The diagnostic and therapeutic role of the stromal cell-derived factor (SDF)-1/CXCR4 axis in breast cancer metastasis

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A thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Dedication

This thesis is dedicated to my wonderful parents who have been ever so supportive and thoughtful, without whom this thesis would have never been accomplished.

To my brother, Ansar, for inspiring me to pursue this thesis, and for guiding me through this journey.

To my dear, late uncle Salar, who suffered from a constant acknowledgement of a failing body. He exemplified patience and persistence.

Abstract

Breast cancer kills through the process of metastasis. In order to improve the prognosis of patients with breast cancer, a better understanding of the underlying factors driving the metastatic process in patients is required. One theory that helps explain the metastatic process suggests that chemokines, such as stromal cell-derived factor (SDF)-1, are overexpressed in specific distant metastatic organs, such as lung, liver, and bone, and serve to home in cancer cells that express their receptors, like CXCR4. The hypothesis of this thesis is that the SDF-1/CXCR4 axis plays an important role in the process of metastasis in breast cancer, and that this ligand/receptor axis can be exploited in the diagnostics and therapy of breast cancer. The first objective of this thesis was to determine if circulating levels of SDF-1 could predict breast cancer metastasis. We found low levels of plasma SDF-1 to be a strong independent prognostic marker, suggesting that the concentration gradient of low plasma SDF-1 and high SDF-1 expressed in the metastatic organ may be critical in driving cancer cells from the circulation to the target organ. We further determined that the levels of plasma SDF-1 were tumor-independent, identifying the first host-derived blood marker predictive of distant metastasis. The second objective was to determine if tumor expression of CXCR4 could modulate the prognostic effect of plasma SDF-1 levels. We found that patients with tumors that highly expressed the activated form of the receptor, phosphorylated-CXCR4, and low plasma SDF-1 levels had a much poorer prognosis than those patients with either risk factor alone. These results highlighted the importance of the dysfunctional relationship between the tumor and the host in the metastatic process. The third objective assessed the therapeutic potential of targeting CXCR4 with a peptide antagonist in a transgenic mouse model. In combination with an anti-angiogenic agent, targeting CXCR4 resulted in a 40% decrease in primary tumor volume and 75% reduction in distant metastasis. Together, these results suggest the potential role for both plasma SDF-1 as a prognostic tool that may assist in the selection of adjuvant therapy, and tumor CXCR4, as a promising druggable target.

Résumé

Le cancer du sein tue par le processus des métastases. Dans le but d'améliorer le pronostic des patients atteints du cancer du sein, une meilleure compréhension des facteurs sous-jacents qui conduisent à la transformation métastatique est nécessaire. Une théorie qui explique la transformation métastatique propose que les chimiokines, telles que le *stromal cell-derived factor (SDF)-1*, sont surexprimées dans des organes métastatiques distants spécifiques, tels que le poumon, le foie, et les os et servent à attirer les cellules cancéreuses qui expriment leurs récepteurs, tels CXCR4. L'hypothèse de cette thèse est donc que l'axe SDF-1/CXCR4 joue un rôle important dans la transformation métastatique dans le cancer du sein, et que cet axe ligand/récepteur peut être exploité dans le diagnostic et la thérapie du cancer du sein. Le premier objectif de cette thèse était de déterminer si les niveaux circulants de SDF-1 peuvent prédire la présence de métastases du cancer du sein. Nous avons découvert que des niveaux peu élevés de SDF-1 dans le plasma représentent un bon déterminant pronostique indépendant, suggérant que le gradient de concentration de faible niveaux de SDF-1 dans le plasma et de niveaux élevés de SDF-1 dans l'organe métastasé peut être un événement critique dans le transfert des cellules cancéreuses de la circulation sanguine jusqu'à l'organe-cible. Nous avons de plus déterminé que les niveaux plasmatiques de SDF-1 sont indépendants des tumeurs, identifiant le premier marqueur sanguin, dérivé de l'hôte, de prédiction de métastases éloignées. Le second objectif était de déterminer si l'expression tumorale de CXCR4 pourrait moduler l'effet pronostique des niveaux plasmatiques de SDF-1. Nous avons découvert que les patients dont les tumeurs expriment de façon élevée la forme activée du récepteur, CXCR4 phosphorylé, et des niveaux plasmatiques faibles de SDF-1, constituaient la cohorte exhibant un mauvais pronostic en comparaison avec les patients présentant l'un ou l'autre facteur de risque isolément. Ces résultats soulignent l'importance de la relation dysfonctionnelle entre la tumeur et l'hôte au cours de la transformation métastatique. Le troisième objectif était d'évaluer le potentiel thérapeutique en ciblant CXCR4 au moyen d'un peptide antagoniste dans un modèle de souris transgénique. Avec l'ajout d'un agent antiangiogenique, le ciblage de CXCR4 a conduit à une réduction de 40% du volume de la tumeur primaire et à une réduction de 75% des métastases éloignées. Ensemble, ces

résultats suggèrent le rôle essentiel à la fois de SDF-1, en tant qu'outil pronostique pouvant aider au choix d'un traitement d'appoint, et de CXCR4 tumoral en tant que cible pharmaceutique prometteuse.

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Abbreviations

A: adenine

17-AAG: 17-allylamino demethoxygeldanamycin

AHR: adjusted hazard ratio

AIDS: acquired immunodeficiency syndrome

AIP4: atrophin-1 interacting protein 4

BCNU: 1,3-bis(2-chloroethyl)-1-nitrosurea

bFGF: basic fibroblast growth factor

C: cysteine

C3a: C3 complement protein cleavage fragment a

CA: cancer antigen

cAMP: cyclic adenosine 5' monophosphate

CC: cysteine, cysteine

CD: cluster of differentiation

CEA: carcinoembryonic antigen

CHUM: Centre Hospitalier de l'Université de Montréal

CI: confidence interval

CTCs: circulating tumor cells

CXC: cysteine, amino acid, cysteine

CX_3C: cysteine, amino acid, amino acid, amino acid, cysteine

CXCL12/SDF-1: CXC ligand 12/stromal cell-derived factor-1

CXCR4: G-protein coupled receptor, chemokine receptor for SDF-1

DCIS: ductal carcinoma in-situ

DDFS: distant disease-free survival

DFS: disease-free survival

4E-BP1: 4E-binding protein 1

ECM: extracellular matrix

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

ELISA: enzyme-linked immunosorbent assay

ELR: tripeptide motif consisting of glutamic acid, leucine, arginine

EPCs: endothelial progenitor cells

ER: estrogen receptor

ERK: extracellular signal-regulated kinase

FAK: focal adhesion kinase

FDA: Food and Drug Administration

G: guanine

G proteins: guanine nucleotide binding proteins

GC: guanine-cytosine

G-CSF: granulocyte-colony stimulating factor

GDP: guanosine diphosphate

GPCR: G-protein coupled receptor

GRK: G-protein coupled receptor kinase

GTP: guanosine triphosphate

HER2: Human epidermal growth factor receptor-2

HIF: hypoxia–inducible factor

HIV: human immunodeficiency virus

HR: hazard ratio

HSP90: heat shock protein 90

HUVECs: human umbilical vein endothelial cells

IFN: interferon

IL: interleukin

ILK: integrin-linked kinase

I.P.: intraperitoneally

JAK: janus kinase

JNK/SNAP: c-jun NH_2-terminal kinase/stress-activated protein kinase

KBPs: kilo base pairs

LI: labelling index

LPS: lipopolysaccharide

MAPK: mitogen activated protein kinase

MIP-1: macrophage inhibiting protein-1

MMPs: matrix metalloproteinases

MMTV-PyMT: murine mammary tumor virus driven by polyoma middle T antigen

mRNA: messenger RNA

mTOR: mammalian target of rapamycin

NF-κB: nuclear factor kappa light-chain enhancer of activated B cells

NK: natural killer

P70S6K: p70 ribosomal protein S6 kinase

p-CXCR4: phosphorylated-CXCR4

PI3K: phosphatidylinositol 3-kinase

PMA: phorbol, 12-myristate, 13-acetate; activator of protein kinase C

PR: progesterone receptor

PS: product score

Rac-1: Ras-related C3 botulinum toxin substrate 1

RAFTK: related adhesion focal tyrosine kinase

RANTES: regulated on activation normal T-cell expressed and secreted, also known as chemokine ligand 5 (CCL5)

RIP-Tag: rat insulin promoter-large SV40-T antigen

SD: standard deviation

SDF-1: stromal cell-derived factor-1

SDF-1-3'A: polymorphism of SDF-1; a guanine to adenine transition in the 3' untranslated region of the SDF-1 gene transcript

SE: standard error

shRNA: short hairpin RNA

sICAM-1: soluble intercellular adhesion molecule-1

Sipa1: signal-induced proliferation-associated gene 1

siRNA: small interfering RNA

sVCAM-1: soluble vascular cell adhesion molecule -1

TATA box: short sequence of thymidine and adenine residues

TGF-β1: transforming growth factor-beta 1

TMA: tissue microarray

TNF: tumor necrosis factor

uPAR: urokinase plasminogen activator receptor

VEGF: vascular endothelial growth factor

VEGFR: vascular endothelial growth factor receptor

X: any amino acid residue

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Preface

i. Format of the thesis

This thesis is comprised of five chapters. The introduction in chapter one presents an overview of breast cancer metastasis and the chemokine/receptor pair: stromal cell-derived factor-1 and CXCR4. The rationale and hypothesis of this thesis are also included in chapter one. Chapters two to four are data chapters that are presented in this thesis as the duplicated text of the published papers or papers submitted for publication in accordance with the "Guidelines for Thesis Preparation". The copyright agreements of Clinical Cancer Research, American Society for Investigative Pathology, and Current Opinion in Biotechnology permit the insertion of these manuscripts in this thesis. Chapter two was published in Clinical Cancer Research; 2008 Jan 15;14(2):446-54. Chapter three was published in The American Journal of Pathology; 2009, 175:66-73 Chapter four is in final preparation, for submission to Breast Cancer Research and Treatment. Chapter five contains a summary and discussion of the results of this thesis and the List of Original Contributions. The appendix contains the original manuscript from the American Journal of Pathology, as requested by Copyright Permissions from the American Society for Investigative Pathology, and a review paper on tissue microarrays published in Current Opinion in Biotechnology; 2008 Feb;19(1):19-25. The appendix also contains the ethics certificates for work on human and animal subjects as well as the copyright permission from the American Society for Investigative Pathology and Elsevier. The American Association of Cancer Research allows use of its articles (from Clinical Cancer Research) for the purpose of dissertations without requesting permission.

ii. Contribution of Authors

Manuscript 1:

Hassan S, *Baccarelli A, Salvucci O, Basik M. Plasma stromal cell-derived factor-1: host derived marker predictive of distant metastasis in breast cancer. Clin Cancer Res. 2008 Jan 15;14(2):446-54.*

AB supervised all statistical analysis. OS assisted in troubleshooting the ELISA experiments. Project design was conceptualized and material accrual was performed by MB. All authors participated in the revision of the final manuscript. All experimental work including creation of the patient database with clinico-pathologic information for 300 patients, ELISAs, and genotyping were performed by the candidate, in addition to statistical analysis and manuscript preparation.

Manuscript 2:

Hassan S, *Ferrario C, Saragovi U, Quenneville L, Gaboury L, Baccarelli A, Salvucci O, Basik M. The Influence of Tumor-Host Interactions in the SDF-1/CXCR4 Ligand/Receptor Axis in Determining Metastatic Risk in Breast Cancer. Am J Pathol. 2009 Jul;175(1):66-73.*

CF assisted in the construction of the tissue microarray and the immunohistochemical staining of Ki67. The laboratory of US synthesized the biotinylated-CXCR4 for detection of CXCR4. LQ and LG assisted in reading the tissue microarrays for each biomarker. AB supervised the statistical analysis. OS performed the western blot for p-CXCR4. The candidate assisted in the conceptualization of the project in collaboration with her supervisor. She was largely responsible for the building of the tissue microarray consisting of 1619 core biopsies. She organized the collection of the paraffin-embedded blocks from two pathology departments. She marked all samples to be biopsied and reviewed them with MB and LQ. She constructed the tissue microarray and performed immunohistochemistry analysis on four biomarkers. She analyzed all her data and consulted her supervisor for data interpretation and revisions of the manuscript.

Manuscript 3:

Hassan S, *Buchanan M, Gaboury L, Muller W, Salvucci O, Basik M. Targeting CXCR4 with a peptide antagonist inhibits distant metastasis, primary tumor growth, and enhances the efficacy of anti-VEGF treatment or docetaxel in a transgenic mouse model of breast cancer. In final preparation, for submission to Breast Cancer Research and Treatment.*

MBu assisted in the genotyping of the mice and necropsies. LG assisted in the reading of micrometastasis and Ki67 Labelling Index. WM provided the initial MMTV-PyMT mice. OS assisted in project design. MB conceptualized and supervised the project. The candidate assisted with the conceptualization of the project, created a colony of mice required for her experiments, genotyped the mice, prepared the pharmacologic agents, and administered these agents. She measured the tumors and performed all necropsies, performed western blot analysis, immunohistochemistry and TUNEL staining on mice tissues. She analyzed all her data and prepared the manuscript.

Manuscript 4:

Hassan S, *Ferrario C, Mamo A, Basik M. Tissue microarrays: emerging standard for biomarker validation. Curr Opin Biotechnol. 2008 Feb;19(1):19-25.*

All authors contributed to the conceptualization and writing of the manuscript. The candidate contributed significantly in the revisions and compilation of the final manuscript.

CHAPTER 1 - INTRODUCTION

1.1 The Breast

The breast is composed of two main components: a glandular and a surrounding stromal component, consisting of fat and connective tissue (Fig. 1-1). The glandular component can be broken down into 15-20 lobules, which produce milk, and ducts, which are tubular structures that transport milk from the lobules to the nipple. The breast has an abundant arterial supply, with venous drainage and lymph vessels that drain to nearby lymph nodes mainly found in the axilla (Fig. 1-1). Whereas the venous system transports blood away from the breast, the lymphatic system functions as a filter or trap for foreign particles such as bacteria or cancer cells (1, 2).

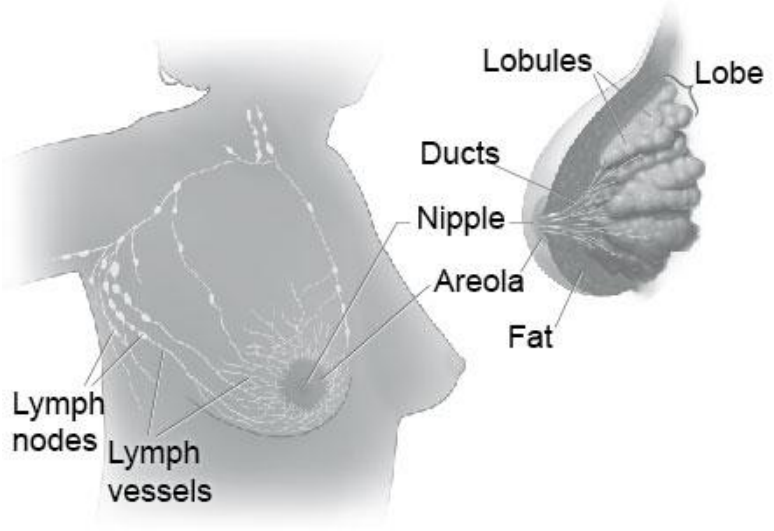


Fig.1-1. Anatomy of the breast. From ref. (2).

1.1.1 Breast Cancer

Breast cancer is the most common non-skin cancer in women, with a lifetime risk of 1/8 women, and an expected incidence of 22 700 new cases in 2009 in Canada (3). Breast cancer can arise from any one of the two main components (glandular or stroma), but more commonly originates from the glandular component. Tumors arise from normal tissues that undergo a progression of cellular changes and proliferation. In fact, tumor progression occurs in steps starting with hyperplasia, dysplasia, pre-invasive ductal carcinoma in-situ (DCIS) (contained within the basement membrane), subsequent

penetration of the basement membrane (microinvasion), and ultimately the formation of invasive adenocarcinoma of the breast (Fig. 1-2) (4).

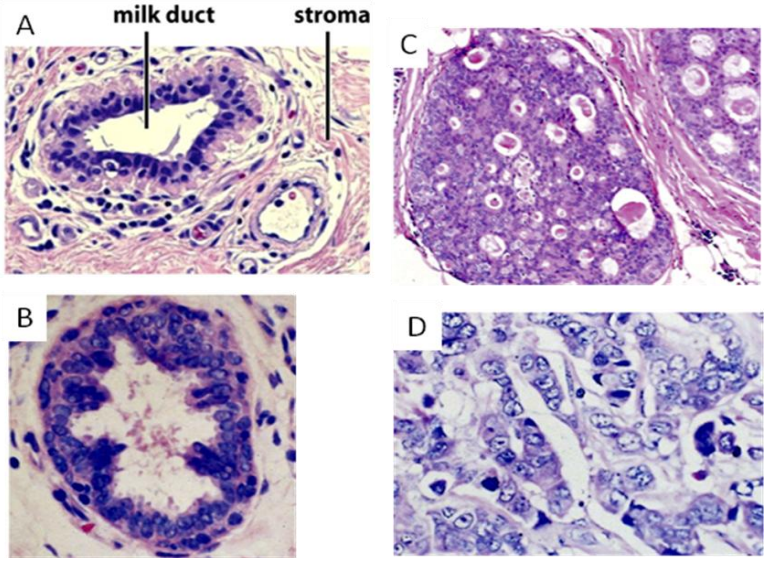


Fig. 1-2. Histologic representation of tumor progression in breast cancer. *A*, Normal epithelial duct. *B*, Hyperplasia. *C*, Ductal carcinoma in-situ. *D*, Invasive adenocarcinoma. Adapted from Weinberg (4).

