12, might function to augment BrCa cell arrest under physiological flow conditions.

Cytoskeleton re-modeling, adhesion and de-adhesion are not only required for cellular motility, but are also linked to proliferation and survival pathways. Integrins elicit a series of transduction signals (e.g., focal adhesion kinase (FAK), Src, ERK1/2, mitogen-activated protein kinase (MAPK), PI3K, etc.) that influence cell proliferation and survival (52, 53). While $\alpha_6\beta_1$ has a high affinity for

fibronectin, $\alpha_v\beta_3$ adhesion depends on vitronectin and osteopontin constituents in bone ECM (37). $\alpha_v\beta_3$ and $\alpha_v\beta_1$ integrin expression by breast tumors correlates with poor prognosis and promotes BrCa cell survival and proliferation under adverse conditions (54-57). G-protein coupled receptor activation has been shown to induce β_3 integrin tyrosine phosphorylation via Src (58). Activation (phosphorylation) of the β_3 integrin subunit has also been shown to augment the interaction with its substrate for tight binding and signaling events (59).

Matrix metalloproteinase (MMP) and cancer progression

Activation of CXCR4 stimulates the production of MMPs (60) potentially facilitating the ability of cancer cells to egress from the primary tumor site and invade secondary sites. Furthermore, CXCL12 signaling is able to enhance integrin activity through cell adhesion under flow conditions (17, 61). Upon entering the blood or lymphatic systems, if CXCR4 truly mediates metastasis, tumors would preferentially migrate and adhere to areas that highly express CXCL12. BrCa cells follow this distinct pattern of metastasis to lymph nodes, lung, liver, and bone marrow all of which express high levels of CXCL12. $\alpha_v\beta_3$ expression by MDA-MB-231 cells promotes bone tumor burden and destruction/invasion (62), in part through MMP upregulation (63).

After adhesion, neoplastic cells must penetrate the basement membrane and invade the interstitial stroma to initiate the metastatic process. Many proteinases are capable of degrading ECM components, but MMPs (e.g., collagenases, gelatinases, and stromelysins) appear to be particularly important

for matrix degradation (64, 65) and cancer cell dissemination (66). Several MMPs and tissue inhibitors of MMPs (TIMPs) are highly expressed by invasive BrCa cell lines (MDA-MB-231) as well as breast tumor tissue. MDA-MB-231 cells express and secrete active MMP-1, -3, -7, -9, -10, -11, and -13, whereas MMP-9 is specifically up-regulated by cell contact (67, 68). Indeed, expression of CXCR4 and MMP-9 by breast tumors correlates with a poor diagnosis of lymph node metastasis (69). Consistent with their role in BrCa progression, high levels of at least MMP-2, -9, -11, and -14 have been found to correlate with poor prognosis of patients with BrCa (70, 71). Accordingly, inhibitors of CXCR4/CXCR7 or siRNA knockdown inhibit metastasis and growth of breast cancer cells (72, 73). Our laboratory has previously shown that CXCL12 can modulate the expression of collagenase, gelatinase, and stromelysin to support cancer cell invasion (74). To this end, studies attempting to correlate mestastatic potential and MMP involvement have yielded mixed results. This dissertation investigated the effects of CXCL12 interactions with CXCR7 and CXCR4 that support active collagenase (MMP-1 and MMP-13) expression.

Chemokine Receptor Signaling

It has become evident that integrations between chemokines and their receptors lead to signal transduction that requires cell-cell adhesion molecules, cell-matrix receptors, and intracellular signaling proteins. A number of kinases have been shown to play a role in leukocyte (as well as cancer cell) adhesion, motility and invasion. Chemokine-induced integrin clustering and affinity

upregulation as well as chemotaxis by lymphocytes via F-actin polymerization and lamelli-podia formation depends on a signaling network involving Rac, Cdc42 and Rap (75-78). Indeed, different signal transduction proteins may regulate multiple invasion events, including adhesion, de-adhesion, motility, and invasion using pathways such as the Src-ERK, FAK/PYK2-ERK, PI3K-Akt-NF-κB, and/or DOCK2-ELMO for Rac and/or Rap activation cascade(s). The current dogma for chemokine receptor signaling involves $G\alpha i$ protein, PI3K (upstream of ERK1/2), and phospholipase C (PLC) activation that leads to Ca^{2+} flux required for Src and FAK activation. There is no doubt that PI3K is a key signaling molecule that is activated after chemokine-chemokine receptor interactions by BrCa cells. Certain isoforms of p110 act to catalyze the formation of *PI* (3,4,5) *P3* and subsequent production of *PI* (4,5) *P2* by PTEN or *PI* (3,4) *P2* by SHIP, which leads to the activation of Akt(s) (subsequent activation of NF-κB) and ERK(s) to regulate cell functions including: proliferation, survival, membrane trafficking and cytoskeletal structures (79).

Src activity is involved in cell invasion (and possibly motility) through its central role in the scaffolding complex of signaling molecules at the focal adhesion signaling organelle (80). Activation by auto-phosphorylation of FAK induces another pathway for motility and/or invasion (80-82). PI3K(s), Src, and FAK/PYK2 can also activate ERK(s) for migration, adhesion and invasion (81, 83-86). It has been suggested that integrin avidity modulation by leukocytes appears to be mediated by ERK1/2.

17

Akt binds to the phospholipids produced by PI3K and recruits this kinase to the plasma membrane, where it is activated by phosphorylation. The non-universal role of PI3K in BrCa cell motility, adhesion, and invasion has important implications for the development of new targets against metastatic cancers. Leukocyte migration seems to be mediated in a predominately PI3K-independent and DOCK2-dependent manner (87). While PI3K and (Src and FAK) events have been shown to play a role in cancer cell motility, adhesion, invasion, and survival (88, 89), the potential of PI3K-independent in CXCR7-mediated events remain uncertain. Most likely, PI3K-dependent and -independent pathways may be involved in CXCR7-triggered integrin activation, for subsequent adhesion and invasion.

Chemokine Receptors and Cell Survival

The expression of chemokine receptors on migrating cells may provide these cells with more than directional cues for metastasis. Chemokine receptor signaling may provide a survival advantage once in a foreign environment. These molecular strategies for survival and growth are often the result of using or reprogramming existing physiological pathways (90). In most cases, survival signals are likely related to the role of chemokine receptor pairs *e.g.,* CXCL12:CXCR4 and CXCL12:CXCR7 in normal development. Other situations for cell survival and proliferation may require redirecting signals from existing migration pathways. For example, studies have shown that metastasized cancer cells have a strong propensity to survive and resist apoptotic stimuli and in some

cases, extracellular survival signals can aide or promote cell survival in the "foreign" microenvironment (91).

Unlike other chemokine receptors (e.g., CXCR3 and CXCR4), CXCR7 does not induce Ca^{2+} flux or migration by BrCa cells (44). However, CXCR7 promotes cell survival and adhesion (92). Studies have also shown that chemokine receptor signaling often leads to activation of Akt and subsequent phosphorylation of multiple targets, e.g., glycogen synthase kinase (GSK)-3β, Forkhead [Drosophila] homolog 1 [rhabdomyosarcoma] (FKHR) and caspase 9 (without caspase 3 induction), which are involved in cell survival (93). The current dogma for chemokine receptor signaling involves Gαi protein, PI3K, and PLC activation that leads to Ca^{2+} flux. There is no doubt that PI3K can be a key signal after CXCR4 and CXCR3 activation on BrCa cells. The prosurvival molecule Akt binds to the phospholipids produced by PI3K through its PH domain and is recruited to the plasma membrane where it is activated by phosphorylation. Phosphorylated Akt in turn phosphorylates Bad, a pro-apoptotic protein belonging to the Bcl-2 family. When phosphorylated, the cytoplasmic protein 14-3-3 sequesters Bad and apoptosis is inhibited.

A second Akt substrate includes FKHR (94-96). Phosphorylation of FKHR prevents translocation of this protein to the nucleus where it regulates the transcription of genes involved in apoptosis (97). ERK1/2 signaling may also contribute to cell survival through these pathways. For example, studies have shown that ERK1/2 via phosphorylation and inhibition of procaspase-9 and BAD may provide signals for cell survival and proliferation (98). Further studies

showed ERK1/2 can localize to the nucleus and activate transcription factors involved in cell-cycle regulation and differentiation that promotes cell proliferation (99). Thus, chemokine receptor signaling can activate transcription factors involved in anti-apoptotic mechanisms, cell-cycle regulation, and growth-factor production. These pro-tumorigenic pathways are likely to be particularly important for the ability of metastatic cancer cells to thrive in foreign microenvironments. These studies provide the rationale for my dissertation, which has demonstrated some of the mechanisms of CXCR7 and CXCL12 interactions and subsequent signaling events that promote BrCa cell invasion and survival.

Apoptosis

Apoptosis is an intrinsic cellular defense mechanism against tumorigenic growth, which, if suppressed, can contribute to development of malignancy (100). A wide variety of cytotoxic agents with different intracellular targets can induce the uniform phenotype of apoptosis (100). This implies that the cytotoxic activity of anti-cancer drugs is not solely dependent on specific drug-target interaction, but also on the activity of apoptotic (cell signaling) machinery of the cancer cell (101, 102). There are numerous downstream effectors and transcription factors of Akt, ERK1/2, and tyrosine kinase signaling that can promote cell survival and proliferation. Chemokine signaling often activates NF-κB, which is commonly downstream of Akt, but can be activated through other pathways, such as PKC (103). NF-κB dimerizes and translocates to the nucleus of the cell upon

activation where it promotes transcription of various apoptosis inhibitors and cell cycle-promoting genes (104). Interestingly, other downstream cell signaling targets through select chemokine receptors activate AKT - NF-κB to promote the phosphorylation of procaspase-9 and BAD, which inhibit cell signals that dictate apoptosis (105). Together, these studies support the notion that CXCR7 (and/or CXCR4) signaling can interfere with apoptosis signals induced by serum starvation. Therefore, I hypothesized that **CXCR7 and its interactions with CXCR4 and CXCL12 promote BrCa cell collagenase expression, alter NF-κB and ERK1/2 localization to the nucleus, and correlates with cell cycle.** This hypothesis was investigated by immunohistochemistry, semi-quantitative RT-PCR, siRNA, Activity Assays, and Amnis ImageStream analyses. These methods were sufficient for determining CXCR7 mRNA and protein levels in BrCa cells. By varying CXCR7 expression with siRNA and inhibitors (pertussis toxin (Gαi) and U-73122 (Gβ/Gγ), I have determined some of the cell signal pathways induced by CXCR4 and CXCR7.

The **Specific Aims** of my dissertation focused on correlating CXCR4 and CXCR7 expression by BrCa cells with cell cycle, collagenase expression, as well as chemokine receptor, NF-kB, and ERK1/2 localization. The results obtained from this study will extend our knowledge concerning CXCR7 and its role in breast cancer progression as well as determine whether or not therapeutic targeting of CXCR4 and/or CXCR7 can alleviate BrCa cell invasion and survival.

Overall Hypothesis: *CXCR7 and its interactions with CXCR4 and CXCL12 promote BrCa cell collagenase expression, alter NF-κB and ERK1/2 localization to the nucleus, and correlates with cell cycle.*

Specific Aim 1: Determined the differential and cell cycle-dependent expression of CXCR7, CXCR4, CXCL12, and collagenase by BrCa cells, which were affected by CXCL12 stimulation..

Specific Aim 2: Characterized some of the CXCL12-dependent mechanisms involved in BrCa progression.

CHAPTER II

MATERIALS AND METHODS

Cell Culture

Human BrCa cell lines, MCF-7 (HTB-22) and MDA-MB-231 (HTB-26),

were purchased from American Type Cell Culture. Primary human mammary

epithelial cells (HMEC) were obtained from LONZA. HMEC, MCF-7, and MDA-

MB-231 cell lines were used for all experiments performed and discussed.

HMEC were maintained in MEGM supplemented with 2ml of BPE, 2ml of

hydrocortisone, 2 ml of human epithelial growth factor (hEGF), 500 l of insulin,

and 2 ml of gentamicin/amphotericin-B. MCF-7 and MDA-MB-231 cells were

maintained in IMEM and DMEM (Gibco-Invitrogen) media, respectively,

supplemented with 10% FBS (Hyclone-Fisher), without phenol red and 5 µg/ml of

penicillin/streptomycin (Gibco) at 37°C with 5% CO_2. Cells were cultured for 3

days in complete culture media then lifted with cell-stripper (Gibco) before being

counted with trypan blue by the Cellometer^{TM} Auto T4 (Nexcelom Biosciences).

Cells were seeded (5 x 10^5 cells per well) in 6-well plates (Corning-Fisher)

containing 1% serum and allowed to acclimate overnight before being treated.

Treatments included no addition, 100 ng/ml of CXCL12 (SCYB12; R&D

Systems), 100 ng/ml of CXCL12 + 100 ng/ml of pertussis toxin (List Biological

Laboratories), 100 ng/ml of CXCL12 + 6 µM of U73122 (Sigma). Cells were treated with inhibitors one hour prior to the addition of CXCL12 and allowed to incubate for 16 hours. Media was collected and stored at -80°C and used for ELISA-based assays. Cells were used for total RNA isolation using the TRIzol method.

Immunohistochemistry

Tissue micro-arrays (TMAs) were deparaffinized in xylene (three changes 5 minutes each) and rehydrated through a graded series of ethanol (100%, 95%, and 70%) for 5 minutes in each and washed in distilled H_2O. Antigen retrieval was performed using 0.01M EDTA (pH 8.0) in a pressure cooker for 5 minutes. Slides were transferred in running water for cooling and then transferred to Tris-buffer (pH 7.6). The endogenous peroxidase activity was blocked, by incubating the slides with 3% H_2O_2 solution for 5 minutes. The slides were then rinsed 3 times with deionized water followed by three washes in Tris-buffer and were incubated with Fc block (Innovex Bioscience) for 30 minutes at room temperature (65°C; RT) in a humidity chamber, then incubated with 3% normal horse serum for 1 hour at RT to block non-specific binding. Next the sections were incubated for 90 minutes in a humidity chamber at RT with mouse anti-CXCR7 antibody. Next, the sections were washed with Tris-buffer and incubated with mouse anti-goat antibody for 20 minutes at RT in a humidity chamber. The slides were then washed with Tris-buffer, treated with streptavidine peroxidase for 20 minutes at RT, washed with Tris-buffer, and treated with 3,3'-diaminobenzidine (DAB) for 2-

24

5 minutes at RT. Subsequently, sections were washed with water, re-hydrated in 70%, 95%, and absolute alcohol for 5 minutes each and passed through xylene three times for 5 minutes; and finally mounted with permount (Sigma).

All transverse sections of tissue were scanned by a ScanScope GL system (Aperio Technologies) using a 40X objective followed by lossless compression and assessment of all immunostainings in identical anatomic regions. After images from the glass slides were digitized, the resulting digital images were automatically analyzed using complex computer algorithms for standardized and unbiased controlled results. Morphometric analyses of sections were performed with the aid of Spectrum Plus software (Aperio Technologies). Automated image analysis was performed Spectrum Plus algorithms for i) *Positive Pixel count and Color Deconvolution* for measuring and quantifying intensity per area for two or more stains; ii) *Immunohistochemistry Membrane* image analysis for detection of membrane-associated staining for individual cells and quantifies intensity and completeness (e.g., change in cytokeratin 5 and 8); iii) *Immunohistochemistry Nuclear* image analysis algorithm detects the nuclear staining for the individual nuclei and quantifies their intensity (e.g., change in the number Ki67^{+} nuclei).

siRNA

Using a 6-well tissue culture plate, 2×10^5 cells per well were seeded in 2ml antibiotic free normal growth media supplemented with 1% FBS. Cells were incubated at 37°C in a CO_2 incubator until the cells were 80% confluent. Cell

25

viability was ensured before transfection with 60 pmoles of siRNA duplexes specific for CXCR7 (mRNA accession number NM_020311: Strand 1-CUACACGCUCUCCUUCAUU, Strand 2- CACUAUUGGUGUACCUUAU, and Strand 3- GGAUGACACUAAUUGUUAG), CXCR4 (mRNA accession number NM_003467: Strand 1- CUAGCUUUCUUCCACUGUU, Strand 2-CAGAGCGUGUAGUGAAUCA, and Strand 3- GAUGGCACUUAUAACCAAA), control siRNA and transfection media were acquired from Santa Cruz, CA) was diluted into 100 µl of FBS-free siRNA transfection medium. 60 pmoles of siRNA transfection reagent was diluted into 100 µl of FBS-free siRNA transfection medium. The siRNA duplex contained a pool of three target-specific 19 nt siRNAs designed to knockdown CXCR7 or CXCR4 gene expression and added directly to the diluted transfection reagent. The mixture was incubated for 30 minutes at RT. Cells were washed with 2 ml of FBS-free siRNA transfection medium. Next, 0.8 ml of siRNA transfection medium was added to each tube containing the siRNA transfection reagent mixture and over-layed onto the washed cells. Cells were incubated for seven hours at 37°C in a CO_2 incubator. After seven hours, 1 ml of normal growth medium was added containing 5% FBS and 1X penicillin/streptomycin without removing the transfection mixture. Cells were incubated for 24 hours and. assayed by RT-PCR or Amnis Imagestream to confirm knockdown of CXCR4 or CXCR7 mRNA or protein expression, respectively.

Primer Design

Human mRNA sequences for CXCR4, CXCR7, CXCL12, MMP-1, MMP-13, and 18S rRNA were obtained from National Center for Biotechnology Information (NCBI, Bethesda, MA) GenBank database. The accession numbers were NM003467, NM020311, NM000609, NM002421, NM005940, and X00686.1, respectively. These sequences were then used to design primers for semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis that generated amplicons of 114, 188, 168, 83, 176 and 149 bp in size for CXCR7, CXCR4, CXCL12, MMP-1, MMP-13 and 18S rRNA respectively. Primers were designed using the Primer 3 software program (http://frodo.wi.mit.edu) from the Whitehead Institute at the Massachusetts Institute of Technology. Thermodynamic analysis of the primers was conducted using Primer Premier[TM] (Integrated DNA Technologies) and MIT Primer III. The resulting primer sets were compared against the entire human genome to confirm specificity and to ensure that the primers flanked mRNA splicing regions.

RNA Isolation and Semi-Quantitative RT-PCR

To determine quantitatively CXCR4, CXCR7, CXCL12, MMP-1, MMP-13, and 18S mRNA expression, cells were grown and treated as mentioned above then analyzed. Total RNA was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol followed by precipitation with High Salt solution (Molecular Research Center). RNA was precipitated and resuspended in 27 µl of RNA Secure (Ambion). Next, 1 µg of total RNA was reverse

27

transcribed to generate cDNA using iScript reagents (BioRad Laboratories) according to the manufacturer's protocol. As described above, cDNA was amplified for gene expression using specific primers for CXCR4, CXCR7, CXCL12, MMP-1, MMP-13, and 18S rRNA. For semi-quantitative PCR, all samples contained a reaction mixture of cDNA; specific primers (10 ng/µl) along with SYBR Green polymerase chain reaction master mix (BioRad) reagents, according to the manufacturer's protocol. All samples were tested for the above listed analysts and amplified using the BioRad iQ5 Real-Time PCR Detection System, following predefined parameters [cycle 1: 95°C for 3 min; cycle 2 95°C for 10 sec, 60°C for 1 min (40 cycles)]. Each experiment contained treatment groups for HMEC, MCF-7, MDA-MB-231, and cDNA standards (10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 copies) for 18S rRNA. The number of copies of mRNA relative to 18S rRNA copies of these targets was evaluated and the number of copies for each target was calculated using a standard curve that was normalized to the number of copies of 18S rRNA expressed in each sample. Results are presented as the number of copies of target per 10^6 copies of 18S rRNA. Samples for gene expression were performed in duplicates and repeated twice.

Active MMP-1 and MMP-13 detection

To determine active MMP-1 (collagenase 1) and MMP-13 (collagenase 3) expression, conditioned media was collected and analyzed for each analyst. Flurokine (MMP-1 and MMP-13; R&D Systems) assay kits were used as described by the manufacturer's protocols.

Amnis Imagestream analysis of protein expression and localization

PE/Cy5 conjugated anti-human CXCR4 antibody (clone#12G5) was

purchased from Biolegend, Inc. Polyclonal rabbit anti-CXCR7 antibody was

purchased from GeneTex, Inc and conjugated with a donkey-anti-rabbit-FITC

antibody (R&D Systems). BrCa cells were seeded in 6-well non-adherent plates

at 10^6 cells/well in media containing 1% FBS and allowed to acclimate 3 hours

before treatment. Cells were treated according to the above-mentioned protocols

and cell suspensions from each well were taken at 0 and 5 minute time-points

and collected for further analysis. Cell suspensions were centrifuged at 200 x g

for 10 minutes at 4°C to pellet cells, supernatants were removed and cells were

resuspended in 500 µl of paraformaldehyde (PFA) / phosphate buffered saline

(PBS) solution for 10 minutes at RT. Again, cells were centrifuged at 200 x g for

10 minutes at 4°C to pellet cells, supernatants were removed and cells were

resuspended in 100 µl of saponin solution for 30 minutes at RT. Cell

suspensions were centrifuged at 200 x g for 10 minutes at 4°C, supernatants

were removed and cells were resuspended in 1 µg (per 10^6 cells) of primary

antibody solution, which consisted of PE-Cy5-conjugated anti-mouse CXCR4,

FITC-conjugated anti-mouse CXCR7, Alexa Fluor 488-conjugated or PE-

conjugated anti-mouse NF-kB p65, PE-conjugated anti-mouse ERK1/2

antibodies and/or 7AAD (BD) for 30 min at room temperature. Next, 1ml of

fluorescence-activated cell sorting (FACS) buffer (1% bovine serum albumin

(BSA) in PBS) to remove any unbound antibodies. Cell suspensions were

centrifuged at 200 x g for 10 minutes at 4°C to pellet cells; supernatants were

removed and cells were resuspended in 100 μl of FACS buffer. Analyses were performed using Amnis ImageStream, which allows for flow cytometry-based image acquisition and analyses with six channels (Bright filed, dark filed, and four channel for different fluorochrome). This flow-based image acquisition device is supported by INSPIRE™ software and statistical results gathered using Amnis IDEAS™ software (Amnis Corporation).

The Amnis ImageStream 100 is the first commercially available imaging flow cytometer. It combines advantages of flow cytometry with those of image analysis (digital imagery of each individual cell, calculation of morphological changes, subcellular localization or co-localization of fluorescent probes). Offering several advantages over flow cytometry analysis or microscopic analysis of fluorescently stained cells. Simply put, the ImageStream is essentially a flow cytometer where photomultiplier tubes have been replaced with an array of sensitive charge coupled device (CCD) cameras to capture ~36X images at 100 cells per second.