1.1.2 Breast Cancer Metastasis

Breast cancer kills by metastatic spread to distant organs. Approximately 5400 women in Canada are expected to die from breast cancer in 2009 (3). Breast cancer can spread locoregionally, that is, mainly to the axillary lymph nodes, and to distant organs, such as lung, liver, bones, and brain through hematogenous dissemination. The process of distant cancer spread, or metastasis, has been dissected into a cascade of events starting with the growth of the primary tumor, entry of tumor cells into the circulation (intravasation), circulation within the bloodstream, adhesion of tumor cells to target organ blood vessels, exit into the distant organ (extravasation), and finally, proliferation at the distant site, that is progression from micrometastasis to macrometastasis (colonization) (Fig.1-3) (4, 5). Yet, the driving force underlying this mechanism is not well understood.

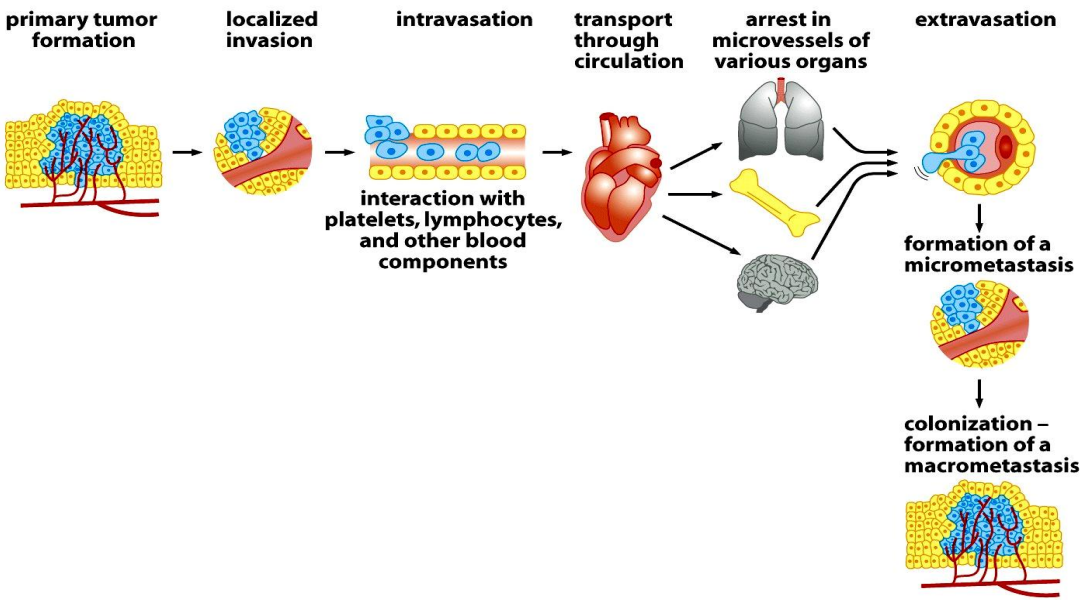


Fig. 1-3. Process of distant metastasis formation. From Weinberg (4).

1.1.2.1 Clinical Challenge of Metastasis

Although recent advances in screening and therapy have helped to improve the prognosis of breast cancer patients, further advances in the diagnosis and therapeutics of breast cancer metastasis are necessary to improve patient survival. Currently, there are a few prognostic markers available to assist the selection of adjuvant therapy (administered after tumor excision) in order to eliminate micrometastatic disease. These include tumor size, grade, estrogen receptor (ER) status, progesterone receptor (PR) status, human epidermal growth factor receptor-2 (HER2) status, and tumor involvement of axillary lymph nodes (6-8). Tumor involvement of lymph nodes is the most powerful prognostic marker for breast cancer. As an indicator of locoregional spread, lymphatic dissemination serves as a surrogate marker for the cancer's propensity to invade into the bloodstream, circulate, and disseminate to distant organs (9). However, studies have demonstrated limitations in the use of axillary lymph node involvement as a prognostic marker due to lack of sensitivity and specificity. About one-third of lymph node-negative breast cancer patients develop distant metastasis, whereas one-third of lymph node-positive patients remain free of distant metastasis ten years after local therapy (10, 11). Therefore, there is a need to identify a better predictive marker for the process of distant metastases. It would be ideal if such a marker could be identified from the blood. In fact, a circulating

biomarker may be a more direct indicator of hematogenous cancer spread, with the added advantage of being easily accessible via a simple blood test. There are currently no serum or plasma markers that are recommended for routine use in the management of breast cancer patients. Although well studied, Cancer Antigen (CA) 15-3, CA 27.29, and carcinoembryonic antigen (CEA) are not validated prognostic markers for staging in breast cancer (12).

In addition to better prognostic markers, therapeutic advances are also required to further improve the survival of breast cancer patients. Until recently, cytotoxic chemotherapies were the mainstay of cancer therapy. Now, targeted therapies are emerging in an attempt to attack the tumor at key signaling pathways to improve efficacy with diminished toxicity. The majority of these therapeutic agents target growth factor receptors or hormone receptors; effectively targeting cellular processes of proliferation or angiogenesis. However, there are very few targeted agents under current investigation that specifically attack the process of metastasis (13). It is plausible that targeting the metastatic process in combination with primary tumor growth may together demonstrate the greatest improvement in patient survival.

1.1.2.2 Biological Theories of Metastasis

The biology underlying the metastatic process is not well understood. There are numerous theories which help explain the metastatic process. A thorough review of these theories is beyond the scope of this thesis (14, 15), and so I will only present a few of the salient models and determinants of metastasis. Until 1889, the prevailing theory underlying cancer cell dissemination was that the spread to distant organs occurred in a random fashion. However, after a review of 900 autopsy records, Stephen Paget, an English surgeon, identified a distinct pattern in the distant organs that were involved by metastasis and thus proposed the "seed and soil" theory. He hypothesized that a certain population of tumor cells (seed) were able to metastasize and colonize in the environment (soil) that was "congenial". In 1929, James Ewing reported that metastasis was more of a mechanical process, one which resulted from the anatomic distribution of blood flow. Subsequent studies in fact continued to support both theories; however, a deeper

understanding of the mechanism underlying tumor dissemination to specific target organs remained incomplete for some time (5).

1.1.2.2.1 Tumor-Derived Determinants of Metastasis

There are two major components which determine the tumor's ability to metastasize: intrinsic factors which originate from the tumor cell proper; and extrinsic factors, derived from the tumor's microenvironment or bone marrow. One important theory from the 1970s modeled the process of tumor progression upon Nowell's hypothesis of clonal genetic evolution. The progression of tumors was postulated to result from the selection of clonal populations of more aggressive tumor cells. These clones demonstrated an increased capacity to proliferate which arose from a series of genetic mutations secondary to genetic instability. Analogously, injection of different melanoma cell line clones resulted in different metastatic potential, suggesting that metastasis originated from a highly metastatic variant from within a primary tumor (16, 17). Recent advances in technology and the mapping of the human genome have allowed for a genome-wide analysis of tumors, enabling greater insight into the timing at which metastatic potential is acquired during tumor progression. Gene-expression profiling of primary human breast tumors has identified genetic signatures that distinguish which tumors will metastasize from those that will remain localized (18-22). Furthermore, genes have been identified that are involved in regulating the metastatic process, such as nm23 (23).

However, recent studies have demonstrated that the tumor cell itself is not the sole instigator determining the tumor's ability to metastasize. Paracrine influences from the tumor microenvironment and endocrine influences originating from the bone marrow have also been shown to play a role in altering metastatic potential. The stromal microenvironment consists of a structural scaffold - the extracellular matrix (ECM) and various cell types, including fibroblasts, myofibroblasts, endothelial cells, macrophages, and lymphocytes (4). The cells in the stroma are a source of both mitogenic growth factors and growth-inhibitory factors. The stroma is also involved in stimulating proteases that can degrade the ECM, inducing endothelial cells to form blood vessels, or angiogenesis, and providing signals for epithelial cells to acquire a more invasive, fibroblast-like phenotype in a phenomenon called epithelial to mesenchymal cell

transition (4). Furthermore, Park's group recently discovered a genetic signature from patients, wherein gene expression of stromal cells alone was predictive of metastasis-free survival (24).

Recent studies have also uncovered a role of the bone marrow and the importance of recruitment of bone-marrow derived progenitor cells for the metastatic process. Kaplan et al. proposed the concept of a "pre-metastatic niche", wherein the arrival of tumor cells at the metastatic site was shown to be preceded by the arrival of a cluster of hematopoietic progenitor cells which express vascular endothelial growth factor receptor (VEGFR)-1. The significance of this pre-metastatic niche was illustrated with the inhibition of metastasis upon blockade of niche formation (25). In addition, bone-marrow derived mesenchymal stem cells have been shown to be involved in augmenting metastatic potential when admixed with breast cancer cell lines of low metastatic capacity (26). Furthermore, the growth of micrometastatic lesions may be facilitated through the recruitment of bone marrow cells (27). In summary, the metastatic process can be considered to result from an interaction of several factors including the genetic make-up of the tumor cell itself, local communication of the tumor with its stromal microenvironment, and systemic communication with the bone marrow.

1.1.2.2.2 Host-Derived Determinants of Metastasis

Very little research has been conducted to date acknowledging the host as a determinant of metastasis. *In-vivo* evidence of the host's role was demonstrated using the highly metastatic transgenic mouse model, MMTV-PyMT, whereby metastatic efficiency was found to be altered in the progeny of mice of this strain when bred with different background strains. Given the constant oncogene, PyMT, differences in metastatic efficiency were attributed to allelic diversity of the background strains. Mapping of the metastasis-modulating loci identified one such gene: *Signal-induced proliferation-associated gene 1 (Sipa1)*. Experimental manipulation of mRNA *Sipa1* levels modulated metastatic potential *in-vivo*. A polymorphism in *Sipa1* was also identified, which altered cellular function *in-vitro,* and correlated with axillary lymph node involvement in human breast cancer (28, 29). Population-genetic studies have also revealed that the metastatic potential of tumors may have a hereditary association. A recent Swedish study

discovered an increased inheritable susceptibility, that is, a worse prognosis, in daughters or sisters of patients with aggressive breast cancer (30). This suggests that there are inheritable factors that may modify the risk of distant metastasis. Thus, further insight into the metastatic process will require a better understanding of the individual determinants from both the tumor and the host, and also the manner in which they interact.

1.1.2.2.3 Chemokine-Receptor Model of Metastasis

In 2001, Muller et al. proposed an intriguing model to explain the manner in which cancer cells metastasize to specific target organs (31). Analogous to the manner in which chemokines were shown to direct the migration of lymphoid cells with their respective receptors to lymph nodes (32), Muller et al. proposed that chemokines expressed at distant target organs can home in breast cancer cells that express their receptors (Fig. 1-4). In order to determine if chemokine receptors were expressed by breast cancers, a panel of breast cancer cell lines was screened to determine the messenger RNA (mRNA) expression of seventeen possible chemokine receptors. A few chemokine receptors were found to be overexpressed in these breast cancer cell lines, of which CXCR4 was the most highly expressed. Overexpression of CXCR4 was confirmed in primary human breast tumors at both the mRNA and protein level. In order to determine if CXCR4 was involved in the metastatic process, expression of its ligand, CXCL12/stromal cell-derived factor (SDF)-1 was determined in various human organs. Increased expression of SDF-1 was identified in lung, lymph nodes, bone marrow, and liver, whereas expression of SDF-1 in kidney, skin, prostate, brain, or muscle tissue was at least 4-fold less. Thus, SDF-1 expression was the highest amongst those distant organs to which breast cancer metastasizes most commonly. In fact, bone, lung, and liver are the most common sites of metastasis that were identified in approximately 60% of autopsy cases of patients with breast cancer (33, 34). Functional studies demonstrated that SDF-1 induced tumor cell migration by inducing F-actin polymerization and pseudopodia formation, and that CXCR4 blockade with a neutralizing anti-CXCR4 antibody inhibited this migration. Furthermore, administration of this antibody in an orthotopic or experimental metastatic breast cancer mouse model resulted in a 60-80% inhibition of lung metastasis. This

study provided the first evidence that the chemokine ligand/receptor pair, SDF-1/CXCR4, plays an important role in breast cancer metastasis, thereby illustrating a novel model to explain the homing of cancer cells to specific metastatic sites, sparking a new field of research in cancer metastasis (35).

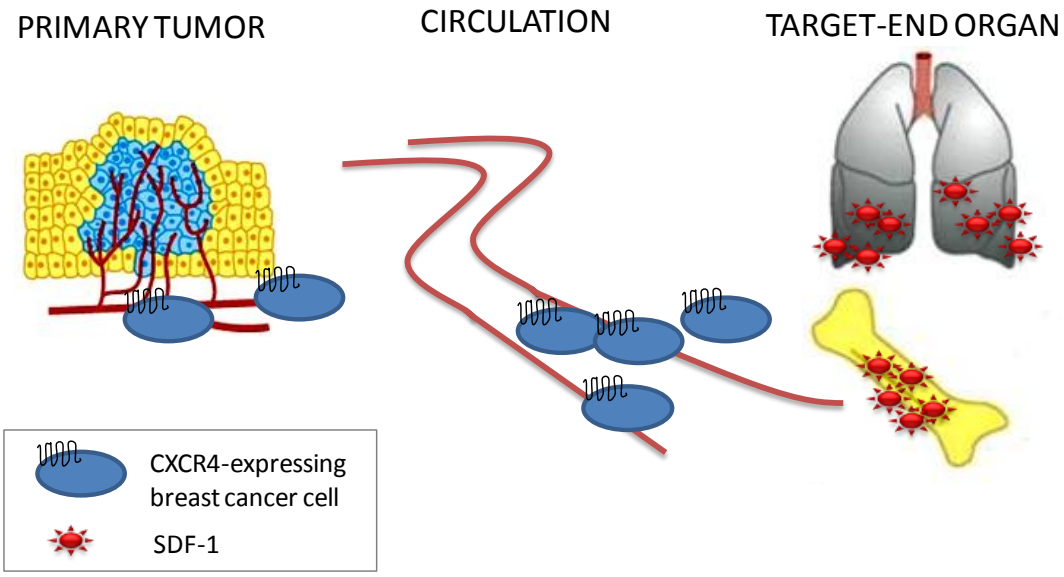


Fig. 1-4. Chemokine-Receptor model of metastasis. Modified from Murphy (36) and Weinberg (4).

1.2 Chemokines

Chemokines are chemoattractant cytokines that are proteins of low molecular weight; ranging from 6-14 kDa, these proteins are either bound to the cell membrane or secreted. Chemokines have been implicated in various physiological and pathological processes, including leukocyte migration, embryogenesis, angiogenesis, hematopoiesis, inflammation, HIV infection, and tumor growth and metastasis (37, 38). Structurally, chemokines have been classified into 4 groups based on the number of cysteine (C) and amino acid (X) residues at the amino terminal: C, CC, CXC, and CX_3C (39). CXC chemokines can be further divided into two families, based upon the presence or absence of a 3-amino acid motif (ELR), consisting of glutamic acid, leucine, and arginine, at the amino-terminal region. ELR+ CXC chemokines are angiogenic and induce proliferation and chemotaxis in endothelial cells, whereas most ELR- CXC chemokines are angiostatic

and inhibit endothelial cell migration (37, 38, 40, 41). Chemokines mediate their effect by binding to G-protein coupled receptors (GPCRs). Based on their physiological function, chemokines can be divided into two main categories: 1) inducible chemokines, as in response to inflammation and 2) homeostatic or housekeeping chemokines (42). Inducible chemokines stimulate the migration of leukocytes to an injured or infected site, and can also activate cells in an immune response or initiate wound healing. Homeostatic chemokines are involved in lymphocyte trafficking, T and B cell development, localization of T cells and B cells within lymphoid tissues and other tissues such as skin and gut (38, 43-46).

1.2.1 SDF-1

SDF-1 is a homeostatic chemokine that belongs to the family of CXC chemokines.