The images were stored in a compensated image file (CIF) for subsequent analysis using IDEAS software which quantifies change in fluorescent probe pixel intensities in: area of mask in pixels, aspect ratio of mask, weighted aspect ratio of mask, mean intensity of pixels outside of mask, standard deviation of intensity of pixels outside of mask, centroid of mask in horizontal axis, intensity-weighted centroid of mask in horizontal axis, centroid of mask in vertical axis, intensity-weighted centroid of mask in vertical axis, total intensity of image using logical "OR" of all six image masks, variance of intensity of pixels within masks,

maximum intensity gradient of pixels within mask, radius mean square (RMS) of

intensity gradient of pixels within mask, background-corrected sum of pixel

intensities within mask, major axis of mask in pixels, intensity-weighted major

axis of mask in pixels, total Intensity of image divided by area of mask, minimum

pixel intensity within mask, minor axis of mask in pixels, intensity-weighted minor

axis of mask in pixels, angle of major axis relative to axis of flow, angle of

intensity-weighted major axis relative to axis of flow, maximum pixel intensity

within mask, number of edge pixels in mask, maximum pixel intensity within large

bright spots, sum of pixel intensities within large bright spots, maximum pixel

intensity within medium-sized bright spots, sum of pixel intensities within

medium-sized bright spots, un-normalized maximum pixel intensity within large

bright spots, sum of un-normalized pixel intensities within large bright spots,

maximum pixel intensity within small bright spots, sum of pixel intensities within

small bright spots, sum of pixel intensities within mask, number of spots detected

in image, area of logical "OR" of all six image masks in pixels, camera line

readout rate in Hertz at time object was imaged, unique object number, pixel

intensity correlation between two images of the same object, and user-defined

algebraic combination of imagery and masks, user-defined masks using (erode,

dilate, threshold, and boolean combinations), and any boolean combination of

user-defined populations.

Statistical Analysis

To interpret our results, significance tests and statistical analysis were used for each experiment. The traditional α-value, i.e., $p = 0.01$, was used to evaluate the statistical significance of each study. The power of these studies was determined by the probability $(1-\beta)$ of detected significant difference (δ) between control and experimental groups. The data were expressed as the mean $\pm$ SEM and compared using a two-tailed paired (or unpaired) student's t-test. The results were analyzed using the SPSS and Microsoft Excel.

CHAPTER III

RESULTS

The Expression of CXCR7 by Breast Tumors

Prior studies in our laboratory and others have shown chemokine receptor expression by various carcinomas (74, 106). I confirmed *in vivo* protein expression of CXCR7 by breast tumor tissue. Immunohistochemistry was performed using deparaffinized TMAs stained with DAB (brown), which is representative of CXCR7, and counterstained with hematoxylin **(Figure 3)**. CXCR7 expression was elevated in breast tumors staged asT1 (n = 10), T2 (n = 40), T3 (n = 15), and T4 (n = 4) and compared with non-neoplastic breast tissue from the same subject (i.e., n = 69). CXCR7 expression was significantly higher in all breast tumor tissue than compared to benign tissue and highest in T4 > T1, T2, and T3 staged tumor tissue. While expression of CXCR7 in breast tumors did not correlate with stage, a trend of cytoplasmic to nucleur localization of CXCR7 expression was observed when comparing early stage with advanced stage cases, respectively.

CXCR7 mRNA Expression in BrCa Cells

To determine whether chemokine/chemokine receptor interactions are involved in BrCa cell progression, I performed comprehensive and quantitative analyses of CXCR7 and CXCR4 expression in two BrCa cell lines, MCF-7 (ER^+,
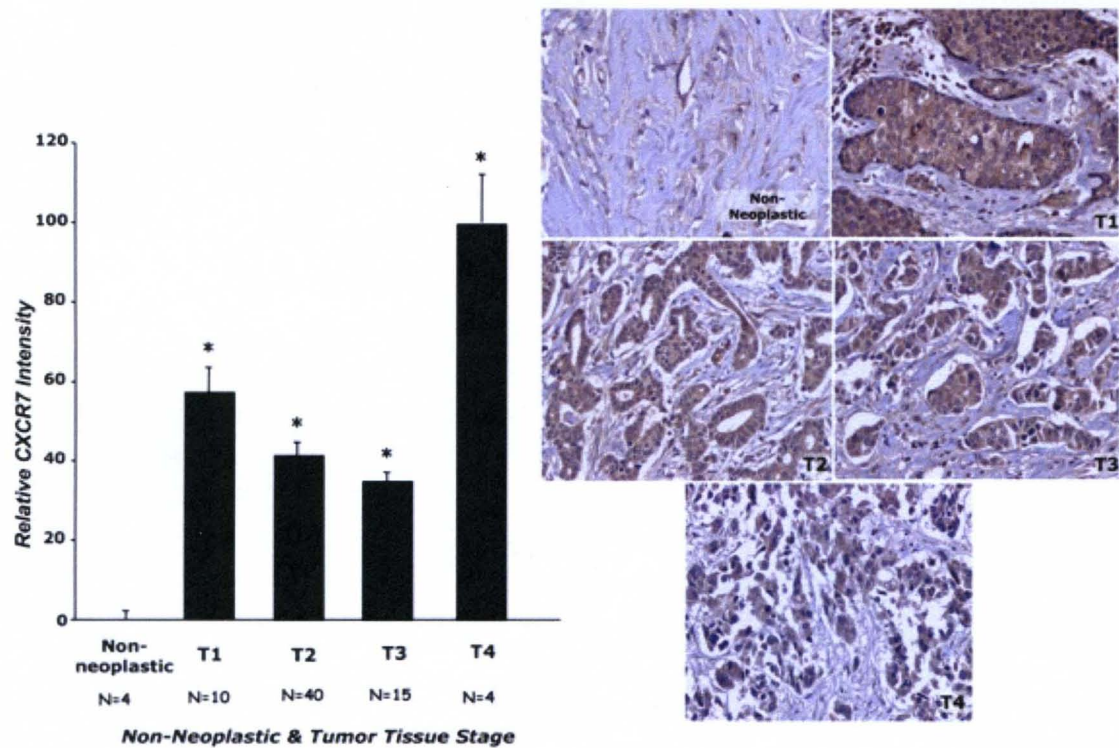
Figure 3. Relative CXCR7 Expression by non-neoplastic and malignant breast tissues. Previously evaluated samples were stained for CXCR7. Relative CXCR7 expression (i.e., colorimetric intensity) ± SD by non-neoplastic (n = 4) breast tissue and T1 (n = 10), T2 (n = 40), T3 (n = 15), and T4 (n = 2) staged tumors are shown in the left panel. Representative CXCR7 (DAB-brown) and hemotoxylin-counter-stained (20X magnification) sections are shown in the right panels.

PR$^+$ and HER2$^+$) and MDA-MB-231 (ER$^-$, PR$^-$ and HER2$^-$) and compared the expression of these chemokine receptors to HMECs. Absolute mRNA levels were determined using semi-quantitative RT-PCR that showed that CXCR7 and CXCR4 were expressed in the BrCa cells (untreated) being investigated. Three different patterns of receptor expression were observed when comparing primary HMECs to the BrCa cell lines. First, both CXCR7 and CXCR4 mRNA expression was relatively low in HMEC **(Figure 4)**. Second, CXCR7 mRNA expression was elevated in MCF-7 cells, with CXCR4 mRNA expression being moderately elevated. With regard to the more aggressive BrCa cell line, MDA-MB-231, CXCR4 mRNA expression was highly elevated, with CXCR7 being low. These findings demonstrated the elevated, yet differential, expression of CXCR7 and CXCR4 mRNAs in BrCa cell lines, compared to normal breast epithelial cell lines.

Amnis ImageStream confirmed strong CXCR7 and CXCR4 protein, cell-surface expression by primary human mammary epithelial cells (HMECs) **(Figure 5)** and MCF-7 and MDA-MB-231 cell lines **(Figures 6)**. Cell density plots, which analyzed both positive and negative events of each receptor, show the percentage of cells that were positive either for CXCR7, CXCR4, or both receptors. The percent distribution displayed in each cell density plot was based on the intensity of each receptor's protein expression. These findings demonstrate that CXCR4 and CXCR7 protein expression does not precisely correlate with mRNA expression in these cell lines. Further, these results
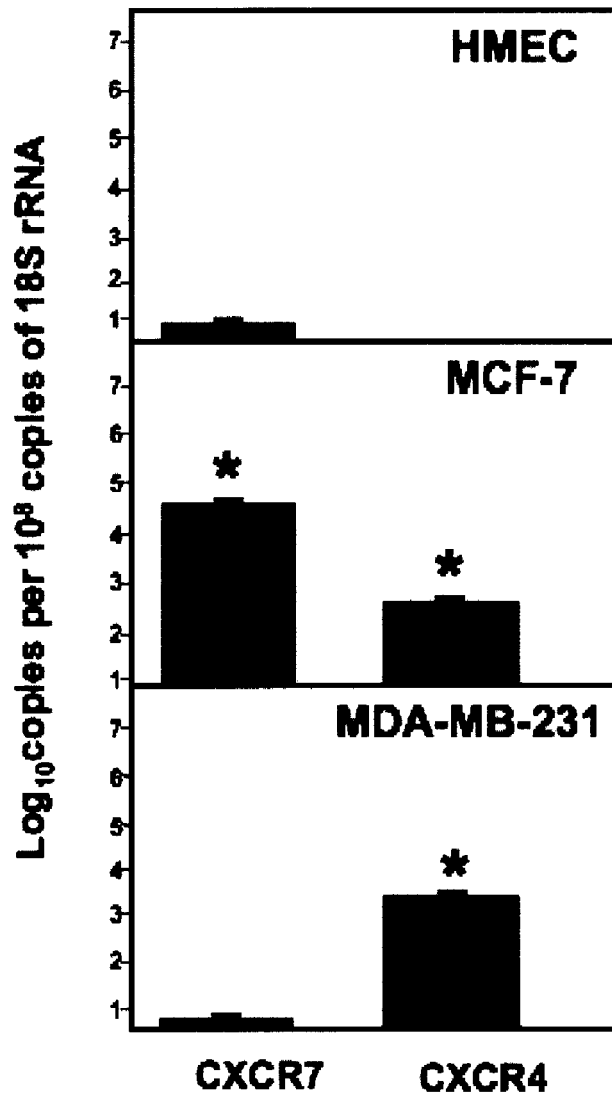
Figure 4. Relative CXCR4 and CXCR7 mRNA expression by BrCa cell lines and HMECs. Total RNA was isolated from primary breast (HMEC) and BrCa cell lines MDA-MB-231 and MCF-7. Semi-quantitative RT-PCR analysis of chemokine receptor mRNA expression was performed in triplicates. The copies of transcripts were expressed relative to actual copies of 18S rRNA. Asterisk(s) indicate statistical significance ($p < 0.05$) between HMEC, MCF-7 and MDA-MB-231 cells.
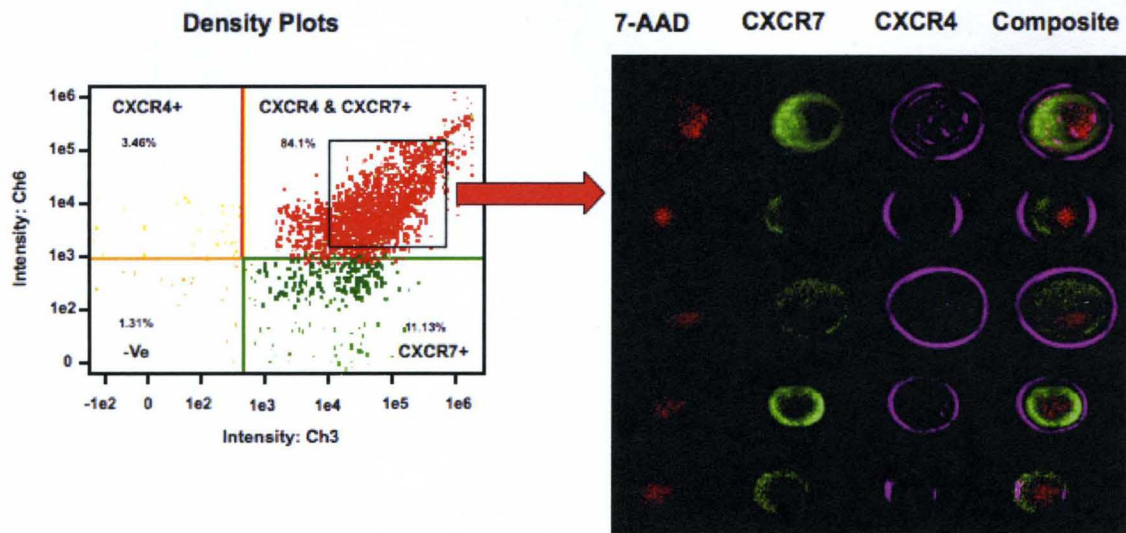
Figure 5. Protein expression and cellular distribution of CXCR4 and CXCR7 by HMECs. Primary breast epithelial cells were stained with polyclonal-rabbit anti-CXCR7 antibody conjugated with donkey-anti-rabbit-FITC and PE/Cy5 anti-human CXCR4 antibody. After staining, cells were washed with FACS buffer and analyzed. 7-AAD was used to stain the nucleus and positive stained cells were quantified by Amnis ImageStream. Image analyses were performed using Image Data Exploration Analysis Software (IDEAS). The panel on the left is a density plot of the cell population that was positively stained for CXCR4 and CXCR7. The panel on the right from left to right is 7-AAD (red), CXCR7 (green), CXCR4 (purple), and composite, respectively.
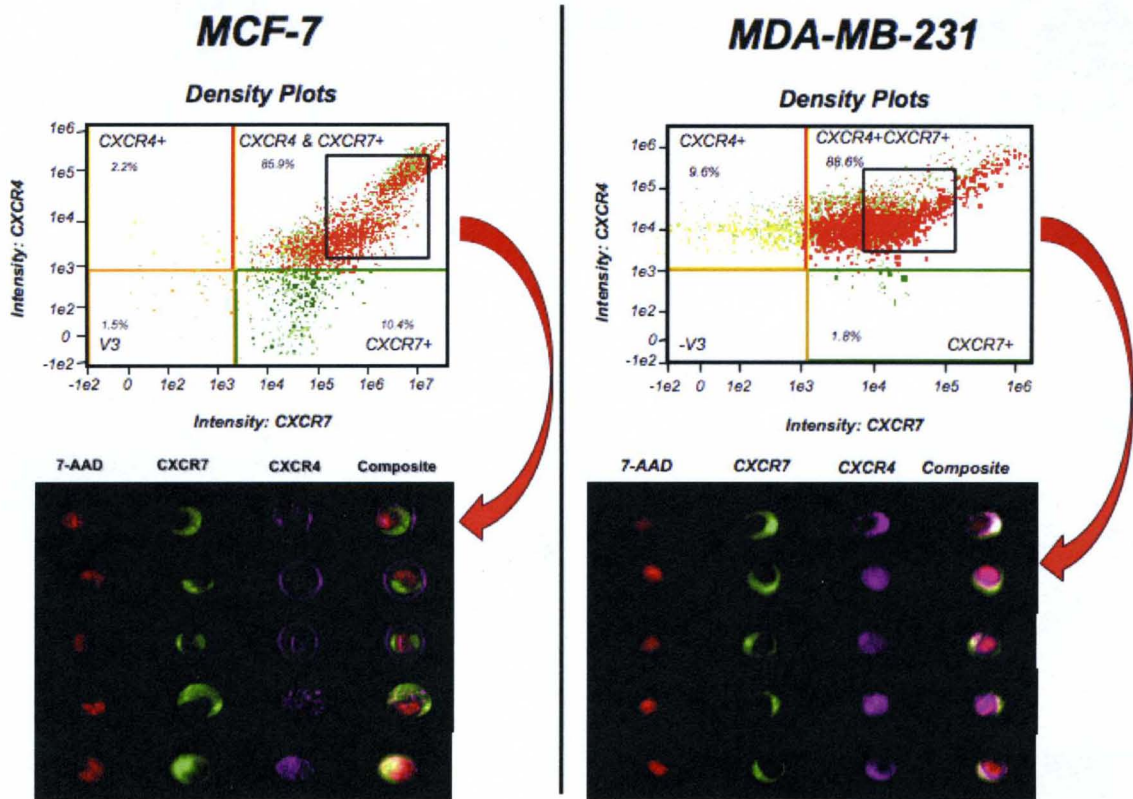
Figure 6. Protein expression and cellular distribution of CXCR4 and CXCR7 by BrCa cell lines. MCF-7 and MDA-MB-231 cells were stained with polyclonal-rabbit anti-CXCR7 antibody conjugated with donkey-anti-rabbit-FITC and PE/Cy5 anti-human CXCR4 antibody. After staining, cells were washed with FACS buffer and analyzed. 7-AAD was used to stain the nucleus and quantified using Amnis Imagestream System. Image analyses were performed using Image Data Exploration Analysis Software (IDEAS). Top panels show density plots of cell populations. Bottom panels from left to right is 7-AAD (red), CXCR7 (green), CXCR4 (purple), and composite respectively.

suggest post-transcriptional and/or -translational modification of chemokine receptors may occur, which would not doubt effect their function.

Receptor expression during the cell cycle as well as translocation was also determined using untreated BrCa cells. MCF-7 and MDA-MB-231 cell lines showed high expression of both CXCR7 and CXCR4 in G_2 phase of the cell cycle, with moderate to low expression for S and comparatively low expression during $G_0/G1$ phases (Figure 7). HMECs showed a similar pattern. Interestingly, this pattern of chemokine receptor expression supports the notion that chemokine receptor signaling during the cell cycle might promote cell survival and proliferation. It is important to mention that while CXCR4 expression is largely confined to the cytoplasm during BrCa progression, the expression of CXCR7 is localized to tumor cell nuclei for advanced BrCa cases (Figure 3). When comparing BrCa cell lines and HMECs, CXCR4 and CXCR7 translocated to the nucleus of cells after CXCL12 stimulation (Figures 8, 9, and 10). Taken together, CXCR4 and CXCR7 expression and cellular distribution correlates with advanced BrCa stage and CXCL12 stimulation of BrCa cells.