1.2.1.1 Structure

The *SDF-1* gene measures 88 kilo base pairs (kbps) and is located on chromosome 10q11.1. The promoter of *SDF-1*, located in the 5' untranslated region, has a large guanine-cytosine (GC)-rich domain and lacks a TATA box (short sequence of thymidine and adenine residues) which helps explain the constitutive expression of SDF-1 (47). Although *SDF-1* was originally believed to have two splice variants: *SDF-1α* and *SDF-1β* of the original *SDF-1* gene, four additional human *SDF-1* splice variants were recently identified: *SDF-1γ, SDF-1δ, SDF-1ε*, and *SDF-1ϕ* (48). The human *SDF-1α* cDNA consists of the first three exons. The human *SDF-1β* cDNA shares the same sequences as the first two exons and 87 bases of the third exon of the *SDF-1* gene, with the third exon spliced onto the fifth exon (Fig. 1-5). At the protein level, SDF-1α encodes an 89 amino acid protein, whereas SDF-1β encodes a similar protein, with 4 additional amino acids (49). SDF-1α is the most abundant isoform. SDF-1α and β are both highly expressed in the liver, spleen, and pancreas. SDF-1 γ is predominantly expressed in the heart, while SDF-1 δ, ε, and ϕ have a similar expression pattern with the highest expression in the pancreas, and moderate expression in the heart, liver, and kidney. A comparison of migrational capacity revealed that SDF-1α elicited the highest chemotactic activity in T lymphoblastic cells *in-vitro* (50).

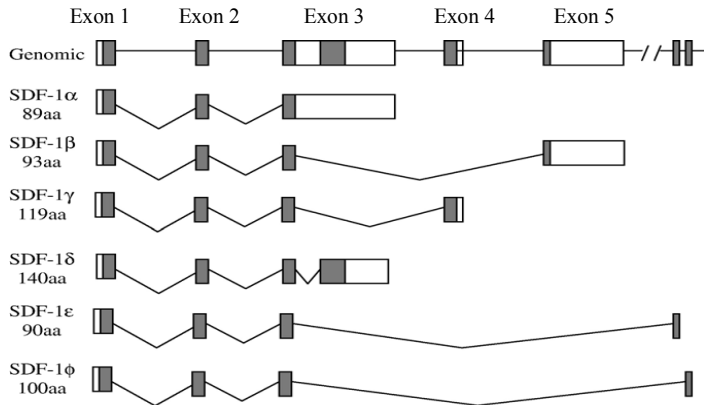


Fig. 1-5. Splice variants of *SDF-1*. Exons are numbered and indicated with the boxes. Filled in boxes are coding regions within the exons. Alternative splicing is indicated by oblique lines. Adapted from Yu et al. (48).

1.2.1.2 Allelic Variant: *SDF-1-3'A*

The SDF-1/CXCR4 ligand/receptor pair was initially studied in the context of human immunodeficiency virus (HIV). Although cluster of differentiation (CD)4 was known to be the primary receptor for HIV-1, the search for a co-factor revealed CXCR4, originally called fusin, as a required "co-conspirator" for the entry of the virus into the cell (51, 52). The role of SDF-1 was deemed to be a protective one; by binding to CXCR4 and inducing internalization, it blocked infection by HIV-1. In order to identify a mechanism that may influence efficacy of HIV-1 pathogenesis, a screening of structural genetic variants in *SDF-1* was initiated in a cohort of 2800 patients (53). A common genetic polymorphism consisting of a guanine to adenine transition was identified in the 3' untranslated region, and is referred to as *SDF-1-3'A* hereafter. The *SDF-1-3'A* allele was observed in 21% of Caucasians, and patients with the homozygous state of the polymorphism (AA genotype) were protected, with a slower progression of acquired immunodeficiency syndrome (AIDS). However, subsequent studies demonstrated contradictory results, whereby the polymorphism was associated with HIV aggressivity and a greater risk of developing non-Hodgkin's lymphoma (54-57). Although the functional significance of the *SDF-1-3'A* polymorphism is not known, recent studies have searched for an association between the polymorphism and the onset of cancer.

Interestingly, two studies have reported that women who have this polymorphism are at greater risk for developing breast cancer (58, 59).

1.2.1.3 Expression

SDF-1 is expressed in several tissues including lung, liver, lymph nodes, bone marrow, adrenal glands, pancreas, spleen, and small intestine (31, 48, 49). At a cellular level, SDF-1 is expressed in stromal cells, lymphocytes, epithelial cells, osteoblasts, pericytes, astrocytes, and endothelial cells (60, 61). Measurement of SDF-1 in blood has been performed in various medical conditions including HIV, rheumatoid arthritis, ischemic heart disease, and in the context of stem cell mobilization (62-69). Although there are a few studies which reported that patients infected with HIV have higher levels of circulating SDF-1 than normal patients, results of correlation analysis between SDF-1 blood levels and disease progression were contradictory (62, 67, 70). Patients with rheumatoid arthritis were found to have elevated plasma SDF-1 levels in comparison to healthy controls (63, 66). Patients with acute coronary syndrome were also found to have increased levels of plasma SDF-1 (68, 69). Plasma SDF-1 levels have also been measured in a few types of cancer. Although patients with multiple myeloma had higher levels of plasma SDF-1 than normal control subjects, a lower level of plasma SDF-1 was identified amongst patients with either B-cell chronic lymphocytic leukemia or colon cancer in comparison to healthy patients (71-73).

1.2.1.4 Regulation

SDF-1 protein levels are dependent upon two main processes: regulation of either protein synthesis or degradation. Little is known about the regulation of SDF-1 protein synthesis, but there have been a few studies examining its transcriptional control in patients infected with HIV. It was hypothesized that the quantity of the *SDF-1* transcript may be modulated by the presence of the *SDF-1-3'A* polymorphism, since the polymorphism is situated at the 3'untranslated region (53). Although one study did identify a positive correlation between the polymorphism and mRNA transcript expression in children with AIDS-related lymphoma (74), the relationship between the *SDF-1-3'A* polymorphism and blood levels of SDF-1 protein is less clear in other studies (62, 70). The *SDF-1* promoter has been shown to have two binding sites to hypoxia-inducible factor (HIF)-1, which,

upon binding, can increase the expression of SDF-1 (75). Other factors which can induce SDF-1 expression include nuclear factor light chain enhancer of activated B cells (NF-κB), irradiation, and the combination of phorbol 12-myristate 13-acetate (PMA) with ionomycin (47, 76). SDF-1 expression in bone marrow osteoblasts was also found to be stimulated by chemotherapeutic agents such as 5-fluorouracil and cyclophosphamide (77). On the contrary, interferon-γ was shown to block activity of the *SDF-1* promoter (47).

As a constitutively expressed chemokine, proteolytic degradation of SDF-1 plays an important role in the regulation of its function. This can be evidenced by the short half life of SDF-1 in the circulation of less than one minute (78). Several enzymes have been implicated in the cleavage of the amino- or carboxy-terminal of SDF-1 such as matrix metalloproteinases (MMPs), CD26/dipeptidylpeptidase, serine proteases, leukocyte elastase, and carboxypeptidase (79, 80). In particular, SDF-1 protein levels have also been shown to be decreased via proteolytic degradation from neutrophil elastase, which can be mediated by granulocyte-colony stimulating factor (G-CSF) (81).

1.3 G-protein coupled receptors

GPCRs are a superfamily of receptors that are involved in mediating cell-cell communication in humans. GPCRs are proteins containing seven transmembrane-spanning domains with interconnecting intracellular and extracellular loops. GPCRs can be further divided into 3 families: A, B, and C (Fig. 1-6). The largest of these is family A and includes the rhodopsin (light receptor) and α2-adrenergic receptor. Families B and C comprise the gastrointestinal peptide hormone family and metabotropic glutamate receptor family respectively, both of which possess much longer extracellular amino-termini in comparison to family A (82). Chemokine receptors belong to Family A of G-protein coupled receptors (83). These receptors have been further classified into subgroups based on the type of chemokine ligand they bind to. CXCR1 through 6, CCR1 through 11, XCR1, and CX_3CR1 bind to their respective CXC, CC, C, or CX_3C ligands (38).

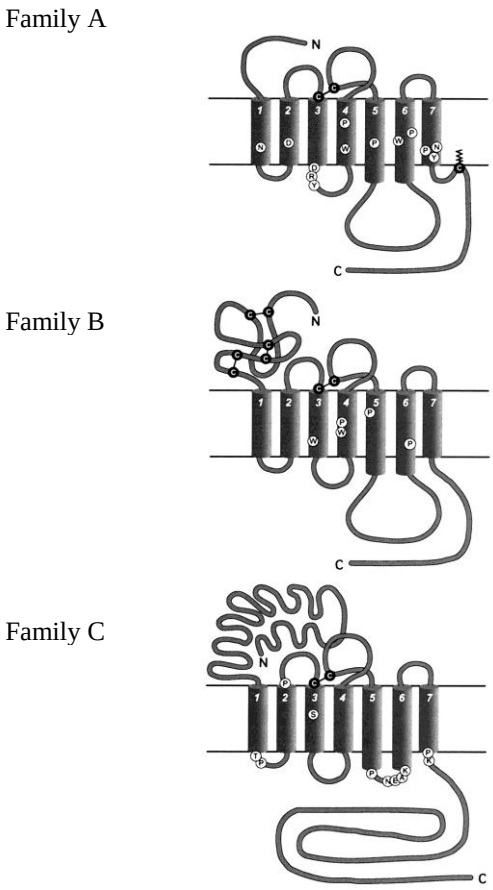


Fig. 1-6. Families of G-protein coupled receptors. N, amino terminal; C, carboxy-terminal; Black letters in white circles refer to highly conserved residues; White letters in black circles refer to disulfide bridges between the second and third extra-cellular loops; , refers to a palmitoylated cysteine; 1-7 refer to transmembrane domain number. Modified from Gether (82).

1.3.1 CXCR4

CXCR4 is a GPCR belonging to the Family A, and bears many of the typical characteristics of GPCRs with regards to ligand binding and signal activation.

1.3.1.1 Structure

The *CXCR4* gene has been localized to chromosome 2q21. It measures 8.7 kbps and is made up of 2 exons, separated by a 2.1 kb intron sequence. Sequence analysis has suggested the promoter, containing a TATA box, is located in the 5'flanking region, upstream to the *CXCR4* open reading frame (84). CXCR4 is a 43 kDa protein, and is subject to various post-translational modifications including glycosylation, sulfation, and

ubiquitination (85, 86). A few studies have demonstrated a role for glycosylation and sulfation in influencing the binding of SDF-1 to CXCR4 (87, 88).

1.3.1.2 Expression

CXCR4 is expressed on various immune cells such as peripheral blood lymphocytes, naïve T cells, monocytes, pre-B cells, plasma cells, macrophages, natural killer (NK) cells, dendritic cells, and mast cells (35). In addition, CXCR4 expression has been found in vascular smooth muscle cells, endothelial cells, thymocytes, splenocytes, microglia, neurons, astrocytes, kidney and tubular cells, and epithelial cells originating from the retina, colon, breast, prostate, and lung (35, 60, 89-93).

1.3.1.3 Regulation

At the genetic level, *CXCR4* expression is regulated in various tissues by transcription factors (94) that bind to the *CXCR4* promoter (76). Expression of CXCR4 has been shown to be upregulated by transcription factors related to tissue damage, stress, or hypoxia such as interleukin (IL)-2, IL-4, or IL-7, NF-κB, glucocorticoids, transforming-growth factor-β1 (TGF-β1), interferon(IFN)-α, HIF-1, and vascular endothelial growth factor (VEGF) (76, 95). Factors which have been shown to inhibit SDF-1/CXCR4 signaling include lipopolysaccharide (LPS), heparin, macrophage inhibiting protein-1 (MIP-1)-α, RANTES (also known as chemokine ligand 5 (CCL5)), and pharmacologic agents (eg. amphotericin B, nystatin, statins) (76, 96). CXCR4 protein activity has also been shown to be regulated by proteolytic activity at the N-terminus. Cleavage of the N-terminal via leukocyte-derived proteases released in inflammation and N-terminal hyposulfation, have been shown to decrease CXCR4 function (96, 97).

1.3.1.4 Functional role of the SDF-1/CXCR4 axis

1.3.1.4.1 Binding of SDF-1 to CXCR4

The binding of SDF-1 to CXCR4 has been proposed as a 2-staged process: the initial "docking step" consists of the binding of amino acids 12-17 of SDF-1 to the N-terminal of CXCR4, followed by binding of the first 11 amino acids of SDF-1 to the extracellular

groove of CXCR4. Binding of the ligand leads to the activation of G proteins (guanine nucleotide binding proteins) – heterotrimers, consisting of α, β, and γ subunits, which then leads to a conformational "switch" in the receptor; the α-subunit releases guanosine diphosphate (GDP), which is then exchanged for guanosine triphosphate (GTP) (Fig.1-7A). The resulting GTP-bound α subunit dissociates from the Gβγ subunit to regulate downstream cytoplasmic enzymes. Adenylyl cyclase is inhibited via the Gα subunit; phospholipase C and phosphatidylinositol 3-kinase (PI3K) are activated by the Gβγ dimer (4, 98-101). As the G-proteins are released, G-protein coupled receptor kinases (GRKs) mediate phosphorylation of the receptor, promoting calcium flux (102-104). Phosphorylation of CXCR4 has been shown to involve two specific motifs - a dileucine motif and a serine motif at positions 338 and 339 (105). Phosphorylation at the cytoplasmic tail subsequently promotes the recruitment of both β-arrestin and adaptin-2 which further recruit clathrin (Fig. 1-7B). Clathrin-coated pits are created, which "pinch off" from the membrane to become vesicles. Uncoating of the clathrin is then associated with entry of the vesicle into the early endosomal compartment. The ligand/receptor pair can subsequently enter the perinuclear compartment and recycle back to the plasma membrane, or it can be further sorted to the lysosomal compartment, where it is mono-ubiquitinated and subsequently degraded (102, 106, 107). Of note, although the functional significance of nuclear CXCR4 has not been described to date, the prognostic significance of nuclear CXCR4 has been reported in several cancers including breast, lung, and colon (89, 108, 109). Following receptor-internalization, the mechanisms underlying the ultimate fate of the receptor (lysosomal degradation versus membrane recycling) are not well understood. Although ubiquitination, mediated by the E3 ubiquitin ligase atrophin-1 interacting protein 4 (AIP4), has been shown to be required for lysosomal degradation (110), recycling of CXCR4 to the plasma membrane appears to be an inefficient phenomenon with less than 30% of the receptor being recycled at best (111).

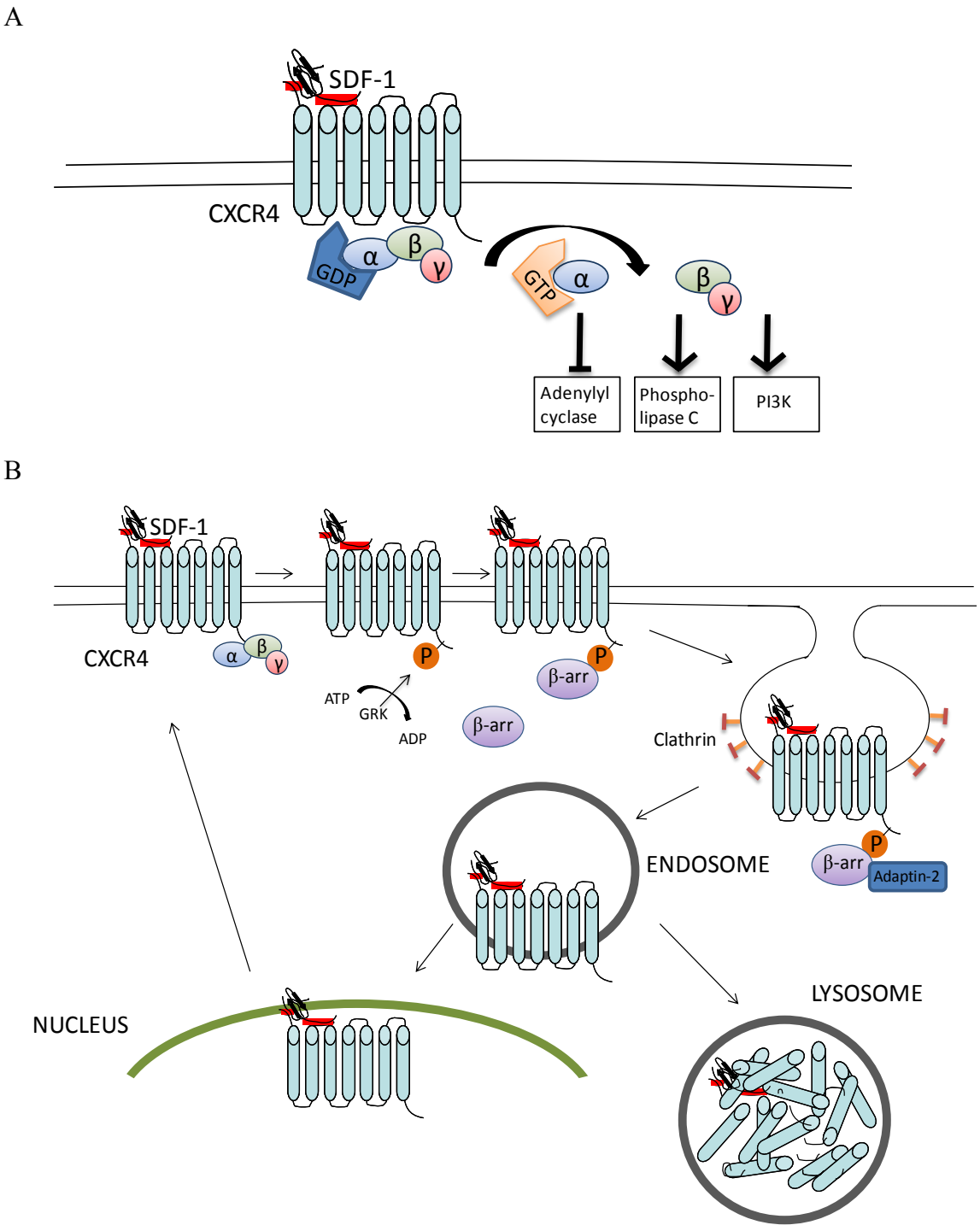


Fig. 1-7. Binding of SDF-1 to CXCR4. *A*, G protein activation and *B*, Receptor internalization. Modified from Hanyaloglu and Zastrow (112).