Regulation of endogenous CXCR4 and CXCR7 expression

A major breakthrough in gene regulation came with the observation that siRNA of 21 nucleotides (nt) in length that mimic Dicer cleavage products can efficiently induce sequence-specific gene silencing when transiently transfected into mammalian cells (107, 108). To genetically knockdown CXCR4 and CXCR7 expression by MCF-7 and MDA-MB-231 cell lines, I used siRNA nucleotides that
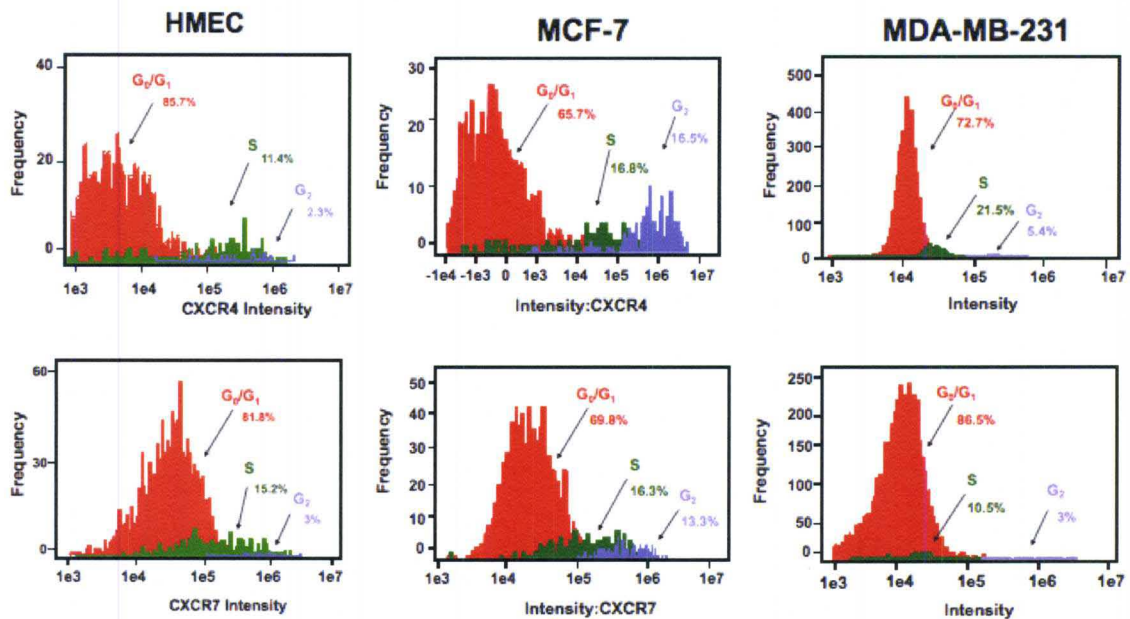
Figure 7. CXCR4 and CXCR7 expression by primary mammary and BrCa cell lines. HMEC cells as well as MCF-7 and MDA-MB-231 cell lines were stained with polyclonal-rabbit anti-CXCR7 antibody conjugated with donkey-anti-rabbit-FITC and PE/Cy5 anti-human CXCR4 antibody. After staining, cells were washed with FACS buffer and analyzed. 7-AAD was used to stain the nucleus and positive stained cells were quantified by Amnis ImageStream. Image analyses were performed using IDEAS. Histograms represent cell populations CXCR4 and CXCR7 intensity.
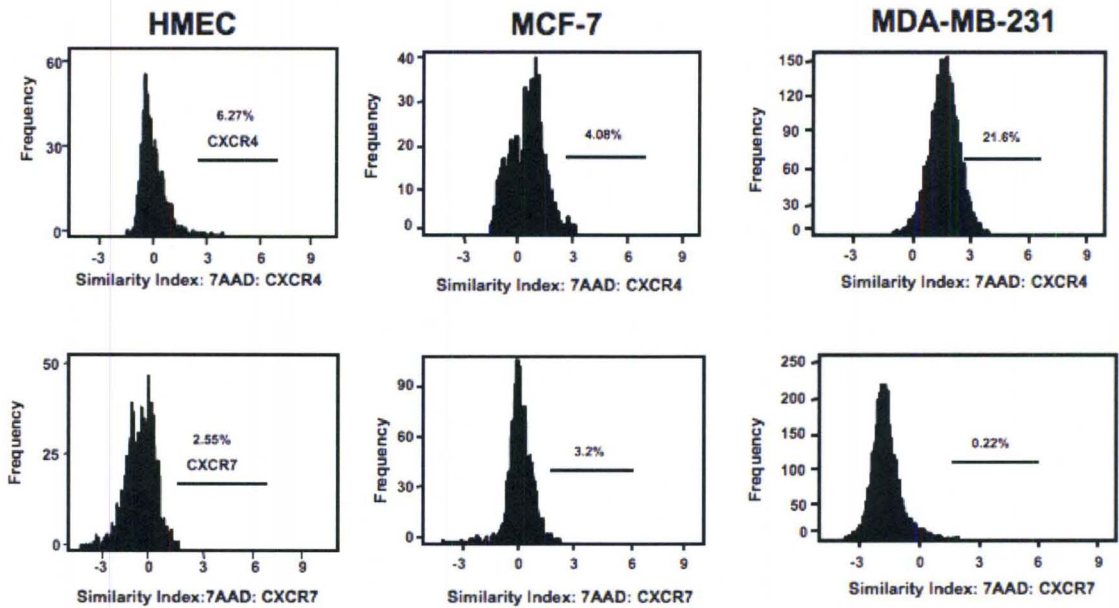
Figure 8. Expression and non-nucleus distribution CXCR4 and CXCR7 in resting primary mammary and BrCa cells. HMEC as well as MCF-7 and MDA-MB-231 cell lines were stained with polyclonal-rabbit anti-CXCR7 antibody, FITC-conjugated donkey-anti-rabbit antibody, and PE/Cy5-conjugated anti-human CXCR4 antibody. After staining, cells were washed with FACS buffer and analyzed. 7-AAD was used to stain the nucleus. Subsequently, cells were quantified by Amnis ImageStream. Image analyses were performed using IDEAS. The left panels show the spatial ratio of chemokine receptor and nuclei intensities as a histogram representing CXCR4 or CXCR7 and nucleus localization or translocation.
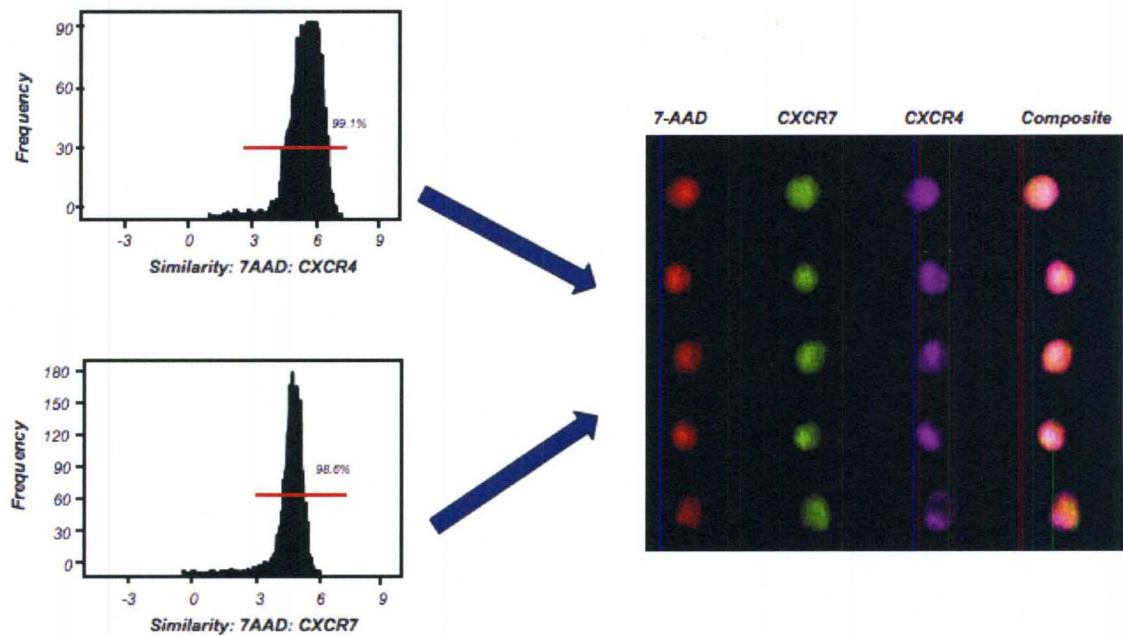
Figure 9. CXCR4 and CXCR7 cytoplasmic to nuclear translocation and expression by MCF-7 cells. MCF-7 cells were treated with 100 ng/ml of CXCL12 for 5 minutes and stained with polyclonal-rabbit anti-CXCR7 antibody, FITC-conjugated donkey-anti-rabbit antibody, and PE/Cy5-conjugated anti-human CXCR4 antibody. After staining, cells were washed with FACS buffer and analyzed. 7-AAD was used to stain the nucleus. Subsequently, cells were quantified by Amnis ImageStream. Image analyses were performed using IDEAS. The left panels show the spatial ratio of chemokine receptor and nuclei intensities as a histogram representing CXCR4 or CXCR7 and nuclear localization or translocation. The right panel shows representative images of the major cell population.
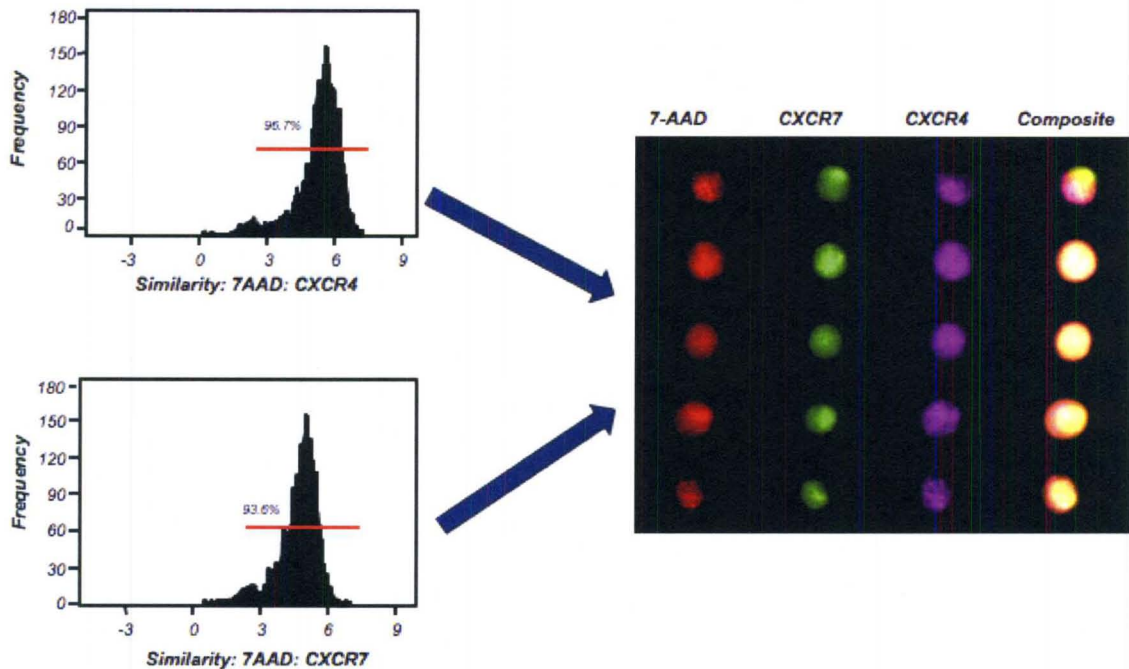
Figure 10. CXCR4 and CXCR7 cytoplasmic to nuclear translocation and expression by MDA-MB-231 cells. MDA-MB-231 cells were treated with 100 ng/ml of CXCL12 for 5 minutes and stained with polyclonal-rabbit anti-CXCR7 antibody, FITC-conjugated donkey-anti-rabbit antibody, and PE/Cy5-conjugated anti-human CXCR4 antibody. After staining, cells were washed with FACS buffer and analyzed. 7-AAD was used to stain the nucleus. Subsequently, cells were quantified by Amnis ImageStream. Image analyses were performed using IDEAS. The left panels show the spatial ratio of chemokine receptor and nuclei intensities as a histogram representing CXCR4 or CXCR7 and nuclear localization or translocation. The right panel shows representative images of the major cell population.

targeted human CXCR4 and CXCR7 mRNA. siRNAs were transiently

transfected into BrCa cells for 24 hours using the above mentioned protocol

before being pre-treated with or without inhibitors (100 ng/ml of pertussis toxin or

6 µM of U-73122) 1 hour prior to 100 ng/ml of CXCL12 for 16 hours. The siRNA

transfection efficiency was determined by Western Blot analyses and Amnis

ImageStream (data not shown). As expected, CXCR4- or CXCR7-specific siRNA

treatment completely abrogated CXCR4 or CXCR7 mRNA expression,

respectively, in both BrCa cell lines **(Figures 11 and 12)**.

CXCL12 treatment significantly increased the expression of CXCR7

mRNA, but not CXCR4 mRNA, by MDA-MB-231 cells without having an effect on

MCF-7 cells. Moreover, CXCL12 treatment did not effect CXCR4 mRNA

expression by either cell line. Pertussis toxin inhibited the CXCL12-mediated

CXCR7 mRNA upregulation; whereas, U-73122 did not **(Figure 13)**. However, in

MDA-MB-231 BrCa cells, both pertussis toxin and U-73122 were able to inhibit or

completely abrogate CXCL12-dependent CXCR7 mRNA expression **(Figure 14)**.

CXCL12 stimulation of MDA-MB-231 cells also enhanced their expression of

CXCL12 mRNA **(Figure 15),** when compared to untreated cells. This

expression; however, was inhibited by CXCR4- and CXCR7-specific siRNA as

well as by pertussis toxin and U-73122 treatment. CXCL12 treatment of MCF-7

cells also resulted in a measurable increase in CXCL12 mRNA expression, but

this effect was enhanced by CXCR4, CXCR7, and Gβ/Gγ protein inhibition, but

hindered by Gαi protein inhibition **(Figure 16)**. These results underline the

CXCR4

Log$_{10}$copies of CXCR4 mRNA per 10^6 of 18S rRNA

6
5
4
3
2
1

BD

*

BD

CXCL12	–	+	+	+	+
siRNA CXCR4	–	–	+	–	+
siRNA CXCR7	–	–	–	+	+

CXCR7

Log$_{10}$copies of CXCR7 mRNA per 10^6 of 18S rRNA

6
5
4
3
2
1

BD

BD

CXCL12	–	+	+	+	+
siRNA CXCR4	–	–	+	–	+
siRNA CXCR7	–	–	–	+	+

Figure 11. CXCR4 and CXCR7 mRNA expression by MCF-7 cells. Total RNA was isolated from BrCa cells pretreated with or without 60 pmoles of CXCR4- or CXCR7-specific siRNA in the presence or absence of 100 ng/ml CXCL12 for 16 hours. Semi-quantitative RT-PCR analysis of CXCR4 and CXCR7 mRNA expression was performed in triplicate. The copies of transcripts were expressed relative to copies of 18S rRNA. Asterisk(s) indicate statistical significance ($p <$ 0.05) between treatment groups.

CXCR7

Log$_{10}$copies per 10^6 of CXCR7 mRNA per 10^6 of 18S rRNA

BD · BD BD BD

CXCL12	–	+	+	+	+
siRNA CXCR4	–	–	+	–	+
siRNA CXCR7	–	–	–	+	+

CXCR4

Log$_{10}$copies per 10^6 of CXCR4 mRNA per 10^6 of 18S rRNA

BD BD

CXCL12	–	+	+	+	+
siRNA CXCR4	–	–	+	–	+
siRNA CXCR7	–	–	–	+	+

Figure 12. CXCR4 and CXCR7 mRNA expression by MDA-MB-231 cells.

Total RNA was isolated from BrCa cells pretreated with or without 60 pmoles of

CXCR4- or CXCR7-specific siRNA in the presence or absence of 100 ng/ml

CXCL12 for 16 hours. Semi-quantitative RT-PCR analysis of CXCR4 and

CXCR7 mRNA expression was performed in triplicate. The copies of transcripts

were expressed relative to copies of 18S rRNA. Asterisk(s) indicate statistical

significance ($p < 0.05$) between treatment groups.

CXCR7

CXCR4

CXCL12	–	+	+	+
siRNA CXCR4	–	–	+	+
Pertussis Toxin	–	–	+	–
U-73122	–	–	–	+

CXCL12	–	+	+	+
siRNA CXCR7	–	–	+	+
Pertussis Toxin	–	–	–	+
U-73122	–	–	+	–

Figure 13. Gα and Gβ/Gγ protein-dependent CXCR7 and CXCR4 mRNA expression by MCF-7 cells. Total RNA was isolated from BrCa cells pretreated with or without CXCR4- or CXCR7-specific siRNA and/or pertussis toxin and U-73122 in the presence or absence of 100 ng/ml CXCL12 for 16 hours. Semi-quantitative RT-PCR analysis of CXCR7 and CXCR4 mRNA expression was performed in triplicates. The copies of transcripts were expressed relative to actual copies of 18S rRNA. Asterisk(s) indicate statistical significance ($p < 0.05$) between treatment groups.

47

CXCR7

CXCR4

CXCL12	−	+	+	+
siRNA CXCR4	−	−	+	+
Pertussis Toxin	−	−	−	+
U-73122	−	−	+	−

CXCL12	−	+	+	+
siRNA CXCR7	−	−	+	+
Pertussis Toxin	−	−	+	−
U-73122	−	−	−	+

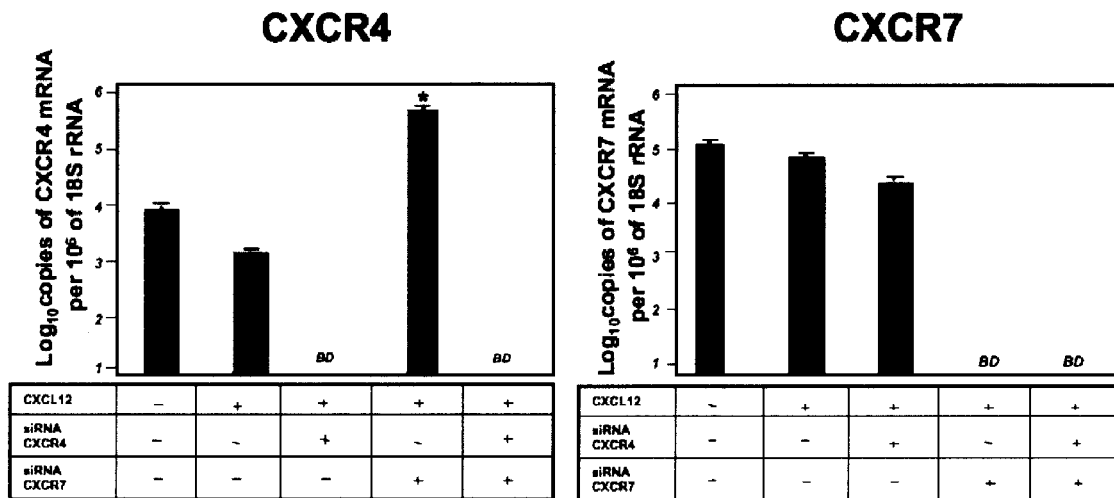**Figure 14. Gα and Gβ/Gγ protein-dependent CXCR7 and CXCR4 mRNA expression by MDA-MB-231 cells.** Total RNA was isolated from BrCa cells pretreated with or without CXCR4- or CXCR7-specific siRNA and/or pertussis toxin and U-73122 in the presence or absence of 100 ng/ml CXCL12 for 16 hours. Semi-quantitative RT-PCR analysis of CXCR7 and CXCR4 mRNA expression was performed in triplicates. The copies of transcripts were expressed relative to actual copies of 18S rRNA. Asterisk(s) indicate statistical significance ($p < 0.05$) between untreated and treated groups.
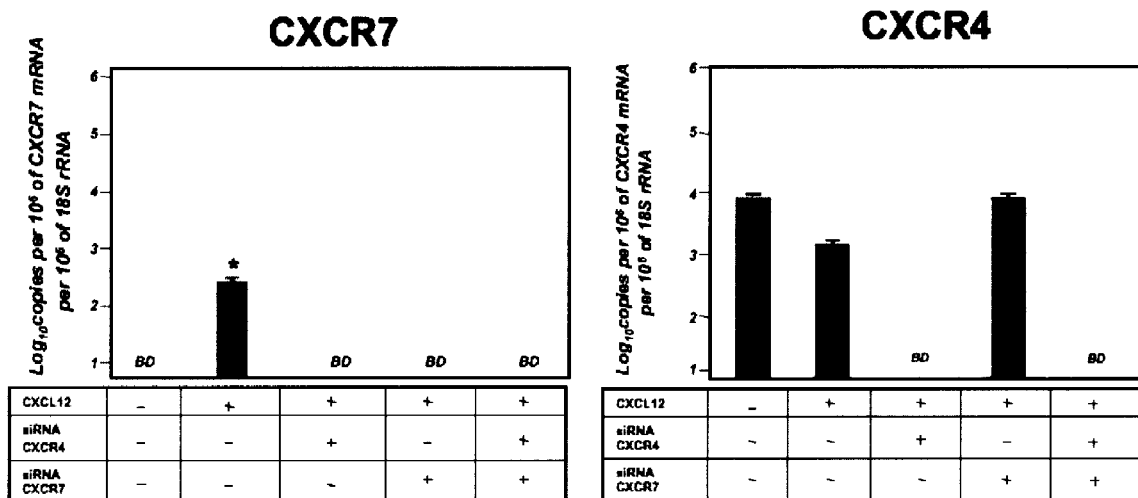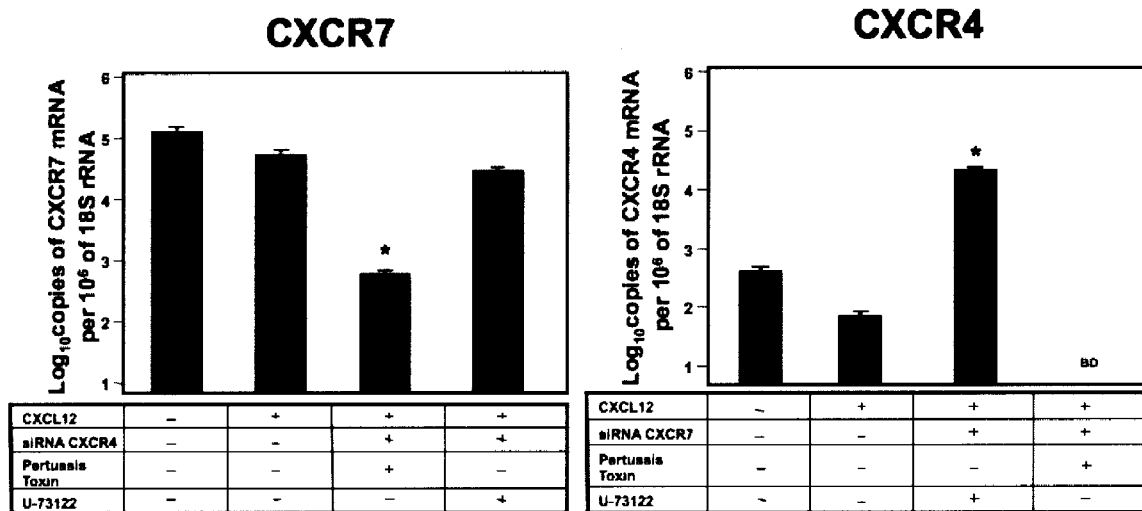
CXCL12	-	+	+	+	+	+	+	+	+	+
siRNA CXCR4	-	-	+	-	+	-	+	-	+	-
siRNA CXCR7	-	-	-	+	+	-	-	+	-	+
Pertussis Toxin	-	-	-	-	-	+	+	+	-	-
U-73122	-	-	-	-	-	-	-	-	+	+

Figure 15. Gα and Gβ/Gγ protein-dependent CXCL12 mRNA expression by MDA-MB-231 cells. Total RNA was isolated from BrCa cells pretreated with or without CXCR4- or CXCR7-specific siRNA and/or pertussis toxin and U-73122 in the presence or absence of 100 ng/ml CXCL12 for 16 hours. Semi-quantitative RT-PCR analysis of CXCL12 mRNA expression was performed in triplicates. The copies of transcripts were expressed relative to actual copies of 18S rRNA.