1.3.1.4.2 Physiologic role

Cancer can be viewed as a process resulting from an evolution or progression from normal cells, or, conversely, as a dysfunctional exploitation of pre-existing physiological processes (4). For example, is vessel formation or cell migration seen in embryonic development similar to that observed in neoangiogenesis and tumor invasion? Furthermore, many similarities in the signaling processes of wound healing and tumor progression have been identified, suggesting the presence of a pre-existing biological program that can be used by the tumor. Hence, a brief review of the physiological role of the SDF-1/CXCR4 axis will precede the discussion of its role in cancer.

1.3.1.4.2.1 Embryonic development

Earlier studies revealed that mice deficient in either SDF-1 or CXCR4 demonstrated a similar phenotype (113-116). By day 17.5, the majority of these knockout mice died (116) secondary to a lack of myelopoiesis in the bone marrow and defects in B lymphocytes, ventricular septum formation in the heart, neuron migration in the cerebellum, or generation of large vessels supplying the gastrointestinal tract (46, 61). The embryonic development of zebrafish is also a model that can be used to study SDF-1 migratory function. The formation of the mechanosensory system consists of the migration of the primordium that deposits seven to eight sense organs called neuromasts along the posterior lateral line (horizontal myoseptum). SDF-1 is present along the pathway that the primordium migrates, and CXCR4 is expressed by the primordial cells themselves. Inactivation of either SDF-1 or CXCR4 results in impaired migration of the primordium with deposition of none to a few neuromasts, suggesting that both SDF-1 and CXCR4 are essential for the development of the posterior lateral line (117, 118).

1.3.1.4.2.2 Bone marrow

SDF-1 was originally characterized in the stroma of the bone marrow as a pre-B cell growth stimulating factor (119). Although a mitogenic effect for SDF-1 was initially described in pre-B cells, subsequent studies demonstrated contradictory results (120, 121). However, the function of SDF-1 as a regulator of stem/progenitor cell trafficking has been better described. The differential concentration gradient of SDF-1 (high SDF-1 in bone marrow, and low SDF-1 in plasma) serves to home in and maintain CXCR4-

expressing stem/progenitor cells in the bone marrow (Fig. 1-8A). Degradation of SDF-1 within the bone marrow by G-CSF decreases the chemotactic force of SDF-1, promoting the egress or mobilization of progenitor cells from the bone marrow to the circulation (Fig. 1-8B) (96). This mobilization is reduced with administration of an SDF-1 or CXCR4 neutralizing agent, suggesting that the concentration gradient of SDF-1 between the bone marrow and circulation is an important determinant of SDF-1 function. The role of SDF-1 in the bone marrow has been exploited therapeutically in the clinic. A CXCR4 antagonist is currently being investigated in a Phase II clinical trial in combination with G-CSF to enhance the mobilization of stem cells into the circulation for potential use in stem cell transplantation (122).

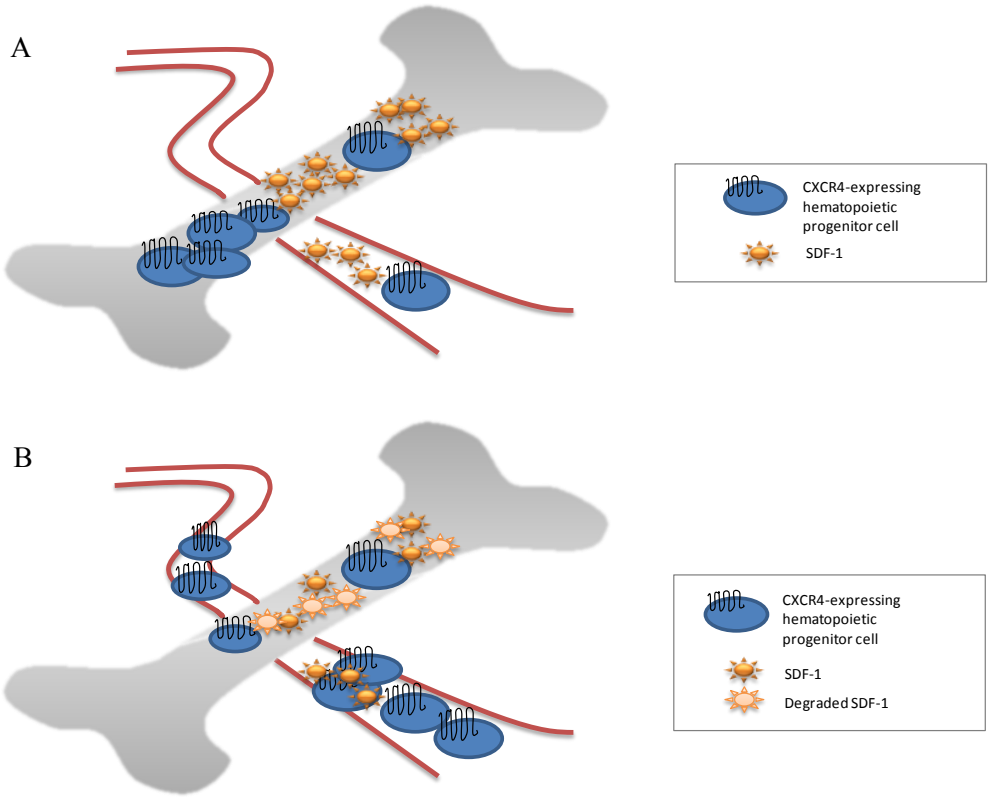


Fig. 1-8. The role of SDF-1 in the bone marrow. *A,* The chemoattractant force of SDF-1 within the bone marrow assists to maintain CXCR4-expressing hematopoietic progenitor cells within the bone marrow. *B*, Administration of G-CSF induces SDF-1 degradation which decreases the chemoattractant ability of SDF-1 and results in the mobilization of CXCR4-expressing hematopoietic progenitor cells to the circulation.

1.3.1.4.2.3 Physiologic angiogenesis

Although lacking the ELR-motif, SDF-1 has been shown to be expressed on endothelial cells and is involved in inducing calcium mobilization, stimulating VEGF secretion, and inducing capillary tube formation (123-125). SDF-1-induced endothelial cell proliferation and chemotaxis have also been described in various studies (126-128). On the other hand, a decrease in SDF-1 expression by tumor necrosis factor (TNF)-α and IFN-γ results in impaired endothelial cell tube formation and angiogenesis (129). Expression of SDF-1 and CXCR4 on endothelial cells has been shown to be promoted by basic fibroblast growth factor (bFGF), VEGF, and HIF-1 via hypoxia (61, 75, 95, 128).

1.3.1.4.2.4 Tissue repair and inflammation

Tissue ischemia has been shown to induce an increase in the expression of SDF-1 in organs such as heart, kidney, or diabetic retina, leading to mobilization and recruitment of progenitor cells to the ischemic site (75, 90, 130-132). One plausible explanation stems from hypoxia induced HIF-1 expression which upregulates SDF-1 expression, promoting homing and engraftment of CXCR4+ endothelial progenitor cells (75). The concept of SDF-1 mediating the recruitment of progenitor cells to an ischemic region has aroused much interest from a therapeutic perspective. Recent studies have found that the administration of SDF-1 in ischemic hearts results in a decrease in infarct size and scar formation, increasing VEGF levels and angiogenesis, and ultimately improving cardiac function in mice (133-135). In summary, promotion of the recruitment of progenitor cells via SDF-1 may be one of the means of facilitating tissue repair.

Unlike other chemokines, the role of SDF-1 in inflammation is less understood. SDF-1 was originally described as a homeostatic chemokine whose expression remained unchanged in inflammatory conditions (136, 137). Although SDF-1 is involved in lymphocyte trafficking and HIV infection, its role in the inflammatory process itself has only been shown in a few studies (35). For instance, in rheumatoid arthritis patients, SDF-1 may be responsible for homing and maintaining CXCR4-expressing CD4+ memory T cells in the rheumatoid arthritis synovium (138). In addition, SDF-1 expressed in the allergic inflamed lung recruits inflammatory leukocytes that express CXCR4, to the

lung. The inflammatory response was also shown to be reduced by neutralizing antibodies of either CXCR4 or SDF-1 (139).

1.3.1.4.3 Role of SDF-1/CXCR4 axis in cancer

1.3.1.4.3.1 Role in metastasis

Analogous to the physiologic role of SDF-1 as a chemoattractant to home in CXCR4-expressing cells to the bone marrow or sites of inflammation, the chemokine-receptor model was proposed to help explain the process of breast cancer metastasis. Muller et al. proposed that the overexpression of SDF-1 in those organs to which breast cancer metastasizes, such as lungs, liver, bone marrow and lymph nodes, serves to home in CXCR4-expressing breast cancer cells from the circulation (31). Blocking CXCR4 activity has been shown to inhibit distant metastasis using different CXCR4 antagonists. Administration of a neutralizing anti-CXCR4 monoclonal antibody in a xenograft model resulted in a marked suppression of lung metastasis in both experimental and orthotopic metastasis models (31) . A similar reduction in metastases was also demonstrated by another group, whereby treatment with a small molecule inhibitor of CXCR4, called TN14003, was started immediately before peripheral injection of a human breast cancer cell line (140). Furthermore, inhibition of metastasis using small interfering RNA (siRNA) knockdown of CXCR4 was also shown to prolong survival in mice. Whereas all the control mice died at 56 days post tumor implantation due to metastatic disease, no evidence of lung metastasis was apparent in the mice with tumors transfected with CXCR4-siRNA, when followed for 90 days post implantation (141). Therefore, CXCR4 blockade reduces distant metastasis and prolongs survival in mice.

In order to better understand the mechanism by which the SDF-1/CXCR4 axis may be implicated in metastasis, *in-vitro* studies have been carried out to assess the role of SDF-1 stimulation in cell adhesion, invasion, and chemotaxis. Various signaling pathways have been implicated in these processes and only those that have been described in cancer cell lines are mentioned below (Fig. 1-9). SDF-1 promotes the adhesion of cancer cells to other cell types, such as endothelial and stromal, and components of the extracellular matrix, including fibronectin, laminin, collagen type I and IV (142, 143). SDF-1 induced adhesion has been shown to be mediated by integrins

(subunits α2, α4, α5, β1), proteins involved in the assembly of integrin complexes called the Rho family of small GTPases (Rho kinase, RhoA, and Rac1), integrin-linked kinase (ILK), focal adhesion kinase (FAK), and paxillin (144-146). The SDF-1/CXCR4 axis is also involved in cell invasion and migration through stimulation of MMPs which degrade the extracellular matrix, activation of FAK, related adhesion focal tyrosine kinase (RAFTK)/Pyk2, Rac, Rho, PI3K, and the Janus Kinase (JAK)/STAT pathway (60, 147, 148). Further downstream, activation of AKT and the mammalian target of rapamycin (mTOR) signaling pathway in addition to Src kinase have also been implicated in mediating migration in response to SDF-1 stimulation (149-151). Activation of VEGF, NFκB, and the mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways, were also observed during SDF-1 induced cell migration (152-154).

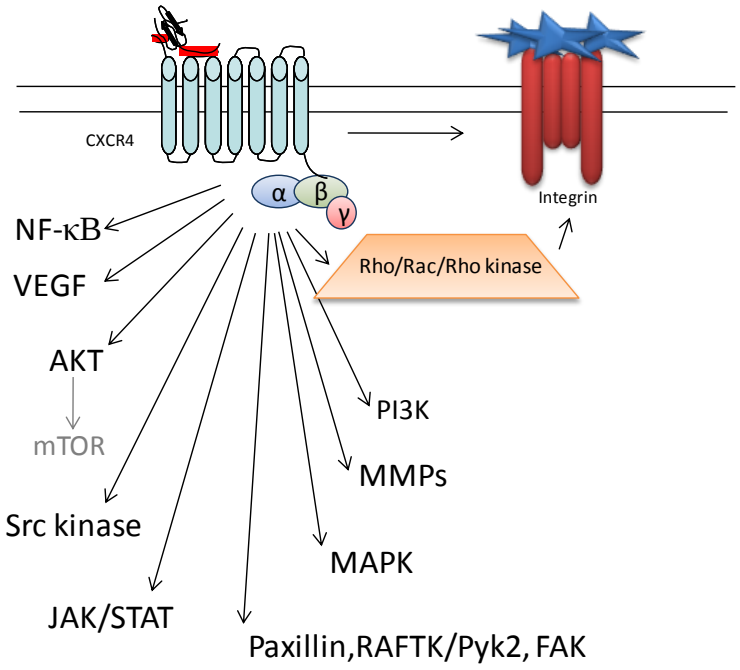


Fig. 1-9. Signaling pathways implicated in SDF-1 mediated cell adhesion, invasion, and migration in cancer. Modified from ref. (155).

The expression of CXCR4 has been identified in various primary cancers such as prostate, renal cell, osteosarcoma, esophageal, colorectal, nasopharyngeal,

neuroblastomas, head and neck (35). Several studies have reported correlations between CXCR4 expression and either local or distant metastasis, yet what has not been established is whether increased CXCR4 activity is actually responsible for the formation of metastasis in human cancers. The fact that CXCR4 expression has been shown to be enriched at the metastatic site compared to its expression at the primary tumor site supports the hypothesis that CXCR4 activity may be required for metastasis in a few different cancers (156-158). Moreover, activated CXCR4 was able to discriminate invasive potential amongst a panel of breast cancer cell lines with uniform expression of CXCR4 (159). Kang et al. also demonstrated that the invasive and migratory potential of a CXCR4-expressing breast cancer cell line was augmented upon overexpression of SDF-1 via stable transfection, suggesting that endogenous SDF-1 may also influence the metastatic capacity of CXCR4-expressing breast cancer cells (160, 161).

The SDF-1/CXCR4 axis has also been shown to play an important role in both the homing of cancer cells specifically to the bone and colonization of cancer cells at the metastatic site. Due to the prominent role of SDF-1 in the trafficking of progenitor cells to the bone marrow, it appeared plausible that CXCR4-expressing cancer cells may have a predilection to metastasize to the bone. Animal experiments have shown that a specific clonal population of breast cancer cells that metastasized rapidly to the bone within five weeks highly expressed three genes including CXCR4 (162). In a cohort of breast cancer patients, CXCR4-expressing tumors demonstrated a higher risk of developing bone metastasis (163). Moreover, in prostate cancer, CXCR4 was responsible for sublocalizing cancer cells to the metaphysis of the bone, the area where growth plates form (164). CXCR4 has also been shown to be involved in the proliferation of tumor cells at the metastatic site. Colon cancer cells lacking functional CXCR4 were able to colonize the lung, but failed to proliferate from micrometastasis to macrometastasis (165). Furthermore, CXCR4 abrogation reduced the number of metastatic lesions when prostate cancer cells were injected directly into the bone (164). Therefore, this is suggestive that CXCR4 blockade may play an important role in inhibiting macrometastasis and micrometastasis.

1.3.1.4.3.2 Role in primary tumor

In addition to its role in metastasis, CXCR4 has also been implicated in primary tumor growth. CXCR4 has been shown to be overexpressed in several primary tumors, including breast (35), and progressively increasing expression of the receptor has been found to correlate with tumor progression. Our laboratory found that CXCR4 expression increased progressively from normal breast epithelial glands to atypical ductal hyperplasia, to ductal carcinoma in situ, and finally to 67% of invasive primary breast cancers, suggestive that CXCR4 is also involved in the earlier stages of tumor progression (89). Furthermore, CXCR4 blockade with siRNA has been shown to slow the rate of tumor development, with at least a two-fold delay in onset of breast cancer in animal models (141). A persistent inhibition of primary tumor growth was also observed after 44 days in neuroblastoma after CXCR4 knockdown with short hairpin RNA (shRNA) (166). Small molecule inhibitors against CXCR4 also demonstrated efficacy in reducing primary tumor volume in brain tumors (167). Analogously, CXCR4 stimulation was also shown to promote tumor growth in neuroblastoma, prostate, and breast cancer (166, 168, 169).