49

CXCL12	-	+	+	+	+	+	+	+	+
siRNA CXCR4	-	-	+	-	+	+	-	+	-
siRNA CXCR7	-	-	-	+	+	-	+	-	+
Pertussis Toxin	-	-	-	-	-	+	+	-	-
U-73122	-	-	-	-	-	-	-	+	+

Figure 16. Gα and Gβ/Gγ protein-dependent CXCL12 mRNA expression by MCF-7 cells. Total RNA was isolated from BrCa cells pretreated with or without CXCR4- or CXCR7-specific siRNA and/or pertussis toxin and U-73122 in the presence or absence of 100 ng/ml CXCL12 for 16 hours. Semi-quantitative RT-PCR analysis of CXCL12 mRNA expression was performed in triplicates. The copies of transcripts were expressed relative to actual copies of 18S rRNA. Asterisk(s) indicate statistical significance ($p < 0.05$) between untreated and treated groups.
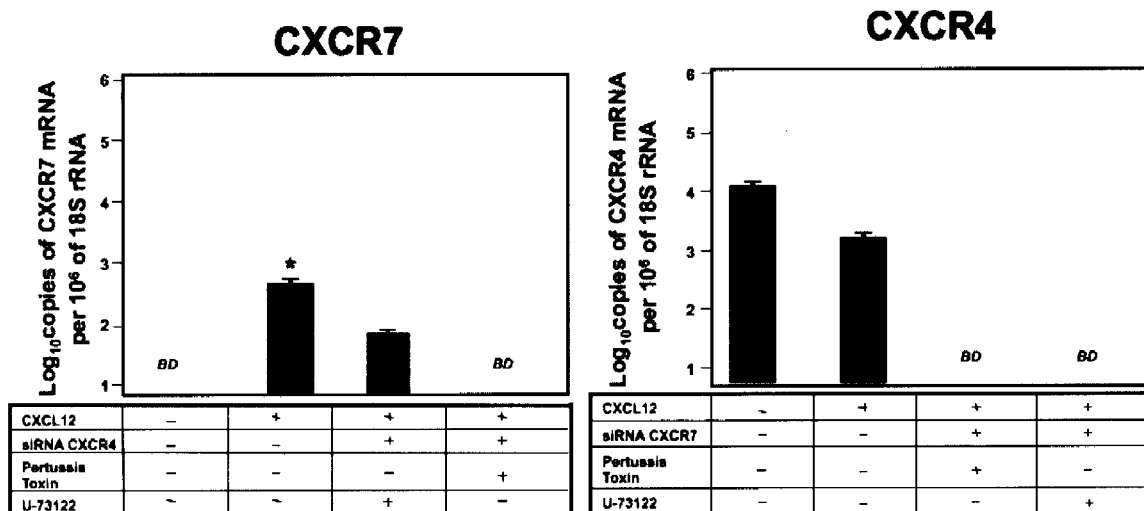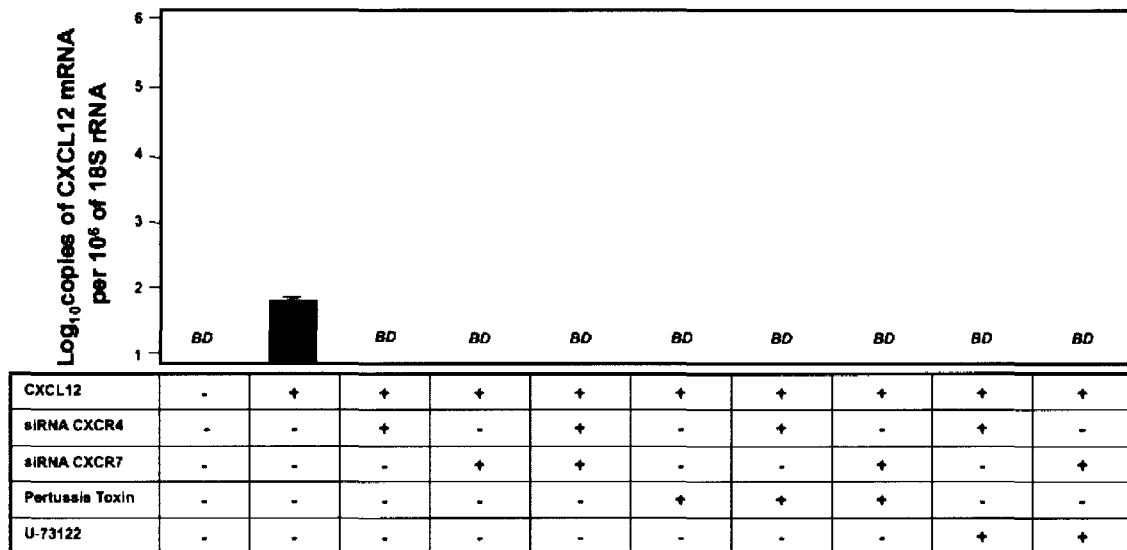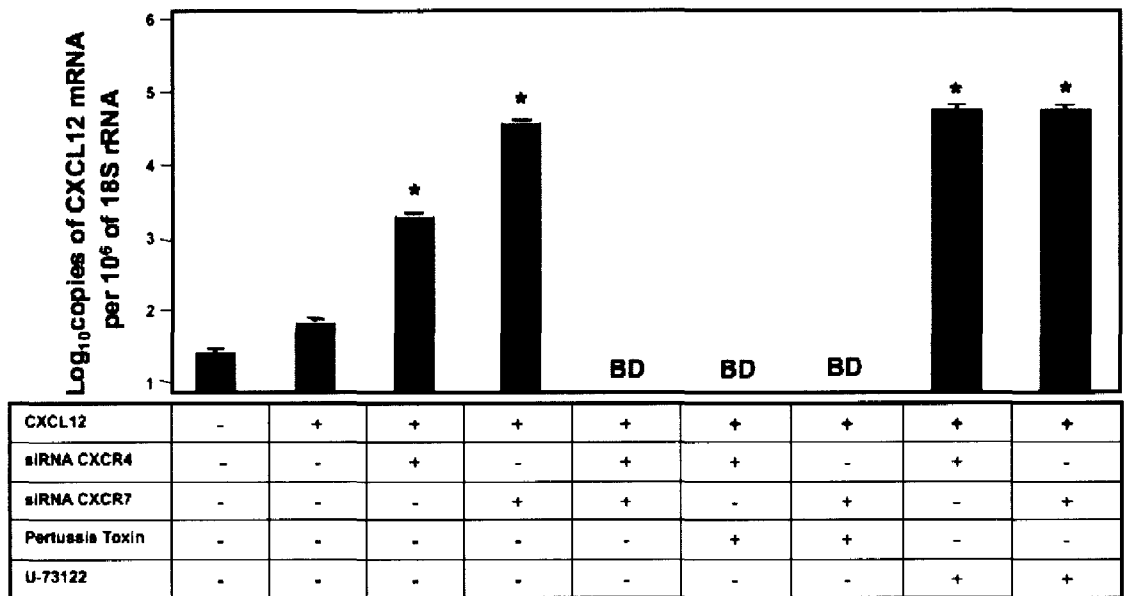
complexity of CXCL12-CXCR4/7 interactions, but demonstrated CXCL12 stimulation of BrCa cells results in the suppression of CXCL12 mRNA expression that was most likely dependent on a Gαi protein.

CXCL12 is a powerful chemo-attractant that stimulates bi-directional migration, invasion, and survival of breast cancer cells (109). I examined whether the interactions of CXCL12-CXCR4/7 are involved in signal-transduction pathways that lead to these events. Previous studies have shown that phosphorylation of ERK1/2 in lung cancer cells is involved in cell migration and invasion (110). Other studies have shown that NF-kB activation is necessary for cell migration and invasion of other cancer cells (111). To determine if ERK1/2 and NF-kB are activated by CXCRL12, *in vitro* assays followed by Amnis ImageStream analysis were performed. Untreated HMECs and BrCa cell lines expressed moderate to high protein levels of phosphorylated ERK1/2 and NF-kB in the cytosol **(Figures 17 and 18)**. After CXCL12 stimulation, both phosphorylated NF-kB and ERK1/2 translocated to the nucleus of BrCa cell lines **(Figures 19 and 20)**. This demonstrates that detection of CXCL12 by CXCR4 and/or CXCR7 transduces cell signals leading to the activation of both ERK1/2 and NF-kB and their translocation to the nucleus.

Liang *et al.* recently demonstrated that silencing of CXCR4 expression in BrCa cells successfully blocks metastasis to various organs. Here, I sought to determine if down regulating CXCR7, CXCR4, or both of their mRNA levels using siRNA would inhibit either activation of ERK1/2 and NFkB or their translocation to cell nuclei. CXCR4- and CXCR7-specific gene silencing lead to complete
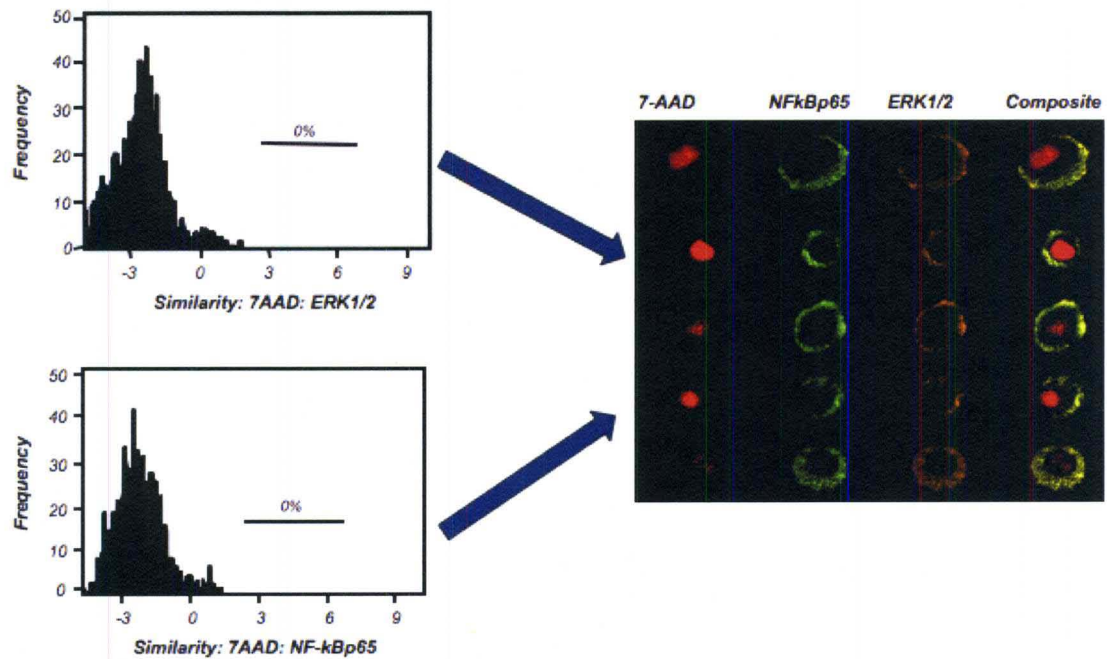
Figure 17. NF-κB and ERK1/2 cytoplasmic to nuclear translocation and expression by HMECs. HMECs were stained with Alexa Fluor 488-conjugated mouse anti-NF-κB p65 and PE-conjugated mouse anti-ERK1/2 antibodies. 7-AAD was used to stain the nucleus. Subsequently, cells were characterized by Amnis ImageStream analysis. Image analyses were performed using IDEAS. The left panels show the spatial ratio of chemokine receptor and nuclei intensities as a histogram representing NF-κB or ERK1/2 and nucleus localization or translocation. The right panel shows representative 7-AAD (red), NF-κB (green), ERK1/2 (orange), and composite images images of the major cell populations.
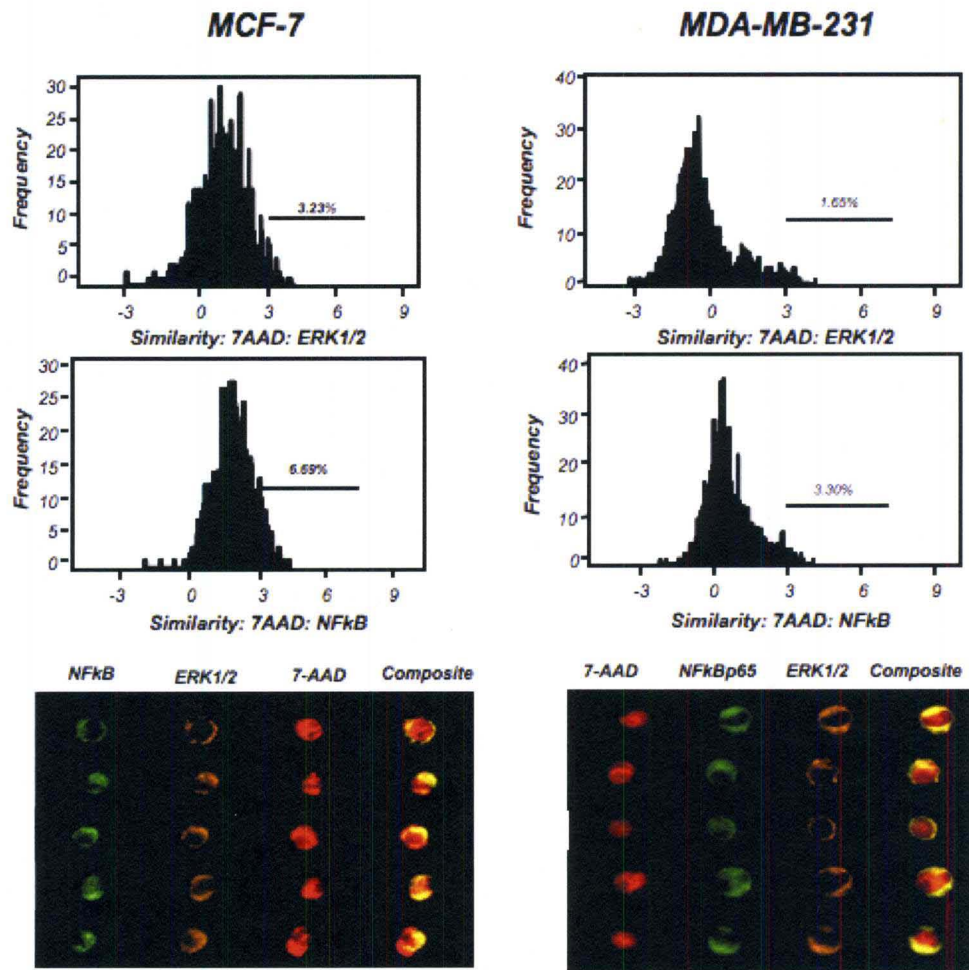
Figure 18. NF-κB and ERK1/2 activation state and cellular distribution by resting BrCa cells. MCF-7 and MDA-MD-231 cell lines were stained with Alexa Fluor 488-conjugated mouse anti-NFκB p65 and PE-conjugated mouse anti-ERK1/2 antibodies. 7-AAD was used to stain the nucleus. Subsequently, images of cells were acquired by Amnis ImageStream and analyzed using IDEAS software. The top panels show the spatial ratio of NFκB, ERK1/2, and nuclei intensities and co-localization as a histogram. The bottom panel shows representative 7-AAD (red), NF-κB (green), ERK1/2 (orange), and composite images of the major cell populations.

Figure 19. NF-κB and ERK1/2 expression and cytoplasmic to nuclear translocation by CXCL12-treated MCF-7 cells. MCF-7 cells were stained with Alexa Fluor 488-conjugated mouse anti-NFκB p65 and PE-conjugated mouse anti-ERK1/2 antibodies. 7-AAD was used to stain the nucleus. Subsequently, images of cells were acquired by Amnis ImageStream and analyzed using IDEAS software. The top panels show the spatial ratio of NFκB, ERK1/2, and nuclei intensities and co-localization as a histogram. The bottom panel shows representative 7-AAD (red), NF-κB (green), ERK1/2 (orange), and composite images of the major cell populations.

Figure 20. NF-κB and ERK1/2 expression and cytoplasmic to nuclear translocation by CXCL12-treated MDA-MB-231 cells. MDA-MB-231 cells were stained with Alexa Fluor 488-conjugated mouse anti-NFκB p65 and PE-conjugated mouse anti-ERK1/2 antibodies. 7-AAD was used to stain the nucleus. Subsequently, images of cells were acquired by Amnis ImageStream and analyzed using IDEAS software. The top panels show the spatial ratio of NFκB, ERK1/2, and nuclei intensities and co-localization as a histogram. The bottom panel shows representative 7-AAD (red), NF-κB (green), ERK1/2 (orange), and composite images of the major cell populations **(Figures 21 and 22).**
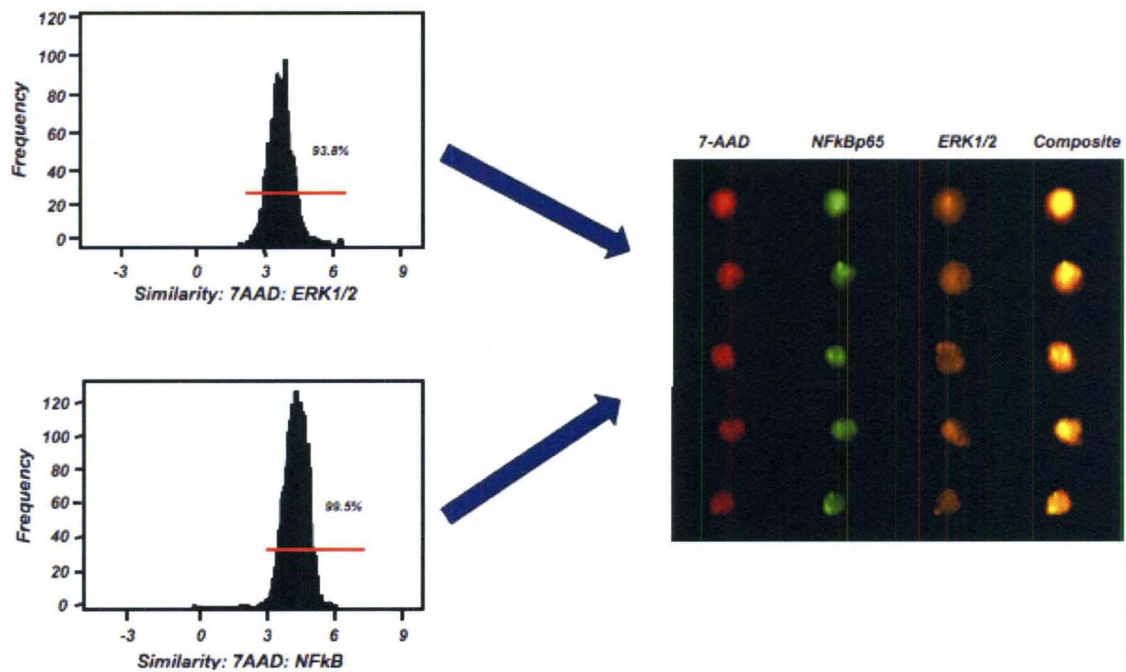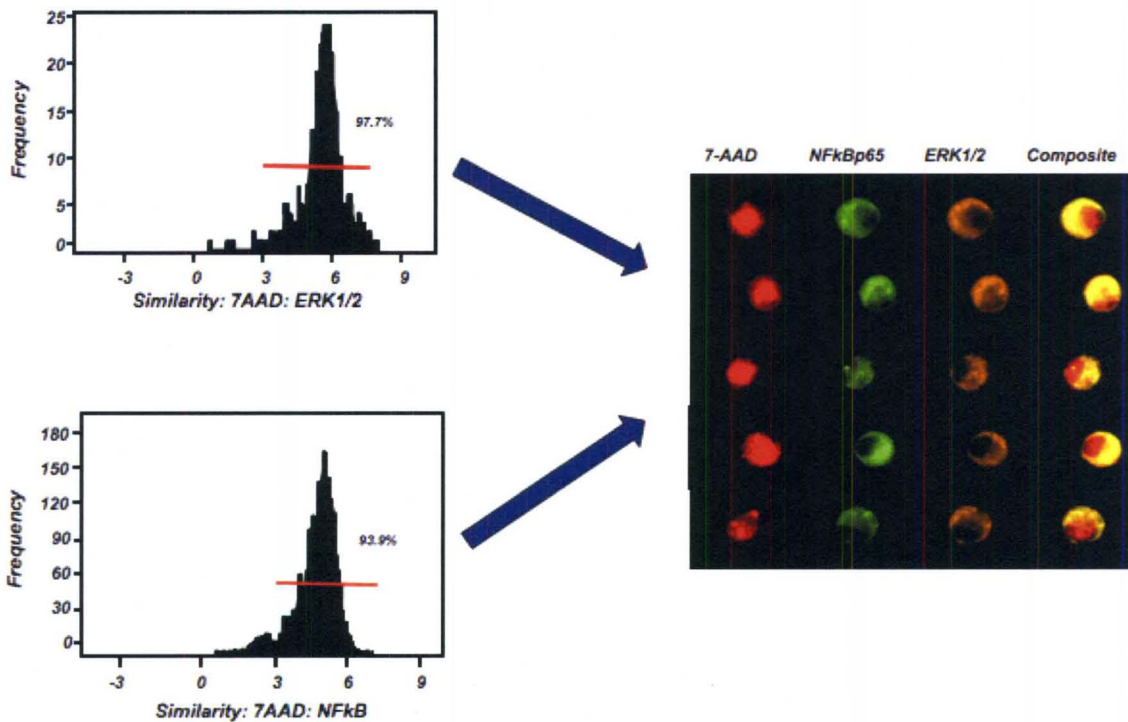
inhibition of CXCL12-mediated ERK1/2 and NF-kB translocation to cell nuclei.

Similarly, Gαi protein inhibition using pertussis toxin and Gβ/Gγ protein inhibition

using U-73122, regardless of CXCR4 or CXCR7 expression also abrogated

ERK1/2 and NF-kB cytoplasmic to nucleur translocation **(Figures 23 and 24)**.

Moreover, these observations were performed in triplicates and at several time

points that demonstrated that the response to CXCL12 is rapid.

CXCL12-CXCR4/CXCR7 interactions mediating MMP-1 and MMP-13 expression

Previous studies in our laboratory demonstrated MMP mRNA expression

and protein activity by prostate cancer cells was modulated by CXCL12-CXCR4

interactions (74). Others have shown that the timely breakdown of the

extracellular matrix (ECM) is essential for breast cancer metastasis (64, 112).

CXCL12 has also been suggested to induce MMP expression in breast cancer

cells through CXCR4 (113). Here, I examined the induction of MMP-1 and MMP-

13 by BrCa cells after CXCL12 stimuli. CXCL12 stimulation of MDA-MB-231

cells caused increases in MMP-1 mRNA and the down regulation of MMP-13

mRNA expression and moderate to low expression of MMP-13 and MMP-1 by

similarly treated MCF-7 cells **(Figure 25)**. Pertussis toxin or U-73122

pretreatment lead to inhibition of both MMP-1 and MMP-13 mRNA expression.

To determine if the observed CXCL12-mediated effects on collagenase

expression and activity were dependent on CXCR4 or CXCR7, these chemokine

receptors were inhibited using siRNA. Interestingly, CXCR4-specific siRNA

Figure 21. CXCR4-dependent NF-κB and ERK1/2 expression and cytoplasmic to nuclear translocation by CXCL12-treated BrCa cell lines. CXCR4-specific siRNA-pretreated MDA-MB-231 and MCF-7 cells were stimulated with CXCL12 and stained with Alexa Fluor 488-conjugated mouse anti-NFκB p65 and PE-conjugated mouse anti-ERK1/2 antibodies. 7-AAD was used to stain the nucleus. Subsequently, images of cells were acquired by Amnis ImageStream and analyzed using IDEAS software. The top panels show the spatial ratio of NFκB, ERK1/2, and nuclei intensities and co-localization as a histogram. The bottom panel shows representative 7-AAD (red), NF-κB (green), ERK1/2 (orange), and composite images of the major cell populations.

MCF-7

MDA-MB-231

Figure 22. CXCR7-dependent NF-κB and ERK1/2 expression and cytoplasmic to nuclear translocation by CXCL12-treated BrCa cell lines. CXCR7-specific siRNA-pretreated MDA-MB-231 and MCF-7 cells were stimulated with CXCL12 and stained with Alexa Fluor 488-conjugated mouse anti-NFκB p65 and PE-conjugated mouse anti-ERK1/2 antibodies. 7-AAD was used to stain the nucleus. Subsequently, images of cells were acquired by Amnis ImageStream and analyzed using IDEAS software. The top panels show the spatial ratio of NFκB, ERK1/2, and nuclei intensities and co-localization as a histogram. The bottom panel shows representative 7-AAD (red), NF-κB (green), ERK1/2 (orange), and composite images of the major cell populations.

58

MCF-7   **MDA-MB-231**

Figure 23. CXCR4-, Gαi protein-, and Gβ protein / Gγ protein-dependent NF-

κB and ERK1/2 expression and cytoplasmic to nuclear translocation by

CXCL12-treated BrCa cell lines. CXCR4-specific siRNA-, pertussis toxin-,

and/or U-73122-pretreated MDA-MB-231 and MCF-7 cells were stimulated with

CXCL12 and stained with Alexa Fluor 488-conjugated mouse anti-NFκB p65 and

PE-conjugated mouse anti-ERK1/2 antibodies. 7-AAD was used to stain the

nucleus. Subsequently, images of cells were acquired by Amnis ImageStream

and analyzed using IDEAS software. The top panels show the spatial ratio of

NFκB, ERK1/2, and nuclei intensities and co-localization as a histogram. The

bottom panel shows representative 7-AAD (red), NF-κB (green), ERK1/2

(orange), and composite images of the major cell populations.