The mechanisms by which CXCR4 mediates primary tumor growth include regulation of angiogenesis and cell survival. Inhibition of CXCR4 activity in breast cancer has been associated with a reduction of microvessel density. In prostate cancer, CXCR4 blockade also resulted in a decrease in vessel perfusion, leading to the development of tumor necrosis after the tumor reached a diameter of 10 mm^3 (168). *In-vitro* and *in-vivo* studies showed that SDF-1 can induce VEGF expression, and stimulate endothelial tube formation (170, 171). Interestingly, SDF-1 overexpressed in cancer-associated fibroblasts from the tumor microenvironment was shown to play an important role in the recruitment of endothelial progenitor cells (EPCs) to induce angiogenesis and promote tumor growth in breast cancer (169). In addition, overexpression of CXCR4 has been shown to augment microvessel density, vessel perfusion, and oxygen delivery efficiency in prostate cancer (168).

Inhibition of primary tumor volume has also been shown to be mediated by SDF-1 via an increase in apoptosis and a decrease in cellular proliferation in glioblastoma and medulloblastoma mouse models. SDF-1 positively regulated the activity of AKT, ERK1/2 and negatively influenced the expression of cyclic adenosine 5'monophosphate

(cAMP) in these tumors (172, 173). In addition, SDF-1 promoted the proliferation of hepatoma cells through the c-jun NH_2-terminal kinase/stress-activated protein kinase (JNK/SNAP) pathway and by triggering quiescent Go cells to enter the cell cycle (174). Proliferation of endometrial cancer cells or meningioma cells was mediated through SDF-1 induced activation of ERK1/2 and/or AKT *in-vitro* (175, 176). Furthermore, estradiol has also been implicated in stimulating SDF-1-mediated cellular proliferation of breast cancer cells *in-vitro* (177, 178). Microarray studies have also demonstrated that SDF-1 is one of the few genes that are significantly induced by estradiol (179, 180).

1.3.2 CXCR7

SDF-1 and CXCR4 were originally reported as a monogamous ligand/receptor pair since deletion of either SDF-1 or CXCR4 in mouse embryos led to similar phenotypes. More recently, a radioligand binding assay found that CXCR7, another G-protein coupled receptor, also binds with high affinity to SDF-1. In zebrafish embryo development, the formation of the posterior lateral line was impaired with knockdown of CXCR7 (181, 182). Deficiencies in both CXCR4 and CXCR7 resulted in a much greater impact upon cell migration than either receptor alone (183-185).

Recent studies have been published addressing the functional role of CXCR7. Although *in-vitro* studies have found that CXCR7 itself does not induce a calcium flux or activate ERK/AKT, heterodimerization of CXCR4 with CXCR7 was found to enhance SDF-1 activity (186-188). In animal models, overexpression of CXCR7 promoted primary tumor growth and metastasis in a breast and lung cancer model, and CXCR7 blockade inhibited primary tumor growth (189). In addition to the epithelial cancer cells, expression of CXCR7 has been identified in tumor-associated vessels. CXCR7 can also regulate the expression of IL-8 and VEGF, two pro-angiogenic factors, and expression of AKT in prostate cancer (190). Therefore, evidence is emerging to suggest that CXCR7 is an important player involved in SDF-1-mediated activity in cancer.

1.4 Pre-clinical testing of therapeutic targets and mouse models

With recent advances in the mapping of the human genome and gene expression technology, there has been an emergence of a huge number of molecular targets that not

only require clinical validation, but also rapid pre-clinical testing for those that are druggable. There are several mouse models that are available to study the process of metastasis; the two main types are xenografts and genetically engineered mice. Xenograft models consist of the transplantation of human cancer cell lines within an immunocompromised mouse. Metastatic xenograft models can be either spontaneous or experimental. Spontaneous metastasis models are either derived from an ectopic or orthotopic implantation of cancer cell lines. In ectopic implantation, cancer cells are injected subcutaneously and progress to metastasis infrequently, while in orthotopic implantation, cancer cells are implanted within its original tissue of origin. With a more favourable microenvironment, tumors in orthotopic models tend to grow faster than their ectopic counterparts, and have demonstrated greater similarities with human tumors in terms of histology, vascularity, and metastatic potential. Experimental mouse models of metastasis entail injection of a cancer cell line peripherally, such as through a tail vein, which then colonize at the distant metastatic site, such as lungs, for instance (191, 192). Thus, orthotopic models allow for the study of metastasis from primary tumor growth to cancer cell dissemination to distant metastasis development, whereas the experimental metastasis model only examines the later stage of metastasis, that is, from the propagation of cancer cells through the circulation to the formation of metastasis.

There are a few drawbacks inherent in the use of xenograft models. First, xenograft models use immunocompromised mice wherein the biology of tumor growth and progression may be different from immunocompetent mice. Second, cancer cell lines are often acquired from metastatic sites and are thus in the later stages of tumor progression. These cell lines may be further modified/selected *in-vitro* through numerous passages prior to being implanted in mice. Therefore, tumors that are grown *in-vivo* from cancer cell lines are biologically different from those that develop in humans. This has been evidenced histologically with differences in tumor architecture and stromal content observed in tumors obtained from patients versus those obtained from xenograft models (4).

Such differences in tumor biology may be part of the reason for which xenograft models have not been able to predict clinical efficacy of therapeutic agents with great accuracy. One review reported that only 15% of agents tested demonstrated some activity

in Phase II clinical trials (193). Furthermore, about 60% of oncologic agents have demonstrated failure at Phase II trials or later, suggesting a need for improvement in pre-clinical testing. As a result, genetically engineered mouse models are now being considered for pre-clinical testing of therapeutic agents. These mice are not only immunocompetent, but also have genetically-driven tumors that progress from primary tumor to metastasis formation in a manner akin to human tumor progression (191, 194). Nonetheless, genetically engineered mice have not been used very commonly for preclinical testing to date mostly due to technical challenges. Variable penetrance (frequency at which a specific genotype is expressed), heterogeneity in tumor development, long latencies from tumor onset to metastasis development, and difficulties in generating large numbers of mice for chemosensitivity testing, are some of the reasons for their decreased popularity. Indeed, only a few models, such as the Murine Mammary Tumor Virus-driven Polyoma Middle-T Antigen (MMTV-PyMT) and the RIP-Tag (rat insulin promoter-large SV40-T antigen) model of pancreatic cancer have a high rate of penetrance, and develop synchronous tumors quickly within two to three months (195-197). In summary, although xenograft models may be easier to use for preclinical testing, transgenic mice offer a greater advantage in terms of similar tumor biology to human tumors, and warrant further use as pre-clinical models.

1.4.1 SDF-1/CXCR4 as a therapeutic target in breast cancer

1.4.1.1 Single agent-targeted therapy

1.4.1.1.1 CXCR4 antagonists

The role of CXCR4 in metastasis and primary tumor growth suggests that CXCR4 has great potential as a therapeutic target. Animal studies have used 3 different modalities to antagonize CXCR4 activity in order to assess inhibition of metastasis or primary tumor growth: a neutralizing antibody, anti-CXCR4 siRNA, and small molecule or peptide inhibitors. Muller et al. first demonstrated the significance of the chemokine receptor model using a neutralizing mouse antibody (31). However, since neutralizing antibodies tend to require pre-mixing with the antigen (cancer cells) to demonstrate efficacy, and a mouse antibody requires chimerization or humanization prior to use in patients, such an

antibody is not yet developed for current use in the clinical setting. Inhibition of tumor growth was also shown in mice by decreasing CXCR4 expression via siRNA (141). Although siRNAs are a good experimental tool which can be transfected in a cancer cell line to be injected *in-vivo*, such an approach cannot be used in patients who present to the clinic with either a radiologically apparent or palpable mass. Moreover, several approaches are currently being investigated to optimize delivery of siRNA *in-vivo*, yet serum stability and off-target effects still remain as some of the concerns regarding *in-vivo* use of siRNA (198). In addition, there are several families of CXCR4 small molecule inhibitors and peptide antagonists that have been recently identified, but I will limit my discussion to the three most studied: AMD3100, the family of T22 and its analogue derivatives, and CTCE-9908.

1.4.1.1.1.1 AMD3100

AMD3100 is a synthetic organic compound belonging to the family of heterocyclic compounds. It is a bicyclam, non-peptide, CXCR4 antagonist (199, 200). The specificity of AMD3100 was demonstrated via several approaches including lack of interaction with other chemokine receptors and inhibition of CXCR4 mediated calcium flux and chemotaxis (201, 202). AMD3100 binds to the second and third extracellular loops and residues of the transmembrane helix of CXCR4 (203, 204). In the context of inhibiting HIV progression, preclinical trials provided evidence for its efficacy in reducing viral load, however, skepticism arose regarding its use when partial agonist activity of AMD3100 was also demonstrated (205). Phase I clinical trials revealed toxicity of AMD3100: cardiac arrhythmias, dose-dependent leukocytosis, and gastrointestinal complaints. These toxicities together with lack of oral bioavailability limited further clinical investigations (206, 207).

1.4.1.1.1.2 Family of T22 and its analogue derivatives

Another family of CXCR4 peptide inhibitors investigated in both the HIV context and in cancer is T22 and its analogues (e.g. T140, TC14003, and TN14003) which originate from polyphemusim II, isolated from horseshoe crab hemocytes (208). T140 binds to transmembrane domains 4 and 5 of CXCR4 (209). One of the derivatives of T140 is TN14003, a 14-residue peptide, found to be stable in serum, which has demonstrated

efficacy in inhibition of metastasis and primary tumor growth in breast cancer (140, 210). Although efficacy of this agent was also shown in other cancers such as pancreas and head and neck, this family of peptides has not been tested in clinical trials to date, and so their safety in humans is yet to be determined (211, 212).

1.4.1.1.1.3 CTCE-9908

A group of recently developed CXCR4 antagonists are the SDF-1-derived peptides, of which CTCE-9908 is the best member of this group. As a peptide inhibitor, there are several advantages and disadvantages of this therapeutic class that need to be taken into consideration. In comparison to small molecule inhibitors, peptides tend to be highly specific and have less potential to cause adverse effects. Peptides often mimic endogenous proteins, and are thus less likely to induce an immune response. On the contrary, protein solubility, serum stability, and metabolism are important factors which may limit the efficacy of such an agent. Enzymatic degradation of protein and protein half-life are contributing factors in determining frequency of administration. Moreover, although peptides can be administered subcutaneously or intravenously, oral administration is much more difficult due to enzymatic breakdown in the stomach (213). Therefore, despite the success of several peptide therapeutics including insulin, coagulation factors, and metabolic enzymes, there are several challenges in the development of such therapeutic agents.

CTCE-9908 consists of a dimer of the first 8 amino acids of SDF-1, with a proline to glycine modification at the second amino acid. This dimer was reported to have a lower binding affinity to CXCR4 compared to native SDF-1, with no detectable induction of chemotaxis. The binding site for the CTCE-9908 dimer has not been identified to date (214, 215). Functional experiments have revealed efficacy of CTCE-9908 in inhibition of cell invasion, migration, and metastasis in xenograft models of osteosarcoma and melanoma. However, in all these experiments, cancer cells were either pre-incubated with the CXCR4 inhibitor or injected into animals after a dose of the compound was administered (142). Thus, the efficacy of CTCE-9908 in a more clinically applicable setting (such as when tumor growth has already begun) is yet to be determined. A single dose Phase I clinical trial showed safety of CTCE-9908 in normal volunteers, and in a

recently completed Phase I/II clinical trial in metastatic patients, no major or minor adverse toxicity was observed except for phlebitis (216). In comparison to other CXCR4 antagonists, CTCE-9908 offers the advantage of having demonstrated safety in patients. Therefore, CTCE-9908 is a promising small molecule competitive inhibitor that requires further pre-clinical testing as an anti-cancer therapeutic.

1.4.1.2 Combination therapy

1.4.1.2.1 Rationale

Blocking CXCR4 activity appears to have great potential in inhibiting tumor growth and metastasis by interfering at various levels of tumorigenesis including cell survival, angiogenesis, invasion, and metastasis. Yet most chemotherapeutic agents, whether cytotoxic or the molecularly targeted ones, have been used in the clinic in combination with other agents in an effort to combat distinct key cellular functions, overcome tumor heterogeneity or pre-empt the emergence of resistant cells to therapy. Different strategies have been employed when devising appropriate combinations in anti-cancer therapy.

Therapeutic agents can be combined to attack the function of a single molecular target. For example, 17-allylamino demethoxygeldanamycin (AAG), an inhibitor of a chaperone protein, heat shock protein 90 (HSP90), blocks the interaction of HSP90 with other proteins, such as HER2, which renders the combination of trastuzumab and 17-AAG a plausible one in inhibiting tumor growth. A more common approach is to hit sequential steps in a single molecular pathway. Various studies have examined the effect of combining upstream growth factor receptors such as epidermal growth factor receptor (EGFR) or HER2 in combination with downstream mediators of the PI3K pathway such as PI3K itself or mTOR in order to enhance the efficacy of knocking down this molecular pathway. Furthermore, anti-cancer therapies can be combined to target two different functional processes. For example, the combination of an anti-angiogenic agent and an agent designed to inhibit cell survival may serve to complement or potentiate one another and thus result in an additive or synergistic effect (217, 218). Preliminary studies with CXCR4 antagonists in combination with other agents have generated interesting results. *In-vitro* studies of either AMD3100 or TC14003 in acute lymphoblastic leukemic cells, revealed that the combination of either agent with dexamethasone or vincristine resulted

in a greater inhibition of cell proliferation or cell viability than with either agent alone (219). In addition, the combination of AMD3100 and BCNU, 1,3-bis(2-chloroethyl)-1-nitrosurea - a cytotoxic chemotherapeutic agent, was shown to inhibit proliferation of glioma cells *in-vitro* and *in-vivo* in a statistically significant greater degree in comparison to either agent alone (220). This suggests that targeting anti-CXCR4 activity has the capacity to potentiate inhibition of cell survival in combination with other therapeutics. The rationale for combining a CXCR4 antagonist with three other anti-cancer therapies is summarized below.

1.4.1.2.2. Anti-HER2 agent

HER2, an epidermal growth factor receptor, is known to activate the AKT/PI3K pathway, resulting in an increase in cell proliferation, anchorage independent growth, *in-vivo* tumor growth and metastasis development (221). Trastuzumab (Herceptin^{TM}), a humanized monoclonal antibody, was approved by the Food and Drug Administration (FDA) in 1998, and has found its place in the clinic potentiating the effect of cytotoxic chemotherapies in the treatment of breast cancer (222-224). There are several reports showing a cross-talk between HER2 and CXCR4, providing rationale for inhibiting both of these molecular targets in combination. High expression of HER2 has been shown to be important for SDF-1 induced invasion, adhesion, migration, and formation of metastasis *in-vivo* (225). SDF-1 transactivates HER2, with CXCR4 blockade inhibiting HER2 phosphorylation (150). On the contrary, CXCR4 expression is also enhanced by HER2, since HER2 can protect CXCR4 from ligand-induced ubiquitination and subsequent degradation (225) (Fig. 1-10). Furthermore, the significance of the cross-talk between CXCR4 and HER2 has been demonstrated in a large cohort of breast cancer patients whereby a strong positive correlation was identified between CXCR4 expression and either HER2 expression or amplification (89). This suggests that knockdown of HER2 activity may compromise CXCR4 function by promoting CXCR4 turnover and limiting the metastatic capacity of CXCR4, whereas blocking of CXCR4 activity may inhibit HER2 activity. Hence, the combination of CXCR4 and HER2 inhibition may potentiate the knockdown of CXCR4 function by targeting the AKT/PI3K pathway from two different upstream membrane receptors.

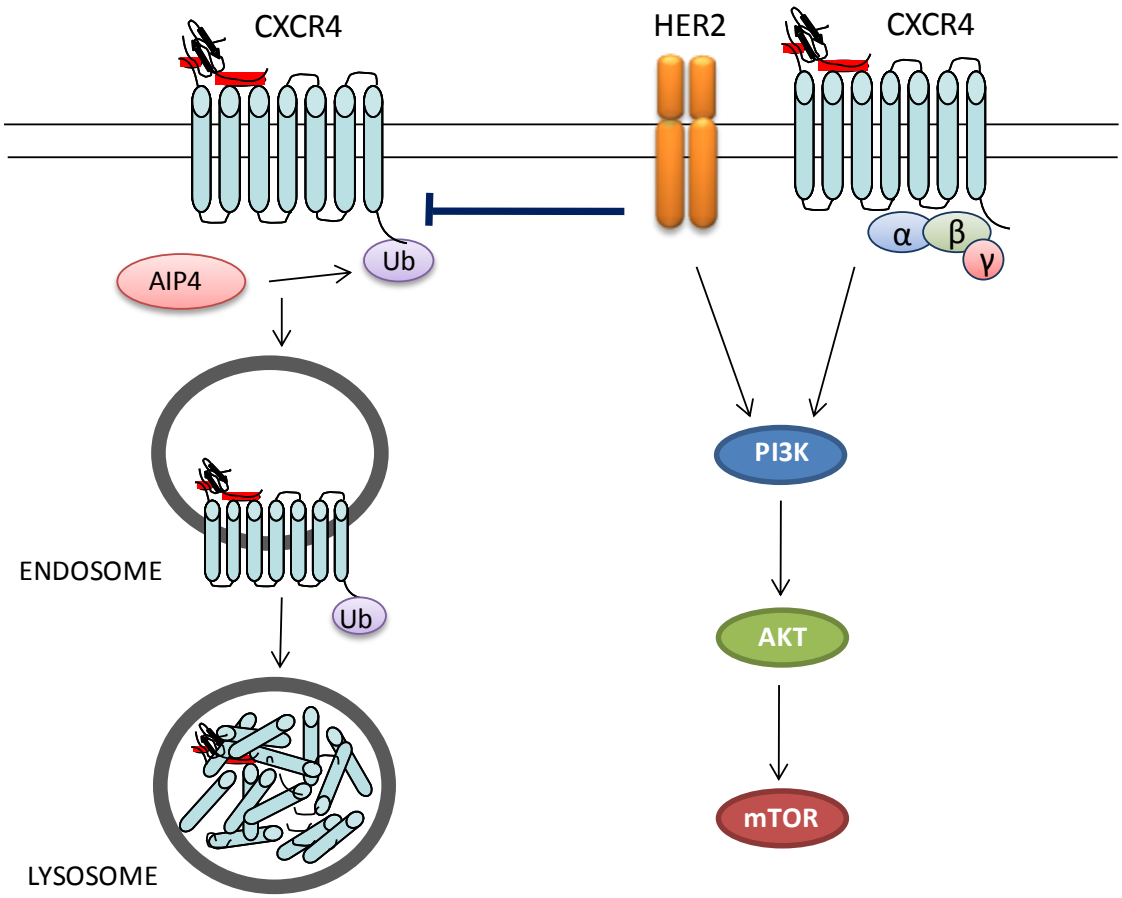


Fig. 1-10. Cross-talk between HER2 and CXCR4. Modified from Li et al. (225).

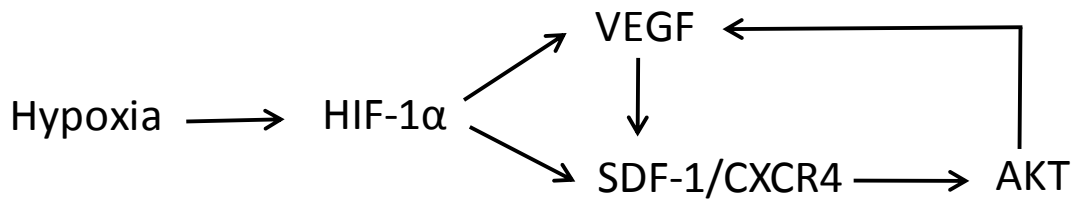
1.4.1.2.3 Anti-angiogenesis agent

There are several angiogenesis inhibitors targeting the VEGF pathway that have demonstrated efficacy in pre-clinical models and in cancer patients. The VEGF pathway inhibitors can be classified into ligand-trapping inhibitors (eg. bevacizumab), receptor inhibitors (eg. DC101 which targets VEGFR2), and tyrosine kinase inhibitors (eg. sorafenib, and sunitinib) (226). Although efficacy of such agents can be evidenced by the rapid approval of bevacizumab, sorafenib, and sunitinib by the FDA, their success in the clinic has been somewhat disappointing. Variations in efficacies have been observed when these agents were administered as monotherapy or in combination with cytotoxics, in different clinical settings, and with different tumor types (227). Transient responses, in terms of tumor stasis or shrinkage, followed by tumor regrowth and progression, have also been frequently observed with angiogenesis inhibitors (228). Furthermore, the

sometimes severe toxicity and mortality due to these agents, when administered both alone and in combination have also been a source of concern (227, 229). It is likely that the shortcomings in clinical efficacy of angiogenesis inhibitors may be due to the fact that the mechanism of resistance of VEGF-pathway inhibitors is not well understood. A better understanding of this mechanism could not only help select a cohort of patients that may benefit from such therapy, but also identify which therapeutic agents may function better in combination to elicit a more sustained inhibition of tumor growth.

The SDF-1/CXCR4 axis has been implicated in angiogenesis through autocrine and endocrine pathways. The autocrine mechanism is based upon a positive feedback loop whereby hypoxia induces the expression of HIF-1α, which can upregulate VEGF, SDF-1, and CXCR4 expression. Endogenous VEGF stimulates expression of CXCR4 in a non-ligand dependent autocrine fashion, and SDF-1/CXCR4 activation stimulates the expression of VEGF via AKT in tumor cells (Fig. 1-11A) (154, 170). Furthermore, SDF-1 has been shown to be involved in the recruitment of endothelial progenitor cells to enhance angiogenesis and primary tumor growth (endocrine mechanism) (169). VEGF may also be implicated in the maturation of such endothelial progenitor cells to endothelial cells (4) (Fig. 1-11B). Thus, inhibiting the VEGF pathway in addition to the SDF-1/CXCR4 axis may abrogate the angiogenic process by two main mechanisms: 1) stifling the CXCR4/VEGF positive feedback loop; and 2) inhibiting the recruitment and maturation of endothelial progenitor cells.

A



B

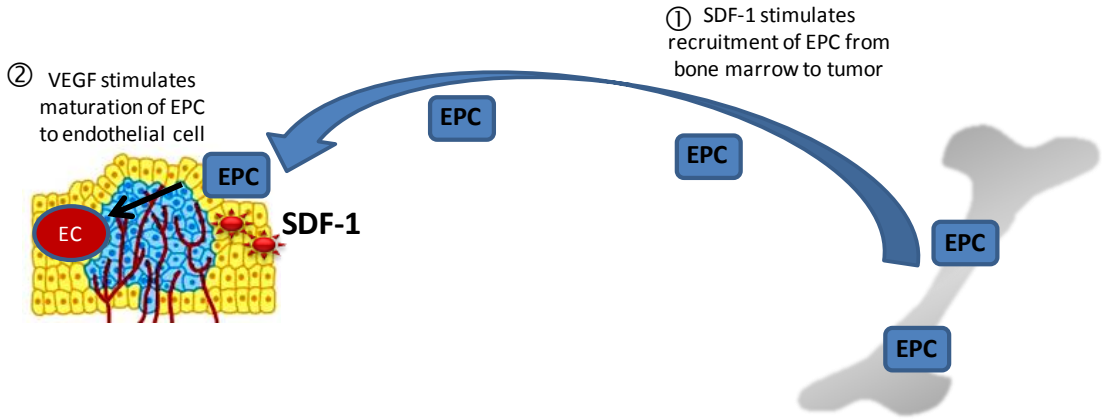


Fig. 1-11. The mechanisms underlying the promotion of tumor angiogenesis by the SDF-1/CXCR4 axis. *A*, Positive feedback loop of SDF-1 and VEGF; *B*, SDF-1 stimulates the recruitment of EPCs and VEGF promotes the maturation of EPCs. Adapted from Weinberg (4).

1.4.1.2.4 Cytotoxic chemotherapeutic agent

Docetaxel, or Taxotere™, comes from the family of therapeutics called taxanes which was originally derived from the bark of the Pacific yew tree, Taxus brevifolia, in 1971. Docetaxel is a microtubule stabilizing agent that binds to β-tubulin and inhibits the disassembly of polymerized tubulin. Docetaxel disrupts centrosome organization, resulting in incomplete mitosis, accumulation of cells in the G_2M phase, and cell death. Apoptosis results from activation of either intrinsic or extrinsic molecular pathways. Whereas the former implicates the mitochondria with regulation of apoptosis via bcl-2, the latter involves numerous pathways including the JNK, Raf/MEK/ERK and PI3K/AKT (230, 231). Indeed, taxane-based regimens have demonstrated a significant survival benefit in comparison to non-taxane based chemotherapy administered in the adjuvant

setting for breast cancer patients (232). Interestingly, administering taxanes in combination with a targeted therapeutic such as trastuzumab has demonstrated an even further improvement of survival and longer time to progression (223, 224). Although HER2+ tumors constitute only 15-25% of the overall population, the addition of trastuzumab to cytotoxic chemotherapy has opened the doors to search for other molecular targeted agents that may further enhance the effect of the latter (233). Since one of the mechanisms by which SDF-1 induces cell motility is via activation of F-actin, we hypothesized that the combination of a microtubule and actin inhibitor may have a synergistic effect upon mitosis and cell migration by attacking two cytoskeletal components.

1.5 Rationale and formulation of question

Breast cancer is the second most common cancer in women. Patients with breast cancer die from metastatic disease. It would be ideal if the process of metastasis could be better predicted, so that patients with breast cancer could be better treated. The identification of better prognostic markers and therapeutic agents could have a major impact on the survival of patients with breast cancer. However, in order for such advances to occur in the clinic, a better understanding of the biology of the metastatic process is necessary. Thus, we turned to a plausible biological theory of metastasis, the chemokine-receptor model, which hypothesized that chemokines, such as SDF-1, are normally overexpressed by specific target metastatic organs, such as lung, liver, and bone, serve to home in tumor cells that express receptors for these chemokines, such as CXCR4. Therefore, the hypothesis of this thesis is that the **measurement of the expression/activity of the SDF-1/CXCR4 ligand/receptor pair can serve as a predictive marker for distant metastasis and as a therapeutic target in breast cancer.** In order to address this hypothesis, I have established the following three objectives:

Objective 1: To determine if plasma SDF- 1 levels can serve as a predictive marker of distant metastasis in breast cancer.

Analogous to the manner in which the concentration gradient of SDF-1 between the bone marrow and plasma played an important role in the trafficking of hematopoietic

progenitors, it is plausible that the concentration gradient of SDF-1, between the metastatic site and circulation, may play a role in driving the cancer cells to the metastatic site. The presence of high SDF-1 at the target organ and low blood levels of SDF-1 may favor the extravasation of cancer cells from the circulation to the metastatic site. Therefore, we hypothesized that low blood levels of SDF-1 may favor the egress of tumor cells to the metastatic site, and thus serve as a predictive blood marker of distant metastasis. In order to better understand the regulation of plasma SDF-1 levels, we proceeded to examine SDF-1 at the genetic level. We wanted to determine if the *SDF-1-3'A* polymorphism could modulate the protein levels of SDF-1 present in the blood of breast cancer patients and if *SDF-1-3'A*, an inherited genetic polymorphism, could influence the tumor's metastatic potential.

Objective 2: To determine if tumor expression of CXCR4 can modulate the prognostic effect of plasma SDF-1 levels.

Since low levels of plasma SDF-1 may favor distant metastasis, it is likely that the concentration gradient of SDF-1, that is, a high level at the metastatic site, and a low level in the plasma, may be an important driving force for the extravasation of cancer cells. It is plausible that a low level of plasma SDF-1 may favor the extravasation of cancer cells that not only express CXCR4, but highly express CXCR4 or the activated form of the receptor, phosphorylated-CXCR4. We therefore determined the tumor cell expression of CXCR4 and p-CXCR4, and their value as prognostic markers. Moreover, we wanted to determine if the prognostic value would be further enhanced amongst patients who demonstrated both of these risk factors: that is, high CXCR4/p-CXCR4 tumor expression and a low plasma SDF-1 level.

Objective 3: To determine if CXCR4 blockade, alone or in combination with other anti-cancer therapies such as trastuzumab, DC101, or docetaxel leads to the inhibition of primary tumor growth and distant metastasis.

In addition to new prognostic markers, further improvement in patient survival requires the discovery of new therapeutic agents. One plausible means of targeting the metastatic process is to target a molecular marker implicated at various stages of the metastatic

process: CXCR4. *In-vitro* and animal studies have demonstrated the significance of CXCR4 in tumor proliferation, angiogenesis, invasion, migration, and the formation of distant metastasis. However, the animal experiments published to date have either not used a CXCR4 antagonist that can be currently used in patients, or have been poorly designed in terms of timing of therapeutic intervention, with limited relevance to the clinical setting (31, 140, 141). Since CXCR4 is overexpressed in breast cancer patients and associated with poor prognosis (89), further investigation of CXCR4 as a therapeutic target is warranted. We wanted to target CXCR4 in a transgenic mouse model, the PyMT model, when the primary tumor became palpable in order to better correspond to the clinical scenario. We selected CTCE-9908 as our CXCR4 antagonist, since initial testing of this peptide has indicated safety in patients. We determined if CXCR4 blockade, alone or in combination with other anti-cancer therapies leads to the inhibition of primary tumor growth and distant metastasis. In the transgenic mouse model, we tested combinations with either one of the following agents: docetaxel or DC101. Docetaxel is a cytotoxic chemotherapeutic agent currently used in the management of breast cancer patients. We selected DC101 as the anti-angiogenic agent since it can target mouse VEGFR2 and has demonstrated efficacy in various cancer mouse models (234-237). In order to target the HER2 pathway, we selected trastuzumab, a humanized anti-HER2 monoclonal antibody, which is currently used in the clinic in combination with other anti-cancer therapies, and tested this agent in a xenograft model using a human cancer cell line, MDA-MB-361, which overexpresses HER2 and CXCR4.

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Connecting Text

The first objective of this thesis aims to better understand the role of SDF-1 in the metastatic process in breast cancer patients. This objective can be divided into three parts: first, to measure the level of plasma SDF-1 and determine its value as a predictor for distant metastasis; second, to study a factor influencing the plasma levels of SDF-1 by identifying the frequency of the SDF-1-3'A polymorphism and correlating it with plasma SDF-1 levels; and third, to determine the prognostic value of the polymorphism alone and in combination with plasma SDF-1 levels.

CHAPTER 2 – MANUSCRIPT 1

Plasma Stromal Cell-Derived Factor-1: Host Derived Marker Predictive of Distant Metastasis in Breast Cancer

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2.1 Abstract

Purpose: Homing of breast cancer cells to metastatic sites may be regulated by the production of stromal cell-derived factor (SDF)-1 by specific target organs, which attracts CXCR4-expressing breast cancer cells. We investigated the value of SDF-1 as a predictive blood marker of distant metastasis in breast cancer, together with a common polymorphism of *SDF-1, SDF-1-3'A*.

Experimental Design: Plasma samples were collected prospectively for 270 consecutive primary breast cancer patients with a median follow-up of 3.3 years. Plasma SDF-1 levels were measured using an ELISA, and the polymorphism was identified via PCR-RFLP analysis.

Results: Plasma SDF-1 levels were divided into two groups, low and high, based on the median SDF-1 value of 2,661pg/mL. Patients with low SDF-1 showed an increased risk of developing distant metastasis (relative risk, 1.94; $P = 0.02$) and poorer breast cancer-specific survival [adjusted hazard ratio (AHR), 3.92; $P = 0.007$]. Patients with both low plasma SDF-1 levels and the *SDF-1-3'A* polymorphism showed a poorer breast cancer - specific survival (AHR, 3.98; $P = 0.001$) and distant disease-free survival (AHR, 2.88; $P = 0.003$). In a separate cohort of 22 breast cancer patients, we found no significant difference in SDF-1 levels before and post tumor resection.

Conclusion: We found that low plasma SDF-1 is an independent host-derived predictive marker of distant metastasis in breast cancer. The prognostic value of the combination of a low plasma SDF-1 level and the *SDF-1-3'A* polymorphism identifies a cohort of patients with an intrinsic susceptibility for poorer survival.

2.2 Introduction

Although the prognosis of patients with breast cancer has improved in recent years due to advancements in adjuvant therapy and earlier diagnosis, ~ 12% of breast cancer patients die of the disease within the first 5 years.[1] Most patients receive adjuvant treatment to eradicate micrometastatic disease, depending on prognostic markers such as tumor size, grade, hormone receptor status, and most importantly, the presence of metastatic disease in axillary lymph nodes (1–3). Lymphatic metastasis is, in fact, a surrogate marker for distant metastatic disease, the major determinant of long-term survival (4, 5). Currently, there is no direct marker predictive of distant metastatic efficiency for individual breast cancers (6). One of the reasons for this is that the study of the metastatic process in humans has lagged behind advances in knowledge about molecular changes of the tumor cell. Although the process of metastasis has been dissected into various components, little is known about the factors that lead to the selection of the tumor cell of a particular organ for metastasis and less about any host factors that may influence the metastatic process (7).

In 2001, Muller et al. (8) proposed a model for metastasis analogous to the manner in which chemokines attract immune cells to sites of inflammation. It was suggested that specific chemokines, secreted by particular target organs to which breast cancer metastasizes, serve to home in circulating breast cancer cells that express receptors for these chemokines. They identified one such chemokine/receptor pair as stromal cell–derived factor (SDF)-1/CXCR4. Tumor cells, including breast cancers, were found to express high levels of the CXCR4 receptor, whereas human organs targeted by metastatic breast tumor cells, in turn, expressed high levels of SDF-1 (8). Moreover, the level of CXCR4 expression in breast cancer cells is predictive of poor prognosis, suggestive of distant metastatic disease, in breast cancer (9). Thus, it seems plausible that the gradient between SDF-1 in the blood and SDF-1 in the target organs may influence the development of distant metastasis in breast cancer. Indeed, SDF-1 is a powerful chemoattractant secreted in the bone marrow, functioning to retain progenitor stem cells within the bone marrow (10). Analogously, we hypothesized that high plasma SDF-1

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levels in the blood will serve to retain tumor cells within the circulation and out of the metastatic organ site, and thus, low plasma SDF-1 levels will serve as a predictive marker for distant metastasis. Moreover, SDF-1 mRNA and protein expression may be regulated by a common polymorphism of SDF-1, a G→A transition at position 801 in the 3' untranslated region of the *SDF-1* gene transcript, also known as *SDF-1-3'A* (11, 12). The *SDF-1-3'A* polymorphism is associated with an increased susceptibility to develop breast cancer (13, 14), although its functional significance in breast cancer is presently unknown (11, 12, 15). Measurement of plasma SDF-1 in breast cancer has not been reported to date, nor has its predictive potential of distant metastasis been described in cancer. We measured SDF-1 levels in the blood and the *SDF-1-3'A* polymorphism in lymphocytes from a cohort of consecutive breast cancer patients. We found that SDF-1 plasma levels and the *SDF-1-3'A* polymorphism are both host-derived markers that can determine an individual's intrinsic susceptibility to develop metastatic disease in breast cancer.