59

MCF-7   **MDA-MB-231**

Figure 24. CXCR7-, Gαi protein-, and Gβ protein / Gγ protein-dependent NF-
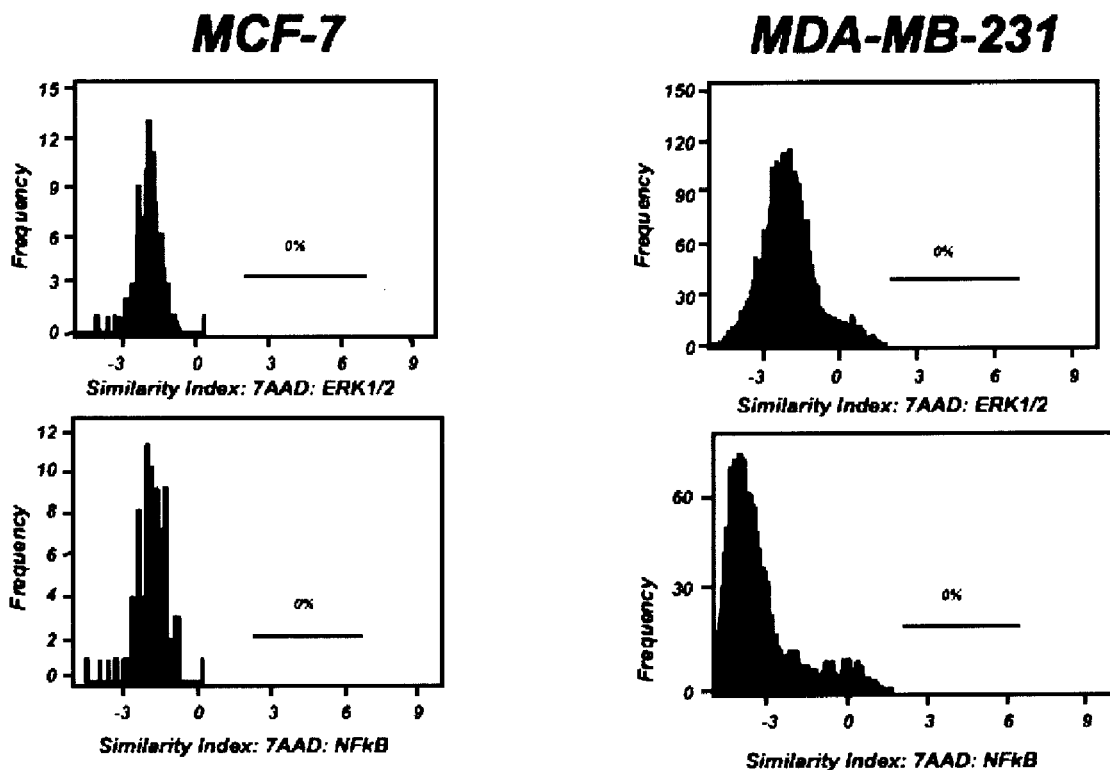
κB and ERK1/2 expression and cytoplasmic to nuclear translocation by

CXCL12-treated BrCa cell lines. CXCR7-specific siRNA-, pertussis toxin-,

and/or U-73122-pretreated MDA-MB-231 and MCF-7 cells were stimulated with

CXCL12 and stained with Alexa Fluor 488-conjugated mouse anti-NFκB p65 and

PE-conjugated mouse anti-ERK1/2 antibodies. 7-AAD was used to stain the

nucleus. Subsequently, images of cells were acquired by Amnis ImageStream

and analyzed using IDEAS software. The top panels show the spatial ratio of

NFκB, ERK1/2, and nuclei intensities and co-localization as a histogram. The

bottom panel shows representative 7-AAD (red), NF-κB (green), ERK1/2

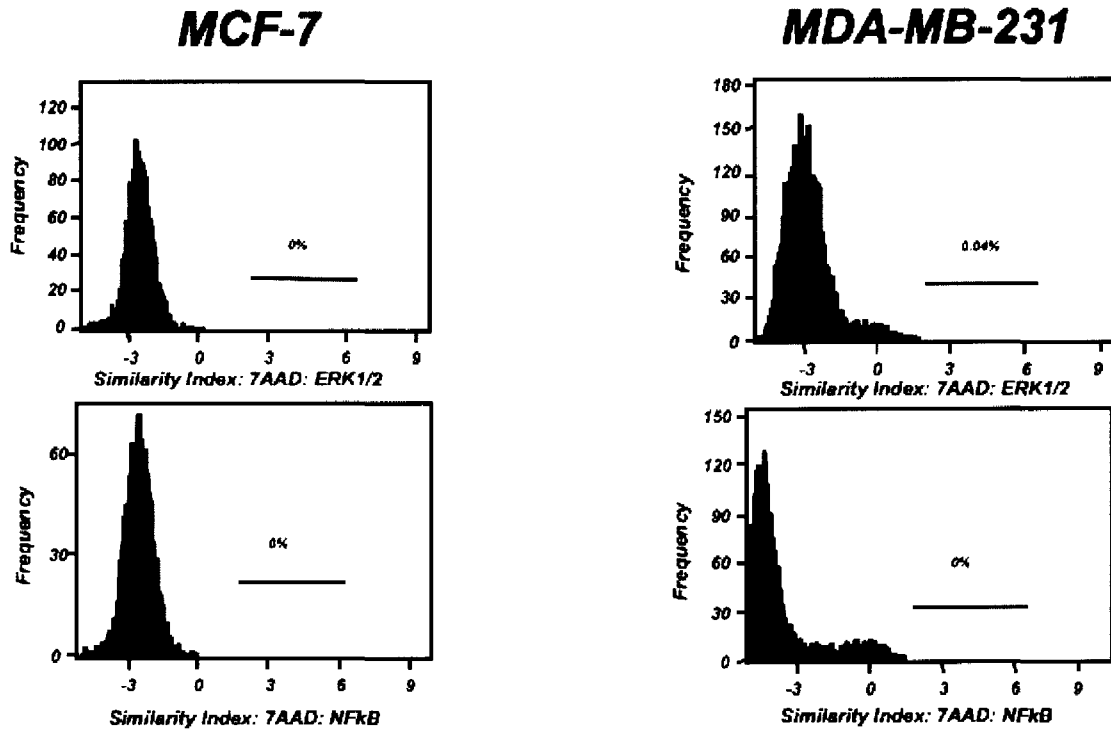(orange), and composite images of the major cell populations.

60

Figure 25. Gα and Gβ/Gγ protein-independent MMP-1 and MMP-13 mRNA expression by BrCa cell lines. Total RNA was isolated from BrCa cells pretreated with or without pertussis toxin and U-73122 in the presence or absence of CXCL12 for 16 hours. Semi-quantitative RT-PCR analysis of CXCL12 mRNA expression was performed in triplicates. The copies of transcripts were expressed relative to actual copies of 18S rRNA. Asterisk(s) indicate statistical significance ($p < 0.05$) between untreated and treated groups.

presumably enhanced CXCL12-dependent induction of MMP-13 mRNA

expression through selective CXCR7 signaling in MCF-7 cells **(Figures 15 and**

26). This CXCL12-CXCR7 interactions were not affected by pertussis toxin, but

U-73122 with selective CXCL12-CXCR7 signaling greatly reduced MMP-13

mRNA expression but enhanced MMP-1 mRNA expression. Interestingly,

CXCR7 silencing, which allowed for selective CXCL12-CXCR4 signaling resulted

in the enhancement of MMP-1 and MMP-13 mRNA expression by MCF-7 cells.

The more aggressive BrCa cell line, MDA-MB-231, produced MMP-13 but

not MMP-1 mRNA **(Figures 16 and 27)**. CXCL12 treatement of these cells

abrogated MMP-13 mRNA expression while greatly enhancing MMP-1 mRNA

expression. CXCR4-specific siRNA abrogated expression of both MMP-1 and

MMP-13, even after CXCL12 treatment. However, CXCR7 inhibition augmented

the endogenous expression of MMP-13 mRNA, but not MMP-1 mRNA. Pertussis

toxin and U-73122 treatment inhibited the ability of CXCL12 to modulate MMP-1

and MMP-13.

MMP-1 and MMP-13 active protein assays were performed to confirm the

above mentioned findings regarding mRNA expression. CXCL12 treatment

increased active MMP-1 and MMP-13 protein expression by MCF-7 and MDA-

MB-231 cells **(Figures 17 and 28)**. CXCR4 inhibition suppressed the functional

activity of MMP-1 and MMP-13 in both BrCa cell lines. However, siRNA silencing

of CXCR7 gene expression resulted in an up regulation of MMP-1 activity by

MCF-7 cells following CXCL12 stimulation. Both pertussis toxin and U-73122

inhibited the increase in active MMP-1 and MMP-13 expressed by MCF-7 and

Log_{10} copies of MMP-1 & 13 mRNA per 10^6 of 18S rRNA

Legend: MMP-13, MMP-1

CXCL12	-	+	+	+	+	+	+	+	+	+
siRNA CXCR4	-	-	+	-	+	-	+	-	+	-
siRNA CXCR7	-	-	-	+	+	-	-	+	-	+
Pertussis Toxin	-	-	-	-	-	+	+	+	-	-
U-73122	-	-	-	-	-	-	-	-	+	+
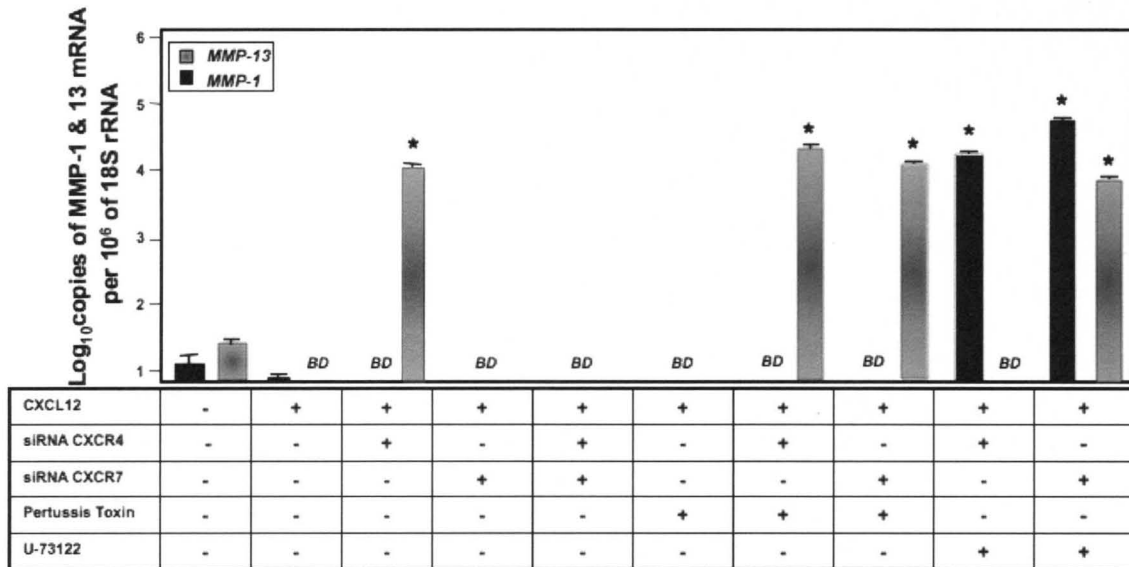
Figure 26. CXCR4- and CXCR7-dependent MMP-1 and MMP-13 mRNA expression by MCF-7 cells. Total RNA was isolated from MCF-7 cells pretreated with or without CXCR4 siRNA, CXCR7 siRNA, pertussis toxin, and U-73122 in the presence or absence of CXCL12 for 16 hours. Semi-quantitative RT-PCR analysis of MMP-1 and MMP-13 mRNA expression was performed in triplicate. Copies of transcripts were expressed relative to copies of 18S rRNA. Asterisk(s) indicate statistical significance ($p < 0.05$) between untreated and treated groups.

CXCL12	-	+	+	+	+	+	+	+	+	+
siRNA CXCR4	-	-	+	-	+	-	+	-	+	-
siRNA CXCR7	-	-	-	+	+	-	-	+	-	+
Pertussis Toxin	-	-	-	-	-	+	+	+	-	-
U-73122	-	-	-	-	-	-	-	-	+	+
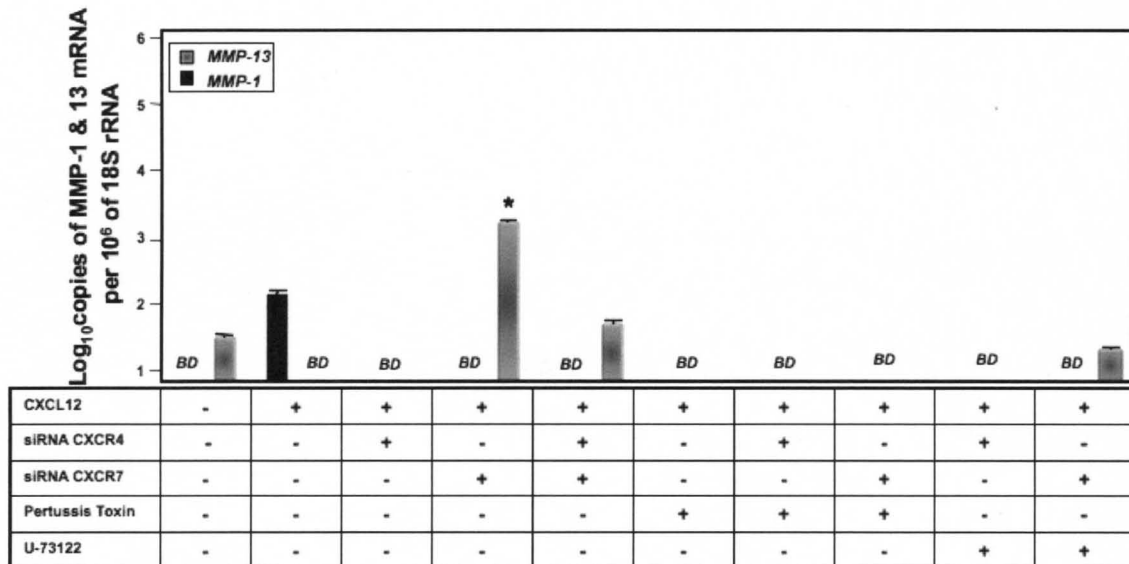
Figure 27. CXCR4- and CXCR7-dependent MMP-1 and MMP-13 mRNA expression by MDA-MB-231 cells. Total RNA was isolated from MDA-MB-231 cells pretreated with or without CXCR4 siRNA, CXCR7 siRNA, pertussis toxin, and U-73122 in the presence or absence of CXCL12 for 16 hours. Semi-quantitative RT-PCR analysis of MMP-1 and MMP-13 mRNA expression was performed in triplicate. Copies of transcripts were expressed relative to copies of 18S rRNA. Asterisk(s) indicate statistical significance ($p < 0.05$) between untreated and treated groups.
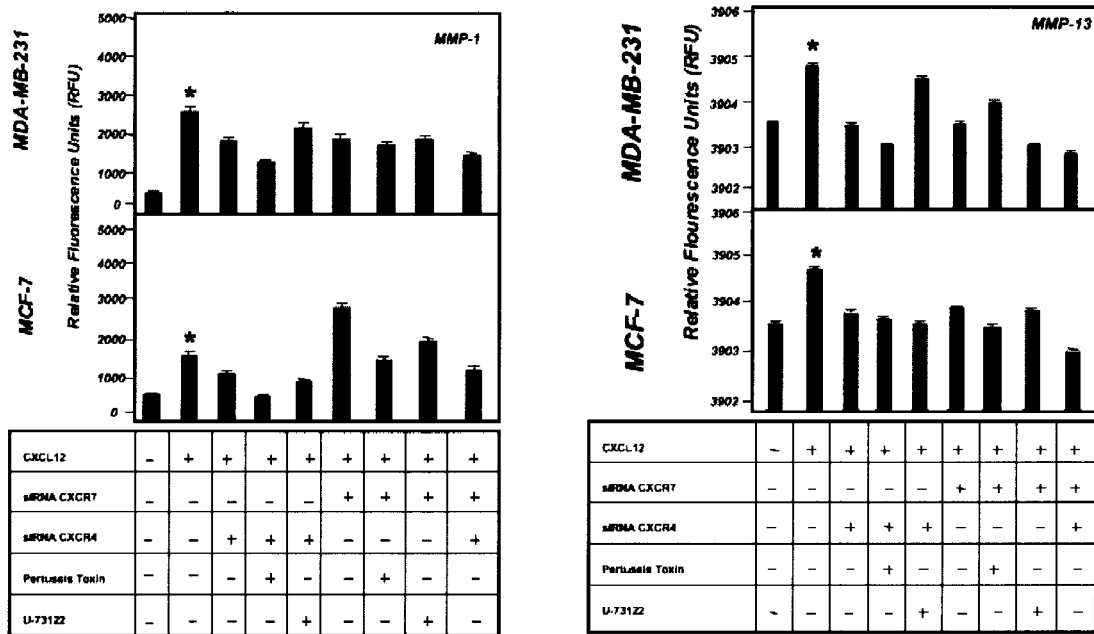
Figure 28. CXCR4- and CXCR7-dependent MMP-1 and MMP-13 active protein expression by BrCa cell lines. Total RNA was isolated from MDA-MB-231 and MCF-7 cells pretreated with or without CXCR4 siRNA, CXCR7 siRNA, pertussis toxin, and U-73122 in the presence or absence of CXCL12 for 16 hours. Culture supernatants were assayed for MMP-1 and MMP-13 activity and performed in duplicate. MMP activity is expressed as relative fluorescence units. Asterisk(s) indicate statistical significance ($p < 0.05$) between untreated and treated groups.

MDA-MB-231 BrCa cells following CXCL12 stimulation. Interestingly, siRNA CXCR4 plus U-73122 pretreatment showed a slight increase that was similar to that of CXCL12 alone.

CHAPTER IV

DISCUSSION

BrCa is a leading cause of cancer morbidity and mortality among women

and with a yearly toll of more than 40,170 deaths in the US alone. Its etiology is

based upon a distinct pattern of metastasis involving regional lymph nodes, bone

marrow, lung and liver (114). Although much has been done to improve the

understanding of the underlying mechanisms for oncogenic transformation that

leads to tumor initiation, much less is known about the processes regulating

BrCa metastasis. However, it has been postulated that BrCa cell metastasis is

highly influenced and facilitated by tumor microenvironmental cues that render an

otherwise non-invading cell metastatic (115). Cell signaling is ostensibly

supplied by the stromal component of breast carcinomas which is made up of a

network of cells including: CAFs, myofibroblasts, lymphocytes, tumor associated

macrophages, pericytes, dendritic cells, mesenchymal cell types, and endothelial

cells (116). At the cellular level, metastasis is an intricate and complex, multi-

step process that is dependent on a variety of factors. Further, a permissive

microenvironment is required for successful metastasis of breast cancer cells.

Specifically, the microenvironment of the primary tumor needs to support tumor

cell dissemination, motility, and invasion into the vasculature. The

microenvironment at the secondary site needs to support cell adhesion,

proliferation, neovascularization, and survival (117). Other mechanisms in which breast tumor microenvironment can influence metastatic potential include the following: remodeling of the extracellular matrix (ECM) and changes in glycoprotein composition which can alter cell adhesion, motility, proliferation, apoptotic rates, degradation of the extracellular matrix via proteinase activity within the stroma which helps with tumor cell migration by disrupting stromal barriers; and lastly, the release of bioactive extracellular fragments and growth factors that can promote or suppress neoplastic progression of both stromal and breast tumor cells (118). In this regard, chemokines and their receptors have been implicated to play critical roles in mediating the mechanisms necessary for breast cancer cell metastasis. Further, cell signaling through chemokine receptors *i.e.* CXCR4 mediates actin polymerization and pseudopodia formation in breast cancer cells and induces chemotactic and invasive responses (114). In addition, it has been shown that organs representing secondary sites of breast cancer metastasis are the most abundant sources of chemokine ligands *e.g.* CXCL12 for their tumor-associated receptors.

The purpose of this study was to investigate the role of CXCR7/CXCR4 and CXCL12 interactions in mediating breast cancer cell progression. The experiments in this study determined the expression of CXCR7 and CXCR4, their responsiveness to CXCL12, and the ability of siRNA interference along with inhibitors pertussis toxin and U-73122 to alter gene expression and/or CXCL12-mediated molecular signals. I performed comprehensive quantitative and qualitative analysis of the expression of CXCR7, CXCR4 as well as CXCL12 in

two different breast cancer cell lines, MCF-7 (ER+, PR+, Her2+) and MDA-MB-231 (ER-, PR-, Her2-). Human primary mammary epithelial cell line (HMEC) was used as the control.

Preliminary immunohistochemistry analysis demonstrated elevated CXCR7 protein expression in breast cancer tumors. Further, its protein expression remained elevated throughout stages 1, 2, and 3 but peaking in tumor stage 4. Also, there was an observation of CXCR7 protein expression moving from the cytoplasm of these tumors to the nucleus. This is suggestive of a role in nuclear signaling for this particular GPCR. The biological importance of this may be considered on various levels. However, the most significant is that studies have shown that only a select group of GPCRs can translocate from the plasma membrane to nuclear membranes upon activation and constitute complexes that sense and transduce distinctive signals in relation to the intracellular levels of their cognate ligand (119). The subsequent nuclear signal may have different or even opposite cellular effects than the original signal. Thus, current research must elucidate the characteristics of GPCR nuclearization and how this separate signaling pathway affects cellular events.

Semi-quantitative RT-PCR analyses showed that breast cancer cells do express chemokine receptors CXCR7 and CXCR4; however, their expression was cell-type specific. For example, in the MCF-7 (ER+, PR+, Her2+) breast cancer cell line, CXCR7 mRNA expression was 2-fold higher than CXCR4 mRNA expression. In the more aggressive breast cancer cell line MDA-MB-231 (ER-, PR-, Her2-), CXCR4 mRNA expression was 3-fold higher than CXCR7 mRNA

expression. With regard to the human primary mammary epithelial cell line, only CXCR7 mRNA was detected.

Having established that BrCa cells expressed functionally active CXCR7 and CXCR4, I evaluated protein expression of CXCR7 and CXCR4 on BrCa cells. Amnis ImageStream analysis demonstrated both CXCR7 and CXCR4 protein expression on primary mammary and BrCa cells. Density plots, which allow for analysis of positive and negative events of cells stained for each receptor, showed that 84.1% of the cells (HMEC) were double positive for both receptors, while 11.13% followed by 3.46% were singly positive for CXCR7 and CXCR4 respectively. Concerning MCF-7, at least 85.9% of the cells were double positive for both receptors while 10.4% followed by 2.2% were singly positive for CXCR7 and CXCR4 respectively. 88.6% of MDA-MB-231 cells were positive for both receptors while 9.6% followed by 1.8% were selectively positive for CXCR4 and CXCR7, respectively. Image analyses of these breast cancer cells showed CXCR7 protein expression just below the cell membrane. These findings are consist with earlier studies that demonstrated intracellular pools of CXCR7 on T lymphocytes (120).

CXCR4 protein expression by MCF-7 was uniformly distributed along the cell surface and in MDA-MB-231 cells; it was concentrated just below the cell membrane. These findings would suggest that cellular distribution of CXCR4/CXCR7 is important for their functional responses as well as signal transduction pathways induced by CXCL12 stimulation. Also, this is consistent with the histology data that demonstrated CXCR7 transition from the cytoplasm

to the nucleus of breast tumors. These site-dependent differences of CXCR4 and CXCR7 distribution might provide key advantages for cellular signaling and intracellular movement involved in breast cancer progression.

The current paradigm suggests that increased CXCR4 expression dictates the ability of breast carcinoma cells to metastasize to organs such as the bone marrow, lung, and liver that express high amounts of CXCL12 (23). CXCL12 engages both CXCR4 and CXCR7 to regulate this pathological process. Here, I demonstrated that both CXCR4 and CXCR7 are expressed on breast cancer cells and how they cooperate to respond to CXCL12 is not well understood. Moreover, it has been shown that CXCR4 occupied by CXCL12 is able to activate heterotrimeric G-proteins that trigger several downstream events, calcium mobilization, actin polymerization, integrin-mediated adhesion, gene transcription, and proliferation. Further, kinetic analysis of CXCR4 activation determined that part of the receptor constitutively interact with inactive, GDP-bound Gα subunits so that G-protein activation arises from conformational changes within preassembled receptor G-protein complexes. These complexes do not disassemble after activation but persist over time (121, 122). Together, these findings suggest CXCR7 constitutively interacts with G-proteins, but fails to activate them or mobilize intracellular calcium once engaged by CXCL12.

In this study, I postulated that CXCR7 cell signaling involved a pathway that was independent of G protein(s) signal transduction. Indeed, CXCR7 contains sequence mutations in chemokine receptors that is conserved for G-protein coupling. To examine this hypothesis, siRNA interference studies along

71

with Gαi inhibitor pertussis toxin and Gβ protein / Gγ protein inhibitor U-73122 were also used.

My results showed that siRNA directed against CXCR7 alone down-regulated its mRNA expression for both MCF-7 and MDA-MB-231 cells. When a combination of siRNA CXCR4 + pertussis toxin or U-73122 was used, only in the presence of pertussis toxin was CXCR7 mRNA significantly inhibited but for U-73122 there was no change. This suggests that CXCR7 cell signaling must involve a pathway independent of its G-protein or Gαi pertussis toxin sensitive. However, since it has been established that CXCR7 activation leads to no calcium flux (123, 124), a pathway independent of its G-protein would be more logical. Moreover, CXCL12 engagement with CXCR7 can transmit a range of cellular responses, such as activation of ERK1/2 and Akt pathways, receptor internalization, cell survival, proliferation, adhesion, and chemotaxis of CXCR4-negative cells (125-128). Hence, CXCL12 does not behave as a neutral ligand for CXCR7; instead, CXCR7 may sequester CXCL12 thereby modifying the chemokine ligand concentration in the extracellular environment. This process is key for cell migration (126).

Here, I also demonstrated that siRNA against CXCR4 down-regulated its mRNA expression by both MCF-7 and MDA-MB-231 cells, but in the presence of CXCR7 siRNA plus U-73122 there was an up-regulation of CXCR4 mRNA. Two key events may account for this finding. First, this particular Gβ protein / Gγ protein inhibitor has been shown to be effective against these subunits, but it has no effect on calcium and cAMP production. Indeed, Ca^{2+} flux and cAMP can act

as intracellular second messengers towards increasing CXCR4 transcription (129) via PKCζ. Second, receptor hetero/homodimer formation in BrCa cells that co-express both receptors. To this end, chemokine receptors can "crosstalk" or signal from one receptor to another via heterodimers so that one promoter can modify the function of the other through trans-conformational changes (130). Therefore, it can be hypothesized that CXCR7 can differentially mediate CXCR4 functions in heterodimer formation through allosteric interactions. This phenomenon has been reported for other chemokine receptors that signal when engaged as heterodimer pairs (131). Conformational changes within CXCR4/Gαi protein complexes in the presence of CXCR7 may represent a molecular mechanism for altering G protein-dependent signaling downstream of CXCR4/CXCR7 heterodimers. In addition to this, pharmacological interference alone may not be sufficient for inhibiting cell signaling events down-stream of the heterodimer. Therefore, siRNA interference is a more efficient way to inhibit CXCR4 and CXCR7 gene expression and alter breast cancer cell metastasis.

CXCL12 plays a major role in BrCa cell metastasis due to its chemoattractant property via CXCR4. The density of CXCR4 on BrCa cells is proportional to the invasiveness of the cancer (132). Furthermore, the expression of CXCR7 by BrCa cells enhances cell growth, survival, and adhesion properties. I sought to determine CXCL12 mRNA expression in the presence of siRNA plus pertussis toxin and/or U-73122 and its stimulatory effect on MCF-7 and MDA-MB-231 cell lines. Semi-quantitative RT-PCR demonstrated that in the presence of siRNA against CXCR4 or CXCR7 as well as U-73122, CXCL12

mRNA expression was up regulated (MCF-7); however, its mRNA was completely abrogated in MDA-MB-231 cells for the same treatments. When both receptors were knockdown and in the presence of pertussis toxin, there was complete abrogation of CXCL12 mRNA. These findings correlate with earlier findings that demonstrated siRNA interference of CXCR4 inhibits BrCa metastasis. The least aggressive BrCa cell line, MCF-7, expressed 1.5 fold higher CXCL12 mRNA after CXCL12 protein stimulation than similarly treated MDA-MB-231 cells. This finding is also consist with a study from Wendt *et al.*, showing that BrCa cells can undergo epigenetic silencing of CXCL12, which results in increased metastasis as well as CXCR4 mRNA expression. They also noted that several Sp1-binding sites are critical for CXCL12 promoter activity when heavily methylated in cells lacking marked CXCL12 expression, *i.e.,* MDA-MB-231. However, when these cells were re-established with endogenous CXCL12, there was hyper-responsiveness to exogenous CXCL12, which contributed to increased metastatic potential of these BrCa cells independent of CXCR4 expression regulation (133). My results are consistent with this previous observation, since CXCR4 inhibition resulted increased CXCL12 mRNA expression.

When MCF-7 and MDA-MB-231 cell lines were stimulated with CXCL12, both CXCR4 and CXCR7 translocated to the majority of cell nuclei. Similarly, advance breast tumors also displayed more CXCR7 protein expression in nuclei than cytoplasm. This is a significant finding because only a select few of GPCRs can translocate from the cell membrane to the nucleus.

For the first time, I demonstrated CXCR4 and CXCR7 expression levels vary with cell cycle. Both CXCR4 and CXCR7 expression were the highest in cell cycle phase G_2. HMECs showed a similar pattern of CXCR4 and CXCR7 expression during various phases of the cell cycle. These findings suggest that expression of these chemokine receptors correlates with cell cycle phases to possibly support cell proliferation. Indeed, crosstalk between CXCR7 and CXCR4 is involved in up-regulating genes, *i.e.* Cyclin B, Cyclin A, Cdc29, that are needed for cell cycle progression as well as down-regulating the activity of p53 (134) **(Figure 29)**.

Neoplastic epithelial cells engage in reciprocal molecular dialogue with surrounding stromal cells, including inflammatory cells, vascular cells, and fibroblasts which results in the production of stromal-derived tumor aiding factors *e.g.*, growth factors, chemokines, cytokines, proteases, and vascular-stimulating factors (135). BrCa represents a carcinoma in which collaborative efforts from malignant epithelial cells and stromal cells within the primary tumor coordinate carcinogenesis and ultimately metastasis to distant organs (136). In this regard, BrCa cells must penetrate the basement membrane and invade the interstitial stroma to initiate the metastatic process. Many proteinases are capable of degrading the extracellular matrix components but matrix metalloproteinases (MMPs) appear to be particularly important for matrix degradation/remodeling and cancer cell dissemination (64, 65). Furthermore, abnormal production of these proteinases is implicated in a number of pathological conditions (66). Collagenases (MMP-1 and −13) degrade several native fibril collagens, including
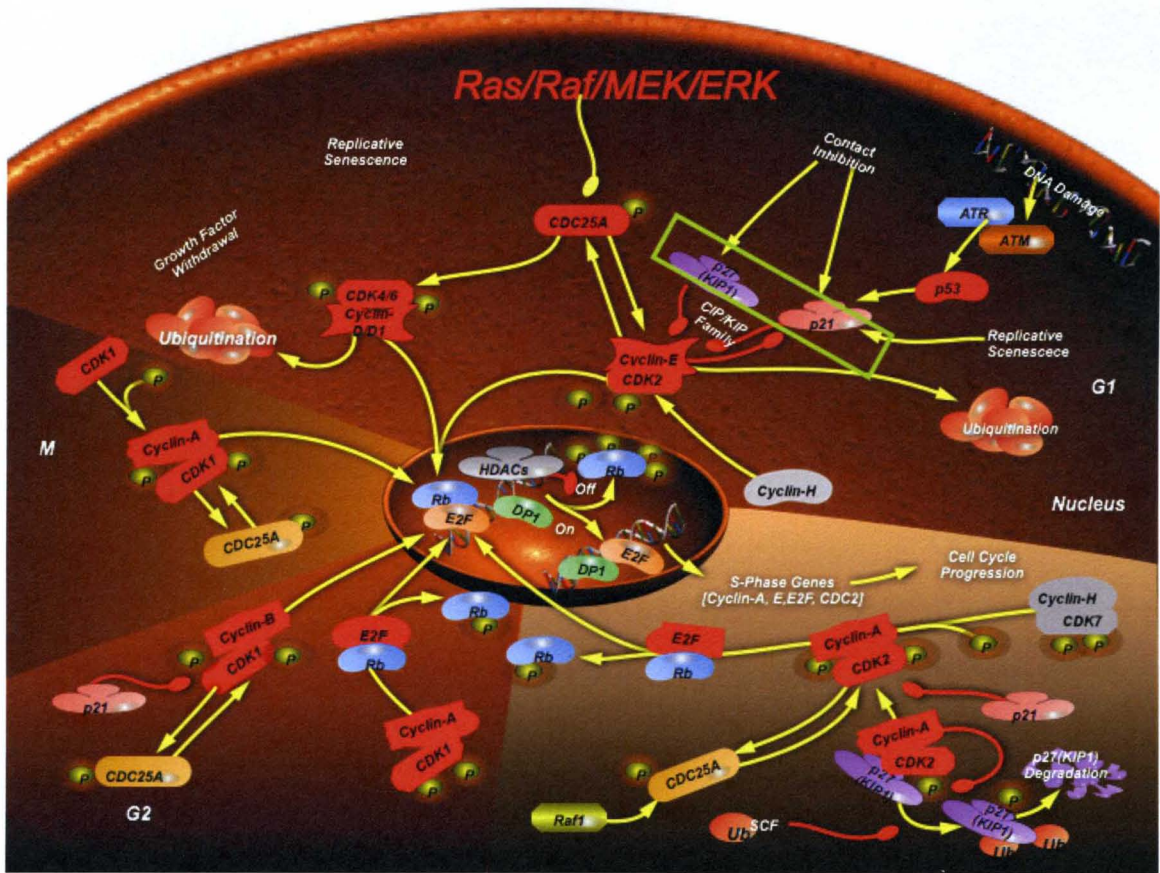
Figure 29. Cyclins, cell cycle regulators, and genes possibly mediated by CXCR4 and CXCR7 cross-talk following CXCL12 stimulation. Crosstalk between CXCR4 and CXCR7 has been shown to regulate cell cycle cyclins *i.e.,* Cyclin A (S, G_2, and M phase), Cyclin B (G_2 phase) as well as cell cycle regulators E2F and p53. These cell cycle components are critical factors for cell proliferation and survival during breast cancer progression.

types I, II and III, which result in their cleavage.

In this study, I demonstrated BrCa cells express moderate to low levels of MMP-1 and MMP-13. After CXCL12 stimulation, there was an up-regulation of MMP-1 and a down-regulation of MMP-13 mRNA expression respectively. For MCF-7 cells there was no change in either MMP mRNA expression after CXCL12 stimulation. In normal physiology, the basal expression of most MMPs, including MMP-1 and −13, is low. Other investigators have shown that MMPs are over-expressed in BrCa and play important roles in cell invasion and metastasis (137). Furthermore, the up-regulation of MMP-1 mRNA does inversely correlate with ER status as shown here concerning MDA-MB-231 cells. It has been demonstrated that as the presence of estrogen receptor predicts a low risk of relapse and good overall survival, MMP-1 mRNA up-regulation is a potential reason for poor outcome of patients with ER-negative breast cancers (138). Increased MMP-1 protein expression has been associated with malignancy or invasiveness of breast cancer. siRNA interference against both receptors altered MMP-1 and −13 induction. This variance is a result of related, yet different induction pathways.

Studies have demonstrated that MMP-13 induction requires p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) activation as well as NF-kB translocation (139). In contrast, MMP-1 induction involves p38 MAPK and MAPK of the extracellular signal-regulated kinase (MEK) activation, but not JNK activation or NF-kB translocation. While p38 MAPK and MEK activity is increased by CXCL12 stimulation of hematopoietic progenitor and

lymphocytes, JNK is not activated by this chemokine (140, 141). The modulation of NF-kB translocation by CXCL12 is not as certain, but my data suggest that CXCL12 stimulation can simultaneously increase p38 MAPK and MEK activity, while lowering and/or not affecting JNK activity or NF-kB translocation which would presumably lead to higher increases in MMP-1 expression when compared to MMP-13. Here, CXCL12 induction through CXCR4 and/or CXCR7, increased MMP-1 mRNA and protein expression by MCF-7 and MDA-MB-231 cell lines. siRNA against CXCR4 and/or CXCR7 along with Gαi inhibitor pertussis toxin was able to inhibit this induction. The Gβ protein /Gγ protein inhibitor U-73122 selectively inhibited MMP-1 induction. These findings highlight the significance of MMP gene expression regulated at the transcriptional level by CXCL12-CXCR4/CXCR7 interactions.

MMP promoters harbor cis-elements allowing for the regulation of MMP gene expression by a diverse set of trans-activators including AP-1, PEA3, Sp-1, β-catenin/Tcf-4, and NF-kB (142). It is possible that restricted BrCa cell-type expression of transcriptional factors is needed for optimal MMP induction. Notwithstanding, the mechanism(s) governing BrCa tissue/cell-type specific MMP expression are poorly understood. However, it can be postulated that BrCa cell growth without motility, *i.e.* epithelial cell growth, is supported by MMP-1 but not MMP-13, while tumor cell motility and invasion requires selective MMP-1 and MMP-13 activity.

CXCL12 and its receptors CXCR4 and CXCR7 have recently sparked substantial interest because of their role in tumorigenesis including tumor growth,

invasion, and metastasis (114, 143). This chemokine and its receptors are able to mediate multiple signal transduction pathways and a variety of cellular functions such as cell migration, proliferation, and survival. Unfortunately, there are few studies linking the cellular functions and individual signaling pathways mediated by CXCL12, CXCR4, and CXCR7 interactions with BrCa cells. In this study, I show for the first time that HMECs express phosphorylated or active NF-kB and ERK1/2 in the cytosol. Further, MCF-7 and MDA-MB-231 cells express significant pools of activated NF-kB and ERK1/2 within the cytosol.

CXCR4 is a Gαi-protein coupled receptor and studies have shown that upon high affinity binding of CXCL12, it causes mobilization of calcium, decrease of cyclic AMP within the cells, and activation of multiple signaling pathways. These pathways include: PI3K, phospholipase C-γ/protein kinase C, and MAP kinases ERK1/2 (144). I hypothesized that CXCR7 cell signaling involved a G protein-independent pathway, which depended on its association with JAT/STAT through PI3K activation and the induction of downstream signaling mediators AKT/NF-kB and RAS/Raf/MEK/ERK1/2. However, nearly all previous studies concerning these molecules were performed using leukocytes or stem cells (145). Due to the important roles of CXCR4/CXCL12 in BrCa cell metastasis, I investigated CXCL12/CXCR4/CXCR7 regulated cell signaling in BrCa cells. For this purpose, MCF-7 and MDA-MB-231 cell lines were treated with CXCL12 and analyzed for changes in active ERK1/2 and NF-kB translocation to the nucleus. Further, CXCL12 stimulation lead to the activation of ERK1/2 and NF-kBp65 in BrCa cells. Similarity indexes and image analyses for protein expression also

showed significant translocation of ERK1/2 and NF-kB to the nucleus of cells (~90% of the cells stained positive for ERK1/2 phosphorylation at residues T202 and Y204 and NF-kBp65 phosphorylation at residue S529).

These findings correlate with other studies that showed ERK1/2 directly phosphorylates many transcriptional factors including Ets-1, c-Jun and c-myc that are important for BrCa progression (146). Active ERK1/2 can enter the nucleus itself and phosphorylate many transcription factors and proteins involved in cell cycle regulation (146). Others have demonstrated that through an indirect mechanism, ERK1/2 can activate NF-kB through phosphorylation of IKK (147). Active NF-kB translocation to the nucleus is key for the promotion of NF-kBp65-dependent transcription (148). Crosstalk between the CXCL12-CXCR4/CXCR7 most likely induces RAS/Raf/MEK/ERK1/2 and PI3K/AKT pathways for cell survival and/or phosphorylation of BAD and caspase-9 to suppress apoptosis. These pathways have diverse effects that could regulate cell cycle progression, apoptosis and differentiation of BrCa cells (148).

CXCL12-CXCR4/CXCR7 cell signaling is a very complex network. CXCR4 and CXCR7 siRNA interference lead to a decrease in ERK1/2 and NF-kB activation and translocation in BrCa cells. Further, pertussis toxin, which inhibits both Gαi protein-dependent and G protein-independent signaling, lead to suppression of both CXCR4 and CXCR7 activation and translocation after CXCL12 stimulation. U-73122 was able to modulate the functional response of CXCR4, CXCR7, ERK1/2 and NF-kB presumably due to Gβ protein / Gγ protein inhibition, but it has no effect on calcium or cAMP production. When siRNA was

used to inhibit CXCR7 expression, CXCL12 stimulatoin failed to induce ERK1/2 or NF-kB nucleus translocation. Hence, ERK1/2 and NF-kB activation is dependent on PI3K induction and other G protein-dependent events (144). Taken together, this dissertation supports that CXCL12/CXCR4/CXCR7 interactions are able to induce multiple cell signaling pathways for BrCa cell migration, invasion, and survival.

CHAPTER V

CONCLUSIONS

This study directly investigated the hypothesis that CXCR7 and its interactions with CXCR4 and CXCL12 promote BrCa cell collagenase expression, alter NF-κB and ERK1/2 localization to the nucleus, and correlates with cell cycle. The experiments detailed in this study determined mRNA and protein expression of CXCR7 and CXCR4 along with their functional responses to their ligand, CXCL12. It was demonstrated that primary mammary epithelial cells and MCF-7 and MDA-MB-231 cell lines express CXCR4 and CXCR7 mRNA as well as protein.

The overall purpose of this study was to yield significant and substantive results that would reveal new insights into the role of this chemokine and its receptors involved in BrCa cell progression. Further, to determine what cell signaling pathways i.e. RAS/Raf/MEK/ERK1/2; AKT/NF-kB may be involved in CXCR7/CXCR4 and CXCL12 interactions as well as the ability of siRNA interference along with the Gαi protein inhibitor, pertussis toxin, and the Gβ protein / Gγ protein inhibitor, U-73122, to mitigate BrCa cell progression. I also conclude that primary breast epithelial and cancer cells express significant pools of active NF-kBp65 and ERK1/2 in the cytosol. After CXCL12 stimulation and

activation of CXCR4/CXCR7, these multiple cell signaling components translocate to cell nuclei.

It is important to understand whether or not CXCR7 is able to signal via a G protein-independent pathway. Here, I postulated that this receptor was able to signal via the association of JAK/STAT with its C-terminal tail upon GRK phosphorylation due to cross-talk after CXCR4-CXCL12 interactions. Related studies have indicated that CXCR7 cell signaling pathways involve no significant Ca^{2+} flux or cell migration; perhaps through heterodimer interactions with CXCR4, CXCR7 is able to mitigate cell migration, invasion and survival. Chemokine receptor heterodimers can signal just as efficiently as homodimers; moreover, receptor number and expression is dependent on specific cell-types and/or involves translation as well as post-translational modifications for receptor function. Further studies will be needed to determine if these ideas hold true.

Lastly, chemokines and their receptors are able to mediate other biological activities besides cancer cell migration, invasion and survival. These interactions may represent major obstacles for therapeutic strategies. However, deciphering signaling pathways activated by chemokines in various cancer cells will be critical towards understanding how chemokines influence disease progression and may reveal potential downstream therapeutic targets.

REFERENCES

1. O'Hayre, M., C. Salanga, T. Handel, and S. Allen. 2008. Chemokines and Cancer: Migration, Intracellular Signaling and Intercellular Communication in the Microenvironment. *Biochemical Journal* 409:635-649.

2. Schedin, P., and A. Elias. 2004. Multistep Tumorigenesis and The Microenvironment. *Breast Cancer Research* 6:93-101.

3. Sporn, M., and N. Suh. 2002. Chemoprevention: An Essential Approach to Controlling Cancer. *Nat Rev Cancer* 2:537-543.

4. Ramaswamy, S., K. Ross, E. Lander, and T. Golub. 2003. A Molecular Signature of Metastasis in Primary Solid Tumors. *Nat Genet* 33:49- 54.

5. Bernards, R., and R. Weinberg. 2002. A Progression Puzzle. *Nature* 418:823.

6. Fearon, E., and B. Vogelstein. 1990. A Genetic Model for Colorectal Tumorigenesis. *Cell* 61:759-767.

7. Debnath, J., K. Mills, N. Collins, M. Reginato, S. Muthuswamy, and J. Brugge. 2002. The Role of Apoptosis in Creating and Maintaining Luminal Space Within Normal and Oncogene-Expressing Mammary Acini. *Cell* 111:29-40.

8. Weaver, V., O. Petersen, F. Wang, C. Larabell, P. Briand, C. Damsky, and M. Bissell. 1997. Reversion of the Malignant Phenotype of Human Breast Cells in Three-Dimensional Culture and In Vivo by Integrin Blocking Antibodies. *J Cell Biol* 137:231-245.