2.3 Materials and Methods

Patients. From 2000 to 2003, blood samples from 270 consecutive patients with primary stage I, II, and III breast tumors were collected prospectively at the Centre Hospitalier de l'Université de Montréal (CHUM) as part of a provincially supported tumor bank (Fonds de la recherche en santé du Québec), in which all patients signed informed consent as part of the approval by the Research Ethics Committee of the CHUM. Baseline characteristics for all patients can be found in Table 2-1. A small proportion (13.7%) of patients received neoadjuvant hormonal or chemotherapy. As no postoperative blood collection was done in this cohort, a second cohort of 22 consecutive primary breast cancer patients at the Sir Mortimer B. Davis Jewish General Hospital, McGill University, underwent plasma collection in K-EDTA tubes from 2004 to 2006, approved by the Research Ethics Committee of the Sir Mortimer B. Davis Jewish General Hospital, both before surgery, and at a median follow-up of 7 months after surgical resection.

Plasma SDF-1 measurement and SDF-1-3'A genotyping. Plasma was collected for all patients on the day of surgery in K-EDTA tubes, processed with Ficoll-Paque Plus (Amersham Biosciences) to obtain lymphocytes, and stored at -80ºC. Plasma SDF-1 levels were measured using Quantikine Colorimetric ELISA kits for CXCL12 (R&D

Systems), in duplicate trials in triplicate wells. The mean coefficient of variation of the ELISA assay for six replicates was 6.8%. From the six values obtained for each patient, the median value was taken. SDF-1-3'A genotyping was done with DNA extracted from lymphocytes using DNAzol (Invitrogen). Primers used were as follows: (F) CAGTCAACCTGGGCAAAGCC and (R) AGCTTTGGTCCTGAGAGTCC (11). PCR amplification was carried out as per protocol (14, 16), except the annealing temperature was modified to 62ºC to decrease nonspecific annealing. PCR products were digested with *Msp*I restriction enzyme at 37ºC for at least 4 h; fragments were then visualized on a 3% agarose gel with ethidium bromide. The PCR products for the three genotypes were as follows: wild-type GG, two discrete fragments at 100 and 200 bps; homozygous AA, one fragment at 300 bps, and heterozygous AG; 3 fragments at 100, 200, and 300 bps (16).

Statistical analysis. χ^2 analysis and Fisher's exact test were done to determine associations between SDF-1 plasma levels and polymorphism with clinicopathologic properties; Fisher's exact test was used when the number of samples was ≤ 5. To further understand directionality of the correlations and an estimation of their magnitude, Spearman's rank correlation was also done, wherein plasma SDF-1 was treated as a continuous variable and the SDF-1 polymorphism as an ordinal variable; the coefficient of correlation is represented by the ρ value. Correlation between plasma SDF-1 levels and the genotype was done using one-way ANOVA. Survival analysis of plasma SDF-1 was carried out by examining the plasma levels as both a dichotomous variable, using the median SDF-1 value as an arbitrary dividing point, and as a continuous variable. To obtain cutpoint(s) of plasma SDF-1 levels that may be more clinically relevant, prognosis-derived cutpoints were also determined using X-tile, Version 3.6.1 (Robert Camp, Yale University of New Haven, CT; ref. 17). Survival intervals were measured from the time of surgery to the time of death or the first clinical or radiographic evidence of local recurrence or distant metastasis. The primary end points included overall survival, breast cancer – specific survival, disease-free survival (DFS; local or distant metastasis), and distant disease-free survival (DDFS). Because this is the first report of plasma SDF-1 levels in breast cancer patients, an *a priori* sample size could not be determined. Kaplan-Meier survival curves were constructed for univariate analysis (n =

270). To examine the effect of multiple covariates, a Cox proportional hazards regression model was used (18) and adjustment for age, tumor size, lymph nodes, tumor grade, estrogen receptor (ER), progesterone receptor (PR), and therapy (neoadjuvant or adjuvant hormonal or chemotherapy) was done. In the multivariate model, the categorical form of lymph node status, tumor grade, ER, PR, and therapy was used, whereas a linear trend was used for age and tumor size. Patients for whom lymphadenectomies were not done were included as a separate category. Fifty patients were excluded in multivariate analysis due to incomplete information regarding clinicopathologic characteristics. A survival analysis was done to compare the outcome of the patients with missing variables to those patients with complete information. No statistically significant difference between these groups was identified for overall survival, breast cancer – specific survival, DFS, or DDFS. Proportional hazards assumptions for all Cox models were assessed using Schoenfeld residuals, and goodness of fit was graphically estimated using Cox-Snell residuals. The preoperative and postoperative blood study was designed to have a 90% power to detect a difference of 239 pg/mL before and post tumor excision, based on the difference observed between those patients who did and did not develop metastasis from the first cohort (see below). An unpaired *t* test was used to compare the preoperative plasma SDF-1 levels between the two cohorts. A paired *t* test compared the difference between the preoperative and postoperative plasma SDF-1 values. All reported *P* values were two sided. *P* values <0.05 were considered statistically significant. All statistical analysis was done using STATA Version 9.2.

2.4 Results

Plasma SDF-1 levels. Two hundred seventy consecutive patients with primary breast cancer underwent surgery at the CHUM and were followed-up for a median of 3.3 years. Ten patients (3.7%) developed local recurrence, whereas 47 patients (17.4%) developed distant metastasis during follow-up. Plasma SDF-1 levels in these patients followed a Gaussian distribution and ranged from 726 to 4,238 pg/mL. Plasma SDF-1 levels were divided into two groups, low (<2,661 pg/mL) and high (≥ 2,661 pg/mL), based on the median SDF-1 value of 2,661 pg/mL for the patients in this cohort. Correlations with the clinicopathologic characteristics were done as described in Table 2-2. Low plasma SDF-1

levels were associated with a younger age at the time of surgery ($P = 0.01$). There was a nonsignificant trend of an increased frequency of T_4 tumors in patients with low SDF-1 levels, although only 11 patients had T_4 tumors. A weak but inverse correlation was also identified between plasma SDF-1 and nodal status ($\rho = -0.15$; $P = 0.02$). Patients who eventually developed distant metastasis had a lower mean SDF-1 value than those who did not develop metastasis [mean difference, 237 pg/mL; 95% confidence interval (95% CI), 78-395 pg/mL; $P = 0.004$]. The sensitivity of low plasma SDF-1 levels for distant metastasis was 66%, and the specificity was 53.4%. Low plasma SDF-1 levels were predictive of distant metastasis (relative risk, 1.94; 95% CI, 1.11-3.37; $P = 0.02$). Low plasma SDF-1 level was also an independent prognostic marker for poorer breast cancer – specific survival (adjusted hazard ratio, 3.92; 95% CI, 1.46- 10.51; $P = 0.007$) and DDFS (adjusted hazard ratio, 2.27; 95% CI, 1.12- 4.61; $P = 0.02$) but was not significant for DFS, which includes local and distant recurrence (Table 2-3). When evaluated as a continuous variable, for every decrease in 1,000 pg/mL of plasma SDF-1, the rate of mortality due to breast cancer was 3.22 (95% CI, 1.76-5.88; $P < 0.001$). To identify a cutpoint that may stratify patients into groups that are validated and may have greater clinical application, optimal cutpoint analysis was done using X-tile software. Three groups were identified with the following SDF-1 levels and population proportions: Lo ($\leq$ 2295 pg/mL), 22.2%; Mid (2296-2557 pg/mL), 20.4%; and Hi (>2557 pg/mL), 57.4%. The relative risk of patients dying from breast cancer was found to be 5.17-fold greater in the Lo versus Hi group (Monte Carlo, $P = 0.03$; Cross-validation Hi/Lo, $P = 0.007$; Fig. 2-1). No statistically significant correlation was identified with plasma SDF-1 levels and other comorbid conditions including previous history of cancer, coronary artery disease, arrhythmias, gastroesophageal reflux disease, hypertension, hypercholesterolemia, diabetes mellitus, arthritis, hypothyroidism, or chronic respiratory disease. There was a higher proportion of high plasma SDF-1 levels in patients with coronary artery disease (85.7%; $P = 0.01$), but this condition was only present in 5.2% of patients. When patients with coronary artery disease were excluded, low plasma SDF-1 remained significant for overall survival, breast cancer – specific survival, and DDFS (data not shown).

SDF-1-3'A genotyping. The genotypic and allelic frequencies were in accordance with Hardy-Weinberg equilibrium. The frequencies of the genotypes were as follows: AA,

4.4%; AG, 29.3%; and GG, 66.3%. With the exception of Spearman's rank correlation, the AA and AG genotypes were combined for all analysis due to the low frequency of the AA genotype. No correlation was identified between the genotype and breast tumor characteristics (Table 2-2). Patients with the *SDF-1-3'A* polymorphism showed a poorer breast cancer – specific survival (adjusted hazard ratio, 2.36; 95% CI, 1.05-5.29; P = 0.04) and DFS (adjusted hazard ratio, 2.28; 95% CI, 1.24-4.21; P = 0.008; Table 2-3). There was an increased incidence of patients with the *SDF-1-3'A* polymorphism who had a previous history of cancer excluding breast (63.2%; n = 19; P = 0.005). No statistically significant correlation was identified with any other comorbid condition such as coronary artery disease, arrhythmias, gastroesophageal reflux disease, hypertension, hypercholesterolemia, diabetes mellitus, arthritis, hypothyroidism, or chronic respiratory disease.

SDF-1 plasma levels and SDF-1-3'A genotyping. The means (SD) of plasma SDF-1 level for patients from each genotype were as follows: AA, 2555 pg/mL (271); AG 2620 pg/mL (540); GG, 2666 pg/mL (508). There seemed to be a trend with decreasing plasma SDF-1 levels from the GG to AG to AA genotypes, but this was not statistically significant (P = 0.65). Because of this trend, and the theoretical possibility that the AA polymorphism may affect SDF-1 mRNA levels, patients with both low plasma SDF-1 level and the *SDF-1-3'A* polymorphism (low + AA/AG) were combined (n = 49), also known as LowA, and compared with the other patients who either had a high plasma SDF-1 level (high + AA/AG or high + GG) or a low plasma SDF-1 level with the GG genotype (low + GG), also called the ''Others'' group. There was no statistically significant difference in survival in patients with high + AA/AG, high + GG, or low + GG. Patients with a low plasma SDF-1 level and the *SDF-1-3'A* polymorphism had a greater risk of developing metastasis (relative risk, 2.11; 95% CI, 1.25-3.59; P = 0.007) versus the Others group. In univariate analysis, n = 270, LowA patients showed a high rate of mortality due to breast cancer-related causes (unadjusted hazard ratio, 4.19; 95% CI, 1.99- 8.81; P < 0.001; Fig. 2-2B). The 5-year rate of breast cancer-specific survival for LowA was 70.8% (7.0% SE) versus 92.5% (1.9% SE) for the Others group. LowA patients also exhibited poorer overall survival (unadjusted hazard ratio, 3.15; 95% CI, 1.57-6.33; P = 0.001; Fig.2-2A). The rates of DFS (unadjusted hazard ratio, 2.28; 95%

CI, 1.29-4.03; $P = 0.005$) and DDFS (unadjusted hazard ratio, 2.42; 95% CI, 1.31-4.48; $P = 0.005$) were also significantly lower for LowA patients (Fig. 2-2C and D).

After multivariate analysis, LowA emerged as a powerful independent prognosticator with almost 4-fold higher rate of mortality secondary to breast cancer (Table 2-4). In comparison with other prognostic markers, LowA seemed to be stronger than tumor size and tumor grade. Because a positive correlation was identified between plasma SDF-1 levels and age, adjustment for age alone revealed similar statistical significance and rate of recurrence or mortality (data not shown). Due to the prognostic value of the combination of low plasma SDF-1 level and the SDF-1-3'A polymorphism, an interaction between these two variables was sought. No interaction was identified in unadjusted or adjusted multivariate analysis between the two variables for overall survival, DFS, or DDFS, but a trend was identified for breast cancer-specific survival (unadjusted interaction hazard ratio, 9.77; 95% CI, 0.93-103.03; $P = 0.06$; adjusted hazard ratio, 4.17; 95% CI, 0.36- 47.7; $P = 0.25$).

Tumor contribution to plasma SDF-1. Since it has been reported that tumors can express SDF-1 (19, 20), we verified whether tumor secretion of SDF-1 may significantly contribute to plasma SDF-1 levels by comparing preoperative and postoperative levels, at a median follow-up of 7 months in an independent group of 22 primary breast cancer patients at the Sir Mortimer B. Davis Jewish General Hospital. None of these patients showed evidence of recurrent disease at the time of the second blood collection. The mean age of this group (57.6 years) was comparable with that of the larger group of patients from the CHUM (58.0 years), and there was no statistically significant difference in mean preoperative SDF-1 plasma levels between the two cohorts (mean difference, 154 pg/mL; 95% CI, -69.30-372 pg/mL; $P = 0.17$). Within the Sir Mortimer B. Davis Jewish General Hospital cohort, the mean difference between the preoperative and postoperative SDF-1 levels was 85 pg/mL, which was not statistically significant (SD, 337; $P = 0.26$). Therefore, the contribution of plasma SDF-1 from the tumor can be considered minimal. The difference in plasma SDF-1 levels (i.e., tumor secreted SDF-1) was correlated with age, tumor size, grade, lymph node status, ER, PR, and Her2/neu status, and no association was identified (data not shown).

2.5 Discussion

Although it is commonly accepted that the clinical behavior of tumors depends on the relationship between tumor cells and the host, the majority of molecular studies have identified tumor-derived markers, whereas little is known about the predictive potential of host factors and their potential role in guiding therapy. One approach to uncover host factors is to screen for genetic polymorphisms in cancer-associated genes, or carcinogen-metabolizing genes (21). Such a search has not been very successful to date because these polymorphisms have generally not been clinically validated. However, starting from recent advances in the understanding of tumor biology, and specifically, the metastatic process in breast cancer, we proceeded to directly study a simple idea: that elevated chemokine levels in the blood of cancer patients may act to retain cancer cells in the circulation and prevent them from homing in to their metastatic target sites. Indeed, we discovered that a low plasma SDF-1 level is predictive of distant metastasis and an independent prognostic marker in breast cancer patients. Our data shows that tumor-secreted SDF-1 plays, at most, a minor role in SDF-1 plasma levels, suggesting that plasma SDF-1 is truly a host factor influencing the propensity of breast cancers to metastasize. Moreover, increasing SDF-1 plasma levels with age may be a factor contributing to the better prognosis of older women with breast cancer. In addition, the presence of the *SDF-1* genetic polymorphism contributed to increasing the risk of metastatic disease in patients with low plasma SDF-1.

The origin of plasma SDF-1 is multifarious, including many different cell types such as lymphocytes, endothelial cells, bone marrow, and tumor stromal cells (22–25). This has led to its measurement in plasma in various medical conditions such as HIV, rheumatoid arthritis, and in the context of stem cell mobilization (26–29). Plasma SDF-1 has previously been measured in only a few neoplasms; in both B-cell chronic lymphocytic leukemia and colon cancer, the authors identified a lower level of plasma SDF-1 in cancer patients compared with normal controls (30–32). Furthermore, patients with more advanced stage colon cancer exhibited lower levels of plasma SDF-1 versus patients of earlier stage colon cancer (32). These results are in concordance with the trend that we have reported herein between low plasma levels and increasing tumor size or

nodal involvement. Here, for the first time, we report that a low plasma SDF-1 level in breast cancer is predictive of distant metastasis and poorer survival.

Our study has several strengths. All blood samples from the CHUM were collected prospectively, minutes before surgery in fasting patients, which minimizes variability that may be induced if collected at different time points. A commercially available assay (R&D Systems) was used for measurement of plasma SDF-1 levels in which a small coefficient of variation was obtained; this allows for greater potential for validation of results and, thus, implementation into the clinic. Furthermore, this study used a cohort of consecutive patients such that there was no *a priori* selection of patients; the resulting trends identified across this population of breast cancer patients are thus more generalizable. One of the limitations of this study is that the median follow-up is only 3.3 years; a study with a longer follow-up will be needed to account for distant recurrences that will occur beyond this point.