9. Chauhan, H., A. Abraham, J. Phillips, J. Pringle, R. Walker, and J. Jones. 2003. There is More Than One Kind of Myofibroblasts: Analysis of CD34

Expression in Benign, In Situ, and Invasive Breast Lesions. *J Clin Pathol* 56:271-276.

10. Tuxhorn, J., G. Ayala, M. Smith, V. Smith, T. Dang, and D. Rowley. 2002. Reactive Stroma in Human Prostate Cancer: Induction of Myofibroblasts Phenotype and Extracellular Matrix Remodeling. *Clin Cancer Research* 8:2912-2923.

11. Orimo, A., P. Gupta, D. Sgroi, F. Arenzana-Seisdedos, T. Delaunay, R. Naeem, V. Carey, A. Richardson, and R. Weinberg. 2005. Stromal Fibroblasts Present in Invasive Human Breast Carcinoma Promote Tumour Growth and Angiogenesis Through Elevated SDF-1/CXCL12 Secretion. *Cell* 121:335-348.

12. Silzle, T., M. Kreutz, M. Dobler, G. Brockhoff, R. Knuechel, and L. Kunz-Schughart. 2003. Tumour Associated Fibroblasts Recruit Blood Monocytes into Tumour Tissue. *Eur. J of Immunology* 33:1311-1320.

13. Vandercappellen, J., J. Van Damme, and S. Struyf. 2008. The Role of CXC Chemokines and Their Receptors in Cancer. *Cancer Letter* 267:226-244.

14. Zlotnik, A., and O. Yoshie. 2000. Chemokines: a new classification system and their role in immunity. *Immunity* 12:121-127.

15. Murphy, P., M. Baggiolini, I. Charo, C. Hebert, R. Horuk, K. Matsushima, L. Miller, J. Oppenheim, and C. Power. 2000. Nomenclature for Chemokine Receptors. *Pharmacology Review* 1:145-176.

16. Balkwill, F. 2004. Cancer and the Chemokine Network. *Nat Rev Cancer* 7:540-550.

17. Campbell, J. J., and E. C. Butcher. 2000. Chemokines in tissue-specific and microenvironment-specific lymphocyte homing. *Current Opinion in Immunology* 12:336-341.

18. Butcher, E. C., M. Willima, K. Youngman, L. Rott, and M. Briskin. 1999. Lymphocyte trafficking and regional immunity. *Advances in Immunology* 72:209-253.

19.   Muller, A., B. Homey, H. Soto, N. Ge, D. Catron, M. E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S. N. Wagner, J. L. Barrera, A. Mohar, E. Verastegui, and A. Zlotnik. 2001. Involvement of chemokine receptors in breast cancer metastasis.[comment]. *Nature.* 410:50-56.

20.   Gupta, G., and J. Massague. 2006. Cancer Metastasis: Building a Framework. *Cell* 127:679-695.

21.   Ben-Baruch, A. 2003. Host Microenvironment in Breast Cancer Development: Inflammatory Cells, Cytokines, and Chemokines in Breast Cancer Progression: Reciprocal Tumour-Microenvironment Interactions. *Breast Cancer Research* 5:31-36.

22.   Kulbe, H., N. Levinson, F. Balkwill, and J. Wilson. 2004. The Chemokine Network- Much More Than Directing Cell Movement. *Int. J Dev. Biology* 48:489-496.

23.   Balkwill, F. 2004. The significance of cancer cell expression of the chemokine receptor CXCR4. *Seminars in Cancer Biology* 14:171-179.

24.   Shirozu, M., T. Nakano, J. Inazawa, K. Tashiro, H. Tada, T. Shinohara, and T. Honjo. 1995. Structure and Chromosomal Localization of the Human Stromal Cell-Derived Factor 1. *Genomics* 374:174-179.

25.   Hesselgesser, J., M. Liang, J. Hoxie, M. Greenberg, L. Brass, M. Orsini, D. Taub, and R. Horuk. 1998. Identification and Characterization of the CXCR4 Chemokine Receptor in Human T-Cell Lines. *Gene* 2:877-883.

26.   Feng, Y., C. C. Broder, P. E. Kennedy, and E. A. Berger. 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor.[see comment]. *Science* 272:872-877.

27.   Zlotnik, A. 2006. Involvement of Chemokine Receptors in Organ-specific Metastasis. *Contrib Microbiol* 13:191-199.

28.   Koshiba, T., R. Hosotani, Y. Miyamoto, J. Ida, S. Tsuji, S. Nakajima, M. Kawaguchi, H. Kobayashi, R. Doi, T. Hori, N. Fujii, and M. Imamura. 2000. Expression of stromal cell-derived factor 1 and CXCR4 ligand receptor system in pancreatic cancer: a possible role for tumor progression. *Clinical Cancer Research.* 6:3530-3535.

29.   Scotton, C. J., J. L. Wilson, D. Milliken, G. Stamp, and F. R. Balkwill. 2001. Epithelial cancer cell migration: a role for chemokine receptors? *Cancer Research.* 61:4961-4965.

30.   Marchese, A., and J. Benovic. 2001. Agonist-Promoted Ubiquitination of The G Protein-Coupled Receptor CXCR4 Mediates Lysosomal Sorting. *J Biol Chem* 49:45509-45512.

31.   Li, Y. M., Y. Pan, Y. Wei, X. Cheng, B. P. Zhou, M. Tan, X. Zhou, W. Xia, G. N. Hortobagyi, D. Yu, and M. C. Hung. 2004. Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis.[see comment]. *Cancer Cell* 6:459-469.

32.   Zhang, G. J., and I. Adachi. 1999. Serum interleukin-6 levels correlate to tumor progression and prognosis in metastatic breast carcinoma. *Anticancer Research* 19:1427-1432.

33.   Kang, H., G. Watkins, C. Parr, A. Douglas-Jones, R. E. Mansel, and W. G. Jiang. 2005. Stromal cell derived factor-1: its influence on invasiveness and migration of breast cancer cells in vitro, and its association with prognosis and survival in human breast cancer. *Breast Cancer Research* 7:R402-410.

34.   Proost, P., A. Mortier, T. Loos, J. Vandercappellen, M. Gouwy, I. Ronsse, E. Schutyser, W. Put, M. Parmentier, S. Struyf, and J. Van Damme. 2007. Proteolytic processing of CXCL11 by CD13/aminopeptidase N impairs CXCR3 and CXCR7 binding and signaling and reduces lymphocyte and endothelial cell migration. *Blood* 110:37-44.

35.   Cannistra, S. A., and J. D. Griffin. 1988. Regulation of the production and function of granulocytes and monocytes. *Seminars in Hematology* 25:173-188.

36.   Hillyer, P., E. Mordelet, G. Flynn, and D. Male. 2003. Chemokines, chemokine receptors and adhesion molecules on different human endothelia: discriminating the tissue-specific functions that affect leucocyte migration. *Clinical & Experimental Immunology* 134:431-441.

37.   Cooper, C. R., J. K. Bhatia, H. J. Muenchen, L. McLean, S. Hayasaka, J. Taylor, P. J. Poncza, and K. J. Pienta. 2002. The regulation of prostate cancer cell adhesion to human bone marrow endothelial cell monolayers

by androgen dihydrotestosterone and cytokines. *Clinical & Experimental Metastasis* 19:25-33.

38. Jung, Y., J. Wang, A. Schneider, Y. X. Sun, A. J. Koh-Paige, N. I. Osman, L. K. McCauley, and R. S. Taichman. 2006. Regulation of SDF-1 (CXCL12) production by osteoblasts; a possible mechanism for stem cell homing. *Bone* 38:497-508.

39. Knaut, H., C. Werz, R. Geisler, C. Nusslein-Volhard, and C. Tubingen Screen. 2003. A zebrafish homologue of the chemokine receptor Cxcr4 is a germ-cell guidance receptor.[see comment]. *Nature* 421:279-282.

40. Dambly-Chaudiere, C., N. Cubedo, and A. Ghysen. 2007. Control of cell migration in the development of the posterior lateral line: antagonistic interactions between the chemokine receptors CXCR4 and CXCR7/RDC1. *BMC Developmental Biology* 7:23.

41. Valentin, G., P. Haas, and D. Gilmour. 2007. The chemokine SDF1a coordinates tissue migration through the spatially restricted activation of Cxcr7 and Cxcr4b. *Current Biology* 17:1026-1031.

42. Thelen, M., and S. Thelen. 2008. CXCR7, CXCR4 and CXCL12: An Eccentric Trio? *Journal of Neuroimmunology*:1-5.

43. Schonemeier, B., S. Schulz, V. Hoellt, and R. Stumm. 2008. Enhanced Expression of the CXCL12/SDF-1 Chemokine Receptor CXCR7 After Cerebral Ischemia in the Rat Brain. *Journal of Neuroimmunology*:1-7.

44. Burns, J. M., B. C. Summers, Y. Wang, A. Melikian, R. Berahovich, Z. Miao, M. E. T. Penfold, M. J. Sunshine, D. R. Littman, C. J. Kuo, K. Wei, B. E. McMaster, K. Wright, M. C. Howard, and T. J. Schall. 2006. A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *Journal of Experimental Medicine* 203:2201-2213.

45. Levoye, A., K. Balabanian, F. Baleux, F. Bachelerie, and B. Lagane. 2009. CXCR7 Heterodimerizes with CXCR4 and Regulates CXCL12-Mediated G Protein Signaling. *Blood* 113:6085-6093.

46. Grabovsky, V., S. Feigelson, C. Chen, D. A. Bleijs, A. Peled, G. Cinamon, F. Baleux, F. Arenzana-Seisdedos, T. Lapidot, Y. van Kooyk, R. R. Lobb, and R. Alon. 2000. Subsecond induction of alpha4 integrin clustering by immobilized chemokines stimulates leukocyte tethering and rolling on endothelial vascular cell adhesion molecule 1 under flow conditions. *Journal of Experimental Medicine* 192:495-506.

47. Taub, D. D., A. R. Lloyd, K. Conlon, J. M. Wang, J. R. Ortaldo, A. Harada, K. Matsushima, D. J. Kelvin, and J. J. Oppenheim. 1993. Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. *Journal of Experimental Medicine* 177:1809-1814.

48. Taub, D. D., K. Conlon, A. R. Lloyd, J. J. Oppenheim, and D. J. Kelvin. 1993. Preferential migration of activated CD4+ and CD8+ T cells in response to MIP-1 alpha and MIP-1 beta. *Science* 260:355-358.

49. Tanaka, Y., D. H. Adams, S. Hubscher, H. Hirano, U. Siebenlist, and S. Shaw. 1993. T-cell adhesion induced by proteoglycan-immobilized cytokine MIP-1 beta. *Nature.* 361:79-82.

50. Weber, C., C. F. Lu, J. M. Casasnovas, and T. A. Springer. 1997. Role Of Alpha(L)Beta(2) Integrin Avidity In Transendothelial Chemotaxis Of Mononuclear Cells. *Journal of Immunology* 159:3968-3975.

51. Weber, C., R. Alon, B. Moser, and T. A. Springer. 1996. Sequential regulation of alpha 4 beta 1 and alpha 5 beta 1 integrin avidity by CC chemokines in monocytes: implications for transendothelial chemotaxis. *Journal of Cell Biology* 134:1063-1073.

52. Mauro, L., D. Sisci, M. Bartucci, M. Salerno, J. Kim, T. Tam, M. A. Guvakova, S. Ando, and E. Surmacz. 1999. SHC-alpha5beta1 integrin interactions regulate breast cancer cell adhesion and motility. *Experimental Cell Research* 252:439-448.

53. Vellon, L., J. A. Menendez, and R. Lupu. 2006. A bidirectional "alpha(v)beta(3) integrin-ERK1/ERK2 MAPK" connection regulates the proliferation of breast cancer cells. *Molecular Carcinogenesis* 45:795-804.

54. Gasparini, G., P. C. Brooks, E. Biganzoli, P. B. Vermeulen, E. Bonoldi, L. Y. Dirix, G. Ranieri, R. Miceli, and D. A. Cheresh. 1998. Vascular integrin

alpha(v)beta3: a new prognostic indicator in breast cancer. *Clinical Cancer Research* 4:2625-2634.

55.   Vellon, L., J. A. Menendez, and R. Lupu. 2005. AlphaVbeta3 integrin regulates heregulin (HRG)-induced cell proliferation and survival in breast cancer. *Oncogene* 24:3759-3773.

56.   Spangenberg, C., E. U. Lausch, T. M. Trost, D. Prawitt, A. May, R. Keppler, S. A. Fees, D. Reutzel, C. Bell, S. Schmitt, I. B. Schiffer, A. Weber, W. Brenner, M. Hermes, U. Sahin, O. Tureci, H. Koelbl, J. G. Hengstler, and B. U. Zabel. 2006. ERBB2-mediated transcriptional up-regulation of the alpha5beta1 integrin fibronectin receptor promotes tumor cell survival under adverse conditions. *Cancer Research* 66:3715-3725.

57.   Langsenlehner, U., W. Renner, B. Yazdani-Biuki, T. Eder, T. C. Wascher, B. Paulweber, H. Clar, G. Hofmann, H. Samonigg, and P. Krippl. 2006. Integrin alpha-2 and beta-3 gene polymorphisms and breast cancer risk. *Breast Cancer Research & Treatment* 97:67-72.

58.   Mahabeleshwar, G. H., W. Feng, K. Reddy, E. F. Plow, and T. V. Byzova. 2007. Mechanisms of integrin-vascular endothelial growth factor receptor cross-activation in angiogenesis.[see comment]. *Circulation Research* 101:570-580.

59.   Corbett, S. A., and J. E. Schwarzbauer. 1999. Beta3 integrin activation improves alphavbeta3-mediated retraction of fibrin matrices. *Journal of Surgical Research* 83:27-31.

60.   Samara, G., D. Lawrence, C. Chiarelli, M. Valentino, S. Lyubsky, S. Zucker, and G. Vaday. 2004. CXCR4-Mediated Adhesion and MMP-9 Secretion in Head and Neck Squamous Cell Carcinoma. *Cancer Letter* 2:231-241.

61.   Wright, N., A. Hidalgo, J. Rodriguez-Frade, S. Soriano, M. Mellado, M. Parmo-Cabanas, M. Briskin, and J. Teixido. 2002. The Chemokine Stromal Cell-Derived Factor 1 Alpha Modulates Alpha 4 Beta 7 Integrin-Mediated Lymphocyte Adhesion to Mucosal Addressin Cell Adhesion Molecule-1 and Fibronectin. *J Immunol* 10:5268-5277.

62.   Zhao, Y., R. Bachelier, I. Treilleux, P. Pujuguet, O. Peyruchaud, R. Baron, P. Clement-Lacroix, and P. Clezardin. 2007. Tumor alphavbeta3 integrin is

a therapeutic target for breast cancer bone metastases. *Cancer Research* 67:5821-5830.

63. Baum, O., R. Hlushchuk, A. Forster, R. Greiner, P. Clezardin, Y. Zhao, V. Djonov, and G. Gruber. 2007. Increased invasive potential and up-regulation of MMP-2 in MDA-MB-231 breast cancer cells expressing the beta3 integrin subunit. *International Journal of Oncology* 30:325-332.

64. Nagase, H., and J. F. Woessner, Jr. 1999. Matrix metalloproteinases. *Journal of Biological Chemistry* 274:21491-21494.

65. Hulboy, D. L., L. A. Rudolph, and M. Matrisian. 1997. Matrix metalloproteinases as mediators of reproductive function. *Molecular Human Reproduction.* 3:27-45.

66. Stetler-Stevenson, W. G., S. Aznavoorian, and L. A. Liotta. 1993. Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Annual Review of Cell Biology.* 9:541-573.

67. Balduyck, M., F. Zerimech, V. Gouyer, R. Lemaire, B. Hemon, G. Grard, C. Thiebaut, V. Lemaire, E. Dacquembronne, T. Duhem, A. Lebrun, M. J. Dejonghe, and G. Huet. 2000. Specific expression of matrix metalloproteinases 1, 3, 9 and 13 associated with invasiveness of breast cancer cells in vitro. *Clinical & Experimental Metastasis* 18:171-178.

68. Bachmeier, B. E., A. G. Nerlich, R. Lichtinghagen, and C. P. Sommerhoff. 2001. Matrix metalloproteinases (MMPs) in breast cancer cell lines of different tumorigenicity. *Anticancer Research* 21:3821-3828.

69. Hao, L., C. Zhang, Y. Qiu, L. Wang, Y. Luo, M. Jin, Y. Zhang, T. B. Guo, K. Matsushima, and Y. Zhang. 2007. Recombination of CXCR4, VEGF, and MMP-9 predicting lymph node metastasis in human breast cancer. *Cancer Letters* 253:34-42.

70. Duffy, M. J., T. M. Maguire, A. Hill, E. McDermott, and N. O'Higgins. 2000. Metalloproteinases: role in breast carcinogenesis, invasion and metastasis. *Breast Cancer Research* 2:252-257.

71. Li, H.-C., D.-C. Cao, Y. Liu, Y.-F. Hou, J. Wu, J.-S. Lu, G.-H. Di, G. Liu, F.-M. Li, Z.-L. Ou, C. Jie, Z.-Z. Shen, and Z.-M. Shao. 2004. Prognostic value

of matrix metalloproteinases (MMP-2 and MMP-9) in patients with lymph node-negative breast carcinoma. *Breast Cancer Research & Treatment* 88:75-85.

72. Liang, Z., Y. Yoon, J. Votaw, M. M. Goodman, L. Williams, and H. Shim. 2005. Silencing of CXCR4 blocks breast cancer metastasis. *Cancer Research* 65:967-971.

73. Miao, Z., K. E. Luker, B. C. Summers, R. Berahovich, M. S. Bhojani, A. Rehemtulla, C. G. Kleer, J. J. Essner, A. Nasevicius, G. D. Luker, M. C. Howard, and T. J. Schall. 2007. CXCR7 (RDC1) promotes breast and lung tumor growth in vivo and is expressed on tumor-associated vasculature. *Proceedings of the National Academy of Sciences of the United States of America* 104:15735-15740.

74. Singh, S., U. P. Singh, W. E. Grizzle, and J. W. Lillard. 2004. CXCL12-CXCR4 interactions modulates prostate cancer cell migration, metalloproteinase expression and invasion. *Laboratory Investigation* 84:1666-1676.

75. Etienne-Manneville, S., and A. Hall. 2002. Rho GTPases in cell biology. *Nature* 420:629-635.

76. Laudanna, C., J. Y. Kim, G. Constantin, and E. Butcher. 2002. Rapid leukocyte integrin activation by chemokines. *Immunological Reviews* 186:37-46.

77. Dustin, M. L., T. G. Bivona, and M. R. Philips. 2004. Membranes as messengers in T cell adhesion signaling. *Nature Immunology* 5:363.

78. Giagulli, C., E. Scarpini, L. Ottoboni, S. Narumiya, E. C. Butcher, G. Constantin, and C. Laudanna. 2004. RhoA and zeta PKC control distinct modalities of LFA-1 activation by chemokines: critical role of LFA-1 affinity triggering in lymphocyte in vivo homing. *Immunity* 20:25.

79. Cantrell, D. A. 2001. Phosphoinositide 3-kinase signalling pathways. *Journal of Cell Science* 114:1439-1445.

80. Miyamoto, S., H. Teramoto, J. S. Gutkind, and K. M. Yamada. 1996. Integrins can collaborate with growth factors for phosphorylation of

receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. *Journal of Cell Biology* 135:1633-1642.

81. Braga, V. 2000. The crossroads between cell-cell adhesion and motility. *Nature Cell Biology* 2:E182-184.

82. Ilic, D., Y. Furuta, S. Kanazawa, N. Takeda, K. Sobue, N. Nakatsuji, S. Nomura, J. Fujimoto, M. Okada, and T. Yamamoto. 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature.* 377:539-544.

83. Fashena, S. J., and S. M. Thomas. 2000. Signalling by adhesion receptors. *Nature Cell Biology* 2:E225-229.

84. Xue, L., J. H. Murray, and A. M. Tolkovsky. 2000. The Ras/phosphatidylinositol 3-kinase and Ras/ERK pathways function as independent survival modules each of which inhibits a distinct apoptotic signaling pathway in sympathetic neurons. *Journal of Biological Chemistry* 275:8817-8824.

85. Liotta, L. A., and E. C. Kohn. 1995. Angiogenesis: role of calcium-mediated signal transduction. *Cancer Research* 55:1856-1862.

86. Masiero, L., K. A. Lapidos, I. Ambudkar, and E. C. Kohn. 1999. Regulation of the RhoA pathway in human endothelial cell spreading on type IV collagen: role of calcium influx. *Journal of Cell Science* 112:3205-3213.

87. Nombela-Arrieta, C., R. A. Lacalle, M. C. Montoya, Y. Kunisaki, D. Megias, M. Marques, A. C. Carrera, S. Manes, Y. Fukui, A. C. Martinez, and J. V. Stein. 2004. Differential requirements for DOCK2 and phosphoinositide-3-kinase gamma during T and B lymphocyte homing. *Immunity* 21:429-441.

88. Bonaccorsi, L., S. Marchiani, M. Muratori, V. Carloni, G. Forti, and E. Baldi. 2004. Singaling mechanisms that mediates invasion in prostate cancer cells. *Annals of the New York Academy of Sciences* 1028:4693.

89. Shulby, S. A., N. G. Dolloff, M. E. Stearns, O. Meucci, and A. Fatatis. 2004. CX3CR1-fractalkine expression regulates cellular mechanisms

involved in adhesion, migration, and survival of human prostate cancer cells. *Cancer Research* 64:4693-4698.

90.   Hendrix, M., E. Seftor, R. Seftor, J. Kasemeier-Kulesa, P. Kulesa, and L. Postovit. 2007. Reprogramming Metastatic Tumour Cells with Embryonic Microenvironments. *Nat Rev Cancer* 7:246-255.

91.   Nishio, M., T. Endo, N. Tsukada, J. Ohata, S. Kitada, J. Reed, N. Zvaifler, and T. Kipps. 2005. Nurselike cells Express BAFF and APRIL, Which Can Promote Survival of Chronic Lymphocytic Leukemia Cells via a Paracine Pathway Distinct from That of SDF-1 Alpha. *Blood* 106:1824-1830.

92.   Balabanian, K., B. Lagane, S. Infantino, K. Y. C. Chow, J. Harriague, B. Moepps, F. Arenzana-Seisdedos, M. Thelen, and F. Bachelerie. 2005. The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *Journal of Biological Chemistry* 280:35760-35766.

93.   Datta, S. R., A. Brunet, and M. E. Greenberg. 1999. Cellular survival: a play in three Akts. *Genes & Development* 13:2905-2927.

94.   Schuler, M., W. Li, B. Eggers-Sedlet, W. Lee, P. Tailor, P. Fitzgerald, G. B. Mills, and D. R. Green. 2001. Linking molecular therapeutics to molecular diagnostics: inhibition of the FRAP/RAFT/TOR component of the PI3K pathway preferentially blocks PTEN mutant cells in vitro and in vivo.[comment]. *Journal of Biological Chemistry* 276:34244-34251.

95.   Balsara, B. R., S. Apostolou, S. C. Jhanwar, and J. R. Testa. 2001. AKT plays a central role in tumorigenesis. *Proceeding of National Academy Science of United States of America* 98:10031-10033.

96.   Balsara, B. R., S. Apostolou, S. C. Jhanwar, and J. R. Testa. 2001. AKT plays a central role in tumorigenesis.[comment]. *Oncogene* 20:6245-6249.

97.   Biggs, W. H., 3rd, J. Meisenhelder, T. Hunter, W. K. Cavenee, and K. C. Arden. 1999. Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proceedings of the National Academy of Sciences of the United States of America* 96:7421-7426.

98. Allan, L., N. Morrice, S. Brady, G. Magee, S. Pathak, and P. Clarke. 2003. Inhibition of Caspase-9 through Phosphorylation at Thr 125 by ERK-MAPK. *Nat Cell Biol* 5:647-654.

99. Kyriakis, J. 2000. MAP Kinases and the Regulation of Nuclear Receptors. *Science* 48:PE1.

100. Kerr, J. F., A. H. Wyllie, and A. R. Currie. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer* 26:239-257.

101. Denmeade, S. R., and J. T. Isaacs. 1996. Activation of programmed (apoptotic) cell death for the treatment of prostate cancer. *Advances in Pharmacology* 35:281-306.

102. Borner, M. M., C. E. Myers, O. Sartor, Y. Sei, T. Toko, J. B. Trepel, and E. Schneider. 1995. Drug-induced apoptosis is not necessarily dependent on macromolecular synthesis or proliferation in the p53-negative human prostate cancer cell line PC-3. *Cancer Research* 55:2122-2128.

103. Ye, R. 2001. Regulation of Nuclear Factor -kB Activation by G-Protein Coupled Receptors. *J Leukocyte Biol* 70:839-848.

104. Karin, M. 2006. Nuclear Factor -kB in Cancer Development and Progression. *Nature* 441:431-436.

105. Lee, B. C., T. H. Lee, S. Avraham, and H. K. Avraham. 2004. Involvement of the chemokine receptor CXCR4 and its ligand stromal cell-derived factor 1alpha in breast cancer cell migration through human brain microvascular endothelial cells. *Molecular Cancer Research: MCR* 2:327-338.

106. Singh, S., U. P. Singh, J. K. Stiles, W. E. Grizzle, and J. W. Lillard. 2004. Expression and Functional Role of CCR9 in Prostate Cancer Cell Migration and Invasion. *Clinical Cancer Research* 10:8743-8750.

107. Caplen, N., S. Parrish, F. Imani, A. Fire, and R. Morgan. 2001. Specific Inhibition of Gene Expression by Small Double-Stranded RNAs in Invertebrate and Vertebrate Systems. *Nat Biotechnology* 98:9742-9747.

108. Elbashir, S., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl. 2001. Duplexes of 21-nucleotide RNAs Mediate RNA Interference in Cultured Mammalian Cells. *Nature* 411:494-498.

109. Huang, Y.-C., Y.-C. Hsiao, Y.-J. Chen, Y.-Y. Wei, T.-H. Lai, and C.-H. Tang. 2007. Stromal Cell-Derived Factor-1 Enhances Motility and Integrin Up-regulation Through CXCR4, ERK and NF-kB-Dependent Pathway in Hunman Lung Cancer Cells. *Biochemical Pharmacology* 74:1702-1712.

110. Fernandis, A., A. Prasad, H. Band, R. Klosel, and R. Ganju. 2004. Regulation of CXCR4-Mediated Chemotaxis and Chemoinvasion of Breast Cancer Cells. *Oncogene* 23:157-167.

111. Boukerche, H., Z. Su, L. Emdad, D. Sarkar, and P. Fisher. 2007. Mda-9/Syntenin Regulates the Metastatic Phenotype in Human Melanoma Cells By Activating Nuclear Factor-kappa B. *Cancer Research* 67:1812-1822.

112. Nagase, H. 1997. Activation mechanisms of matrix metalloproteinases. *Biological Chemistry* 378:151-160.

113. Eck, S., A. Cote, W. Winkelman, and C. Brinckerhoff. 2009. CXCR4 and Matrix Metalloproteinase-1 Are Elevated in Breast Carcinoma- Associated Fibroblasts and in Normal Mammary Fibroblasts Exposed to Factors Secreted by Breast Cancer Cells. *Mol Cancer Res* 7:1033-1044.

114. Muller, A., B. Homey, H. Soto, N. Ge, D. Catron, M. E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S. N. Wagner, J. L. Barrera, A. Mohar, E. Verastegui, and A. Zlotnik. 2001. Involvement of chemokine receptors in breast cancer metastasis.[see comment]. *Nature* 410:50-56.

115. Jemal, A., T. Murray, E. Ward, A. Samuels, R. C. Tiwari, A. Ghafoor, E. J. Feuer, and M. J. Thun. 2005. Cancer statistic. *CA Cancer J Clin* 55:10-30.

116. Luster, A., and A. Viola. 2008. Chemokines and Their Receptors: Drug Targets in Immunity and Inflammation. *Annual Review of Pharmacology and Toxicology* 48:171-197.

117. Fidler, U. 2003. The Pathogenesis of Cancer Metastasis: The Seed and Soil Hypothesis Revisited. *Nat Rev Cancer* 3:453-458.

118. Bissell, M., and D. Radisky. 2001. Putting Tumours In context. *Nat Rev Cancer* 1:46-54.

119. Goetzl, E. 2007. Diverse Pathways for Nuclear Signaling by G Protein-coupled Receptors and Their Ligands. *FASEB Journal* 21:638-642.

120. Hartmann, T., V. Grabovsky, R. Pasvolsky, Z. Shulman, E. Buss, A. Spiegel, A. Nagler, T. Lapidot, M. Thelen, and R. Alon. 2008. A Crosstalk Between Intracellular CXCR7 and CXCR4 Involved in Rapid CXCL12-Triggered Integrin Activation but Not in Chemokine-Triggered Motility of Human T Lymphocytes and CD34+ Cells. *J of Leukocyte Biology* 84:1130-1140.

121. Gales, C., J. Van Durm, and S. Schaak. 2006. Probing the Activation-Promoted Structural Rearrangements in Preassembled Receptor-G Protein Complexes. *Nat Struct Mol Biol* 13:778-786.

122. Audet, N., C. Gales, and E. Archer-Lahlou. 2008. Bioluminescence Resonance Energy Transfer Assays Reveal Ligand-Specific Conformational Changes Within Preformed Signaling Complexes Containing Delta-Opioid Receptors and Heterotrimeric G Proteins. *J Biol Chem* 283:15078-15088.

123. Infantino, S., B. Moepps, and M. Thelen. 2006. Expression and Regulation of the Orphan Receptor RDC1 and Its Putative Ligand in Human Dendritic and B Cells. *J Immunol* 176:2197-2207.

124. Sierro, F., C. Biben, and L. Martinez-Munoz. 2007. Disrupted Cardiac Development but Normal Hematopoiesis in Mice Deficient in the Second CXCL12/SDF-1 Receptor CXCR7. *Proc Natl Acad Sci USA* 104:14759-14764.

125. Wang, J., Y. Shiozawa, and J. Wang. 2008. The Role of CXCR7/RDC1 As a Chemokine Receptor for CXCL12-SDF-1in Prostate Cancer. *J Biol Chem* 283:4283-4294.

126. Boldajipour, B., H. Mahabaleshwar, and E. Kardash. 2008. Control of Chemokine-Guided Cell Migration By Ligand Sequestration. *Cell* 132:463-473.

127. Meijer, J., J. Ogink, and E. Roos. 2008. Effect of the Chemokine Receptor CXCR7 on Proliferation of Carcinoma Cells In Vitro and In Vivo. *Breast Cancer Research* 99:1493-1501.

128. Valentin, G., P. Haas, and D. Gilmour. 2007. The Chemokine SDF-1a Coordinates Tissue Migration Through the Spatially Restricted Activation of CXCR7 and CXCR4b. *Curr Biol* 17:1026-1031.

129. Moriuchi, M., H. Moriuchi, W. Turner, and A. Fauci. 1997. Cloning and Analysis of The Promoter Region of CXCR4, A Coreceptor for HIV-1 Entry. *J Immunol* 159:4322-4329.

130. Pin, J., J. Kniazeff, and J. Liu. 2005. Allosteric Functioning of Dimeric Class C G-protein Coupled Receptors. *FEBS* 272:2947-2955.

131. Springael, J., E. Urizar, and M. Parmentier. 2005. Dimerization of Chemokine Receptors and Its Functional Consequences. *Cytokine Growth Factor Rev* 16:611-623.

132. Kato, M., J. Kitayama, S. Kazama, and H. Nagawa. 2003. Expression pattern of CXC chemokine receptor-4 is correlated with lymph node metastasis in human invasive ductal carcinoma. *Breast Cancer Research* 5:R144-150.

133. Wendt, M., A. Cooper, and M. Dwinell. 2008. Epigenetic Silencing of CXCL12 Increases the Metastatic Potential of Mammary Carcinoma Cells. *Oncogene* 27:1461-1471.

134. Yoshida, D., R. Nomura, and A. Teramoto. 2009. Signaling Pathway Mediated by CXCR7, An Alternative Chemokine Receeptor For Stromal Cell Derived Factor-1 Alpha in AtT20 Mouse Adrenocorticotrophic Hormone-Secreting Pituitary Adenoma Cells. *J Neuroendo* 21:481-488.

135. Mueller, M., and N. Fusenig. 2004. Friends or Foes-Bipolar Effects of the Tumour Stroma In Cancer. *Nat Rev Cancer* 4:839-849.

136. Parkin, D., F. Bray, J. Ferlay, and P. Pisani. 2002. Global Cancer Statistics. *Cancer J Clin* 55:74-108.

137. Miyata, Y., T. Iwata, and K. Ohba. 2006. Expression of Matrix Metalloproteinase-7 on Cancer Cells and Tissue Endothelial Cells in Renal Cell Carcinoma: Prognostic Implications and Clinical Significance for Invasion and Metastasis. *Clin Cancer Research* 12:6998.

138. Clark, G., and W. McGuire. 1988. Sterooid Receptors and Other Prognostic Factors in Primary Breast Cancer. *Semin Oncol* 15:20.

139. Mengshol, J. A., M. P. Vincenti, C. I. Coon, A. Barchowsky, and C. E. Brinckerhoff. 2000. Interleukin-1 induction of collagenase 3 (matrix metalloproteinase 13) gene expression in chondrocytes requires p38, c-Jun N-terminal kinase, and nuclear factor kappaB: differential regulation of collagenase 1 and collagenase 3. *Arthritis and Rheumatism* 43:801-811.

140. Ganju, R. K., S. A. Brubaker, J. Meyer, P. Dutt, Y. Yang, S. Qin, W. Newman, and J. E. Groopman. 1998. The alpha-chemokine, stromal cell-derived factor-1alpha, binds to the transmembrane G-protein-coupled CXCR-4 receptor and activates multiple signal transduction pathways. *Journal of Biological Chemistry* 273:23169-23175.

141. Yonezawa, A., T. Hori, and H. Sakaida. 2000. SDF-1 has Costimulatory Effects on Human T Cells: Possible Involvement of MAPK (ERK2) Activation. *Microbial Immunol* 44:135-141.

142. Benbow, U., and C. Brinckerhoff. 1997. The AP-1 Site and MMP Gene Regulation: What is All The Fuss About? *Matrix biol* 15:519-526.

143. Taichman, R. S., C. Cooper, E. T. Keller, K. J. Pienta, N. S. Taichman, and L. K. McCauley. 2002. Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. *Cancer Research.* 62:1832-1837.

144. Sotsios, Y., G. Whittaker, J. Westwick, and S. Ward. 1999. The CXC Chemokine Stromal Cell-Derived Factor Activates a Gi-Coupled Phosphoinositide 3-Kinase in T Lymphocytes. *J Immunol* 163:5954-5963.

145. Guinamard, R., N. Signoret, and M. ishiai. 1999. B cell Antigen Receptor Engagement Inhibits Stromal Cell-Derived Factor SDF-1 Alpha Chemotaxis and Promotes Protein Kinase C Induced Internalization of CXCR4. *Exp Med* 189:1461-1466.

146. Steelman, L., S. Pohnert, J. Shelton, R. Franklin, F. Bertrand, and J. McCubrey. 2004. JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell Cycle Progression and Leukemogenesis. *Leukemia* 18:189-218.

147. Nakano, H., M. Shindo, S. Sakon, S. Nishinaka, M. Mihara, H. Yagita, and K. Okumura. 1998. Differential Regulation of I kappa B Kinase Alpha and Beta By Two Upstream Kinases, NF-kB Inducing Kinase and Mitogen Activated Protein Kinase ERK Kinase 1. *Pro Natl Acad* 95:3537-3542.

148. Viatour, P., M. Merville, V. bours, and A. Chariot. 2005. Phosphorylation of NF-kB and IKB proteins: Implications in Cancer and Inflammation. *Trends Biochem* 30:43-52.

CURRICULUM VITAE

Name: LaSharon D. Mosley

Work Address:

James Graham Brown Cancer Center
580 S. Preston Street, Baxter II Room 311
Louisville, KY 40202
(502) 852-2881

Education:

University of Louisville, Louisville, KY
Ph.D., Pharmacology & Toxicology
Graduation date: November 2009
Dissertation Title: Mechanisms Mediated by CXCL12 Signaling through CXCR4
and CXCR7 in Breast Cancer

University of Louisville, Louisville, KY
M.S., Pharmacology & Toxicology
Graduation date: May 2007
Thesis Title: Role of Estrogen Receptors Alpha and Beta in Thyroid Cancer

Mississippi University for Women, Columbus, MS
B.S., Chemistry & Biology
Graduation date: January 2000

Scientific/Professional Experience:

2007-2009; PhD
Department of Pharmacology & Toxicology, University of Louisville
Research Focus: The Role of CXCR7 in Breast Cancer Cell Progression
Dissertation Advisor: Dr. James W. Lillard, Jr.

2004-2007; M.S.
Department of Pharmacology & Toxicology, University of Louisville
Research Focus: Role of estrogen receptors alpha and beta in normal and
neoplastic thyroid cells

Thesis Advisor: Dr. Richard Goldstein
2000-2003; Tissue Culture Core Director/Research Assistant
Tulane University Center for Gene Therapy
Research Focus: Isolation of adult stem cells from primary cultures to treat brittle bone and Parkinson's diseases
Principal Investigators: Drs. Darwin Prockop and Donald Phinney

2000; Research Assistant
Department of Biochemistry, University of Mississippi
Research Focus: Effects of vinca alkaloids in P-19 and MCF-7 cell lines
Principal Investigators: Drs. Barbara Rogers and Sharon Lobert

1998; Summer Internship
Department of Pharmacology; Tulane University
Research Focus: Effects of phytochemicals in breast cancer cell line(s) growth and progression
Principal Investigator: Dr. Nancy Hopkins

Teaching Experience:

2009	Judge, Nativity Academy Science Fair
2005, 2006	Judge, Newburg Middle School Science Fair
2000-2002	Reading Tutor, Union Kemper Springs Missionary Baptist Church
1997-1998	Organic Chemistry Teacher Assistant, MUW
1996-1997	General Chemistry Assistant, MUW

Honors and/or Special Awards:

2009	Susan G. Komen Grants Committee Advisor (Local Affiliate)
2009	University of Louisville Graduate Student Travel Award
2009	FASEB/MARC Minority Student Travel Award (AAI)
2009	AACR Minority Scholar in Cancer Research Award
2008	FASEB/MARC Minority Student Travel Award
2008	Susan G. Komen Award (ICC meeting on inclusive policies)
2008	University of Louisville Graduate Student Travel Award
2007-present	Southern Regional Education Board Fellowship
2007	University of Louisville Minority Fellowship
2007	FASEB/MARC Minority Student Travel Award
2005-2006	Surgery Oncology Graduate Fellowship, U of L
2004-2005	IPIBS Fellowship, University of Louisville
2001	Oral abstract presentation award, Mississippi Academy of Science

| 1999 | Most Outstanding Presentation in Biochemistry, Cellular & Molecular Biology, Mississippi Academy of Science |
| 1993- 1994 | Young Woman of the Year Award, Kemper County |

Membership/Academic Service in Professional Societies:

2008-present	American Society for Pharmacology & Experimental Therapeutics
2007-present	Women in Cancer Research
2007-present	Minority in Cancer Research
2007-present	American Association for Cancer Research (AACR)
2007-present	American Association of Immunologists (AAI)
2005-present	BBGSO (Treasurer)- U of L
2006-2007	American Chemical Society
2001-2003	ISSCR (International Society for Stem Cell Research)
1999-2001	Mississippi Academy of Science
1996-1999	Beta Beta Beta Honor Society (MUW)
1996-1999	Alpha Epsilon Delta Honor Society (MUW)

Humanitarian Services:

2009	Nativity Academy (mentor)
2007- present	Susan G. Komen Breast Cancer Foundation (volunteer)
2006	Food Drive for the Poor (Canaan Christian Church)
2005- present	American Cancer Society Relay for Life (BBGSO)
2005- 2006	Goodwill Donation Drive
2005	United Way Hurricane Katrina Fund Drive
2002- 2003	Habitat for Humanity (Tulane)
2000- 2001	Learn to Read Summer Program (Kemper County)
1998- 1999	Habitat for Humanity (MUW)
1998	Feed and Clothe the Poor Drive (MUW)

Seminars and Presentations:

2009	Posters Presentation, American Association of Immunology
2009	Posters Presentation, American Assoc for Cancer Research
2008	Poster Presentation, American Assoc for Cancer Research
2008	Poster Presentation, American Association of Immunology
2008	Susan G. Komen, Louisville Affiliate (Minority Inclusion)
2007	Research Seminar, University of Louisville (Pharm & Tox)
2006	Research Seminar, University of Louisville (Pharm & Tox)
2005	Research Seminar, University of Louisville (Pharm & Tox)
2002	Research Tulane Seminar, Tulane Center for Gene Therapy
2001	Oral Presentation, Mississippi Academy of Science (2nd)
1999	Oral Presentation, Mississippi Academy of Science (won)

Meetings Attended:

2009	American Association of Immunologists
2009	American Association for Cancer Research
2008	SREB Faculty Compact
2008	Intercultural Cancer Council Meeting (local Susan G. Komen Affiliate)
2008	FASEB/AACR Meeting
2008	Experimental Biology Conference
2007	James Graham Brown Cancer Center Retreat
2007	SREB Faculty Compact
2007	AAI Conference
2004, 2005, 2006	Research Louisville
2004, 2005, 2006	James Graham Brown Cancer Center Retreat
2001-2002	ISSCR Meeting
2002	Research Tulane
2001	ISHAGE Meeting
2001	Mississippi Academy of Science
1999	Mississippi Academy of Science

Abstracts

1. **Mosley L**, Sharma P, Singh R, Singh S, Lillard JW Jr. "CXCL11 and CXCL12 mediate matrix metalloproteinase expression by breast cancer cells through CXCR7". 2009 AAI Meeting

2. **Mosley L**, Singh R, Sharma P, Singh S, Lillard JW Jr. "CXCL11 and CXCL12 activates ERK1/2 and NF-κB in breast cancer cells via CXCR3, CXCR4, and CXCR7". 2009 AAI Meeting

3. **Mosley L**, Sharma P, Singh R, Singh S, Lillard JW Jr. "CXCL11 and CXCL12 Modulates Matrix Metalloproteinase Expression by Breast Cancer Cells predominately through CXCR7 Interactions". 2009 AACR Meeting

4. **Mosley L**, Sharma P, Singh R, Singh S, Lillard JW Jr. "CXCL11 and CXCL12 Modulates ERK1/2 and NF-κB activation in Breast Cancer Cells via CXCR3, CXCR4, and CXCR7". 2009 AACR Meeting

5. **Mosley L**, Sharma P, Singh R, Singh S, Lillard JW Jr. "Differential β-3 integrin of CXCR3, CXCR4, and CXCR7 Clustering on Breast Cancer Cells in Response to CXCL11, CXCL12, and Adrenomedullin". 2008 FASEB Meeting

6. **Mosley L**, Sharma P, Singh R, Singh S, Lillard JW Jr. "CXCR7 and CXCR3 interactions promote breast cancer cell migration, adhesion, and survival." 2008 Annual American Association for Cancer Research Meeting, San Diego.

7. Kumar A, **Mosley L**, Klinge C, Goldstein R. "Estradiol and Selective Estrogen Receptor Modulators (SERMs) modulate the proliferation of Thyroid

Cancer Cells: Role of Estrogen Receptors Alpha and Beta". 2007 Annual Endocrine Society Conference

8. Goldstein RE, Klarer A, **Mosley L**, Agrawal A, Joshua J, Stein E, Zeiger M, Kebebew E, Waigel S, Beckers S, Zacharias Z, Ho S, Cheng RY. "Clustering of Papillary Thyroid Carcinomas from African-American and Caucasian Female Subjects into Distinctly Different Microarray Expression Profiles". The International Association of Endocrine Surgeons, Durban, South Africa, August 2005.

9. Propst E, **Mosley L**, Rogers B, Lobert S. "Inhibition of Proliferation by Vinca Alkaloids in P-19 and MCF-7 Cell Lines". The Journal of the Mississippi Academy of Sciences. 2001

10. **Mosley L**, Hopkins NE, Alworth WL. "The Effects of Phytochemical on MCF-7 Cell Growth and Estrogen Receptor Binding". The Journal of the Mississippi Academy of Sciences. 1999

Manuscripts

1. **L. Mosley***, P. Sharma*, R, Singh, S. Singh, and J.W. Lillard, Jr. 2009. CXCL12 activates matrix metalloproteinase expression and activation by breast cancer cells via CXCR4 and CXCR7 interactions. (in preparation)

2. **L. Mosley***, R, Singh*, P. Sharma, S. Singh, and J.W. Lillard, Jr. 2009. CXCL12 mediates ERK1/2 and NF-kB activation in breast cancer cells in a CXCR4 and CXCR7 dependent fashion. (in preparation)

References

1. Dr. James W. Lillard, Jr. (mentor) 502-852-2174 or james.lillard@louisville.edu

2. Dr. Jill Suttles (committee member) 502-852-5144 or jill.suttles@louisville.edu

3. Kathy Schroerlucke (served as executive director of local Susan G. Komen) 502-548-2028