Recent reports have shown that women with the *SDF-1-3'A* polymorphism have an increased susceptibility to develop breast cancer (13, 14). The reasons for this association are presently unknown. Although an association between the polymorphism and distant metastasis was identified in acute myeloid leukemia patients (33), this was not observed in one underpowered study with breast cancer patients (34). In our cohort of 270 patients, we found that the *SDF-1-3'A* polymorphism is an independent prognostic marker for overall survival, breast cancer–specific survival, and DFS. Although we did find a correlative trend between SDF-1 plasma levels and *SDF-1-3'A* polymorphism, it was not significant. To clarify the association between SDF-1 levels and the polymorphism in breast cancer, a larger cohort would be necessary.

The combination of the polymorphism and low plasma SDF-1 level has a strong prognostic value with a 4-fold lower survival rate compared with those patients with either high plasma SDF-1 or the GG genotype. Given that plasma SDF-1 is mostly a host-derived factor and that the *SDF-1-3'A* polymorphism is a germline polymorphism, we have identified a cohort of patients with a poor prognosis due to an intrinsic, tumor-independent susceptibility to develop metastatic disease. As CXCR4 expression has been identified in many different neoplasms (35), it is likely that this intrinsic susceptibility for metastatic disease may not be unique to breast cancer. Because breast tumors widely

express the receptor for SDF-1, CXCR4 (9), this ligand/receptor pair provides an interesting therapeutic target in breast cancer patients. In fact, anti-CXCR4 agents are currently in clinical trials in various cancers including breast cancer[2]. Studies of anti-CXCR4 therapy in breast cancer should consider selecting not only patients whose breast tumors overexpress the CXCR4 receptor, but those patients carrying the *SDF-1-3'A* polymorphism and whose plasma SDF-1 levels are low. Our results point the way to new clinical trials in oncology that will consider both host factors in addition to tumor factors as criteria for selection of therapies. In conclusion, the predictive value of plasma SDF-1 offers a direct view of the physiology of metastatic disease in the blood of cancer patients. The significance of low plasma SDF-1 levels suggests that a clinically important step in the process of breast cancer metastasis occurs at the stage of tumor extravasation driven by the differential concentration gradient of lower SDF-1 levels in the circulation compared with the metastatic organ site.

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Figures

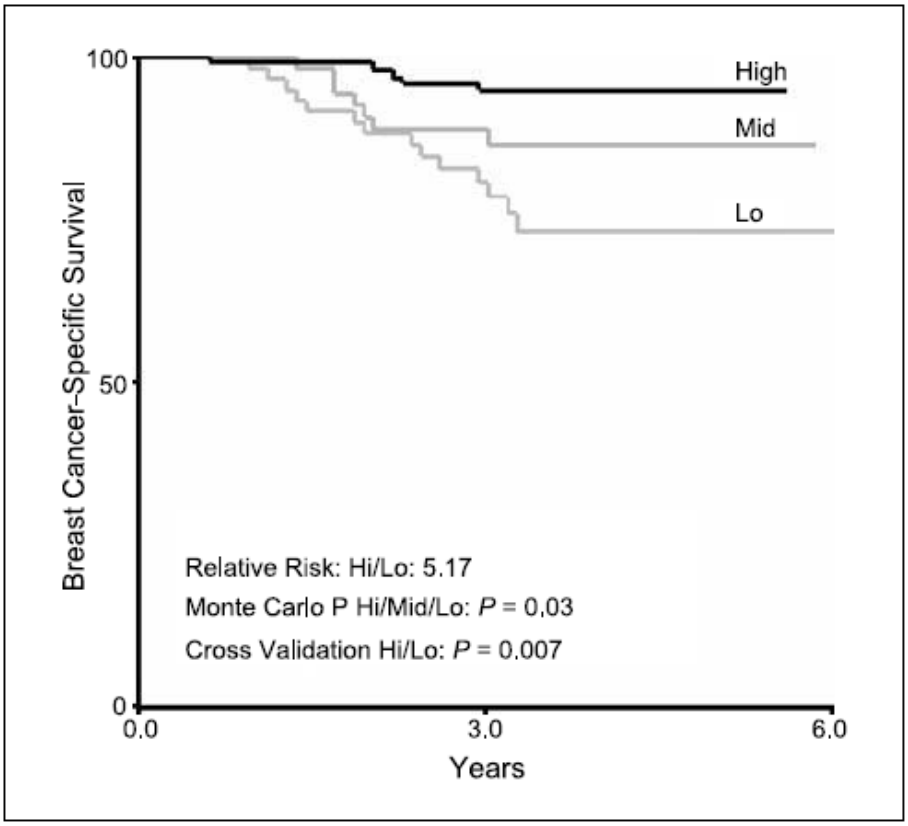

Fig.2-1. Unadjusted Kaplan-Meier survival curve of plasma SDF-1 with Lo, Mid, Hi values derived from optimal cutpoint analysis from X-tile software.

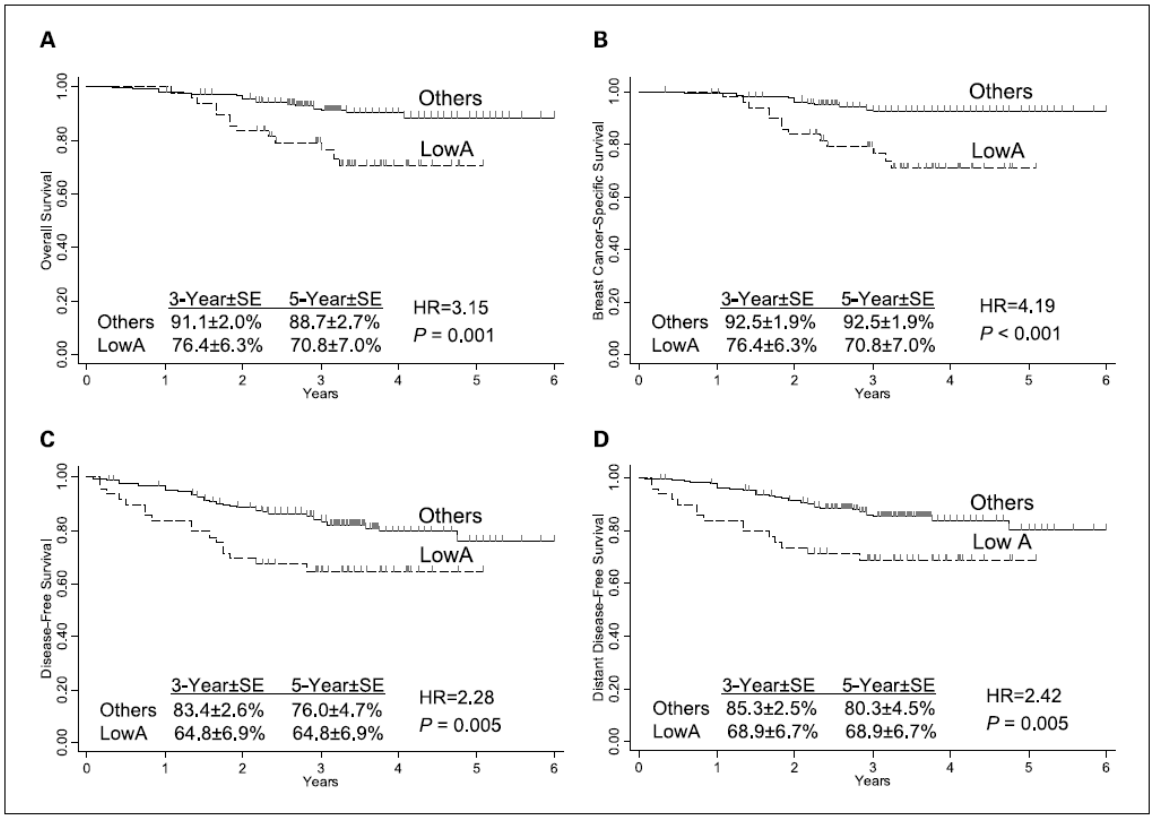

Fig.2-2. Unadjusted Kaplan-Meier survival curves of LowA (low plasma SDF-1 level + AA/AG genotype) versus Others (low plasma SDF-1 + GG, or high plasma SDF-1 + AA/AG, or high plasma SDF-1+GG) (n=270). *A*, Overall survival; *B*, Breast cancer-specific survival; *C*, Disease-free survival; *D,* Distant disease-free survival.

Tables

Table 2-1. Baseline Characteristics of Patients (n=270)

Variable	Overall No. patients %	At diagnosis* No. patients (%)	At time of surgery† No. patients (%)
Age – years			
≤ 40	24 (8.9)		
41-50	80 (29.6)		
51-65	83 (30.7)		
≥ 66	83 (30.7)		
Tumor Grade			
1	38 (14.1)		
2	122 (45.2)		
3	65 (24.1)		
Unavailable	45 (16.7)		
Tumor Size			
T1, ≤ 2 cm		125 (46.3)	135 (50.0)
T2, 2-5 cm		103 (38.1)	101 (37.4)
T3, > 5 cm		27 (10.0)	17 (6.3)
T4		15 (5.6)	15 (5.6)
Unavailable			2 (0.7)
Nodal Status			
N0		126 (46.7)	126 (46.7)
N1 (1-3)		64 (23.7)	64 (23.7)
N2 (4-9)		33 (12.2)	33 (12.2)
N3 (≥ 10)		17 (6.3)	16 (5.9)
Lymphadenectomy not performed		30 (11.1)	31 (11.5)
Stage			
1		79 (29.3)	83 (30.7)
2		101 (37.4)	98 (36.3)
3		61 (22.6)	58 (21.5)
Unavailable		29 (10.7)	31 (11.5)
Estrogen Receptor (ER) Status			
Positive	189 (70.0)		
Negative	78 (28.9)		
Unavailable	3 (1.1)		
Progesterone Receptor (PR) Status			
Positive	172 (63.7)		
Negative	95 (35.2)		
Unavailable	3 (1.1)		
Her2/neu status			
Positive	27 (10.0)		
Negative	161 (59.6)		
Unavailable	82 (30.4)		

*Prior to neoadjuvant chemotherapy or anti-hormonal therapy.
†Characteristic at the time of surgery only, reflecting some downstaging due to neoadjuvant treatment. Blood collection was always performed at the time of surgery.

Table 2-2. Clinicopathologic correlations

	Plasma SDF-1 levels					SDF-1-3'A Polymorphism				
	Categorical variable			Continuous variable		Categorical Variable			Ordinal variable	
	Low	High	*P*	rho	*P*	AA/AG	GG	*P*	rho	*P*
	No. patients (%)	No. patients (%)				No. patients (%)	No. patients (%)			
Age – years			0.01	0.22	<0.001			0.67	0.01	0.88
≤ 40	15 (11.1)	9 (6.7)				7 (7.7)	17 (9.5)			
41-50	46 (34.1)	34 (25.2)				27 (29.7)	53 (29.6)			
51-65	45 (33.3)	38 (28.2)				32 (35.2)	51 (28.5)			
≥ 66	29 (21.5)	54 (40.0)				25 (27.5)	58 (32.4)			
Tumor Grade			0.44	-0.11	0.10			0.74	-0.02	0.81
1	17 (14.9)	21 (18.9)				14 (18.2)	24 (16.2)			
2	60 (52.6)	62 (55.9)				39 (50.7)	83 (56.1)			
3	37 (32.5)	28 (25.2)				24 (31.2)	41 (27.7)			
Tumor Size			0.06	0.01	0.91			0.61	0.05	0.41
T1, ≤ 2 cm	72 (53.3)	63 (47.4)				47 (51.7)	78 (43.6)			
T2, 2-5 cm	42 (31.1)	59 (44.4)				30 (33.0)	73 (40.8)			
T3, > 5 cm	10 (7.4)	7 (5.3)				9 (9.9)	18 (10.1)			
T4	11 (8.2)	4 (3.0)				5 (5.5)	10 (5.6)			
Nodal Status			0.68	-0.15	0.02			0.89	0.02	0.75
N0	60 (49.6)	66 (55.9)				43 (53.8)	83 (51.9)			
N1 (1-3)	34 (28.1)	30 (25.4)				22 (27.5)	43 (26.3)			
N2 (4-9)	17 (14.1)	16 (13.6)				9 (11.3)	24 (15.0)			
N3 (≥ 10)	10 (8.3)	6 (5.1)				6 (7.5)	11 (6.9)			
Stage			0.51	-0.08	0.23			0.52	0.07	0.30
1	39 (32.2)	44 (37.3)				30 (37.5)	49 (30.4)			
2	49 (40.5)	49 (41.5)				32 (40.0)	69 (42.9)			
3	33 (27.3)	25 (21.2)				18 (22.5)	43 (26.7)			
ER Status			0.40	0.08	0.17			0.69	0.02	0.74
Positive	91 (68.4)	98 (73.1)				63 (69.2)	126 (71.6)			
Negative	42 (31.6)	36 (26.9)				28 (30.8)	50 (28.4)			
PR Status			0.76	0.06	0.33			0.87	0.01	0.89
Positive	84 (63.2)	88 (65.7)				58 (63.7)	114 (64.8)			
Negative	49 (36.8)	46 (34.3)				33 (36.3)	62 (35.2)			
Her2/neu status			0.39	-0.07	0.36			0.24	-0.11	0.15
Positive	15 (16.7)	12 (12.2)				12 (18.5)	15 (12.2)			
Negative	75 (83.3)	86 (87.8)				53 (81.5)	108 (87.8)			
Pre-operative hormonal or chemotherapy										
Yes	20 (14.9)	17 (12.5)	0.56	-0.01	0.89	12 (13.2)	25 (14.0)	0.86	0.01	0.90
No	114 (85.1)	119 (87.5)				79 (86.8)	154 (86.0)			
Local Recurrence			0.75	0.03	0.59			1.00	-0.02	0.74
Yes	4 (3.0)	6 (4.4)				3 (3.3)	7 (3.9)			
No	131 (97.0)	129 (95.6)				88 (96.7)	172 (96.1)			
Distant Metastasis			0.02	-0.17	0.01			0.16	0.08	0.19
Yes	31 (23.0)	16 (11.9)				20 (22.0)	27 (15.1)			
No	104 (77.0)	119 (88.2)				71 (78.0)	152 (84.9)			

Table 2-3. Unadjusted and adjusted survival analysis

	Unadjusted		Adjusted*	
	Hazard Ratio (95% CI)	***P***	**Hazard Ratio (95% CI)**	***P***
Overall Survival				
SDF-1 Plasma levels (low vs. high)	2.66 (1.18-6.01)	0.02	3.48 (1.46-8.30)	0.005
SDF-1 Polymorphism (AA/AG vs. GG)	2.45 (1.18-5.09)	0.02	2.42 (1.15-5.12)	0.02
SDF-1 Plasma and Polymorphism (LowA vs. Others)†	3.91 (1.88-8.13)	<0.001	3.40 (1.56-7.39)	0.002
Breast Cancer-Specific Survival				
SDF-1 Plasma levels (low vs. high)	3.21 (1.28-8.04)	0.01	3.92 (1.46-10.51)	0.007
SDF-1 Polymorphism (AA/AG vs. GG)	2.52 (1.14-5.55)	0.02	2.36 (1.05-5.29)	0.04
SDF-1 Plasma and Polymorphism (LowA vs. Others)	5.17 (2.36-11.35)	<0.001	3.98 (1.74-9.12)	0.001
Disease-Free Survival				
SDF-1 Plasma levels (low vs. high)	1.58 (0.86-2.91)	0.14	1.64 (0.85-3.15)	0.14
SDF-1 Polymorphism (AA/AG vs. GG)	2.19 (1.20-3.99)	0.01	2.28 (1.24-4.21)	0.008
SDF-1 Plasma and Polymorphism (LowA vs. Others)	2.47 (1.31- 4.69)	0.005	2.32 (1.19-4.53)	0.01
Distant Disease-Free Survival				
SDF-1 Plasma levels (low vs. high)	2.08 (1.07-4.05)	0.03	2.27 (1.12-4.61)	0.02
SDF-1 Polymorphism (AA/AG vs. GG)	1.80 (0.96-3.37)	0.07	1.90 (1.00-3.61)	0.05
SDF-1 Plasma and Polymorphism (LowA vs. Others)	2.95 (1.53- 5.68)	0.001	2.88 (1.44-5.78)	0.003

NOTE. Cox proportional hazards model was used for unadjusted, univariate and adjusted, multivariate analyses. 50 patients were excluded due to incomplete data regarding tumor characteristics.

*Multivariate analysis included adjustment for age, tumor size, lymph nodes, tumor grade, estrogen receptor, progesterone receptor, and therapy. Plasma SDF-1 levels were adjusted for pathological characteristics from the time of surgery. SDF-1 polymorphism was adjusted for pathological characteristics from initial diagnosis. LowA was adjusted for variables at the time of surgery (See Table 2-1).

†LowA refers to the combination of patients with low plasma SDF-1 levels and *SDF-1-3'A* polymorphism versus Others (low SDF-1 + GG genotype, or high SDF-1 + AA/AG, or high SDF-1 + GG genotype)

Table 2-4. Multivariate variable analysis for LowA versus Others

	Unadjusted		Adjusted*	
	Hazard Ratio (95% CI)	***P***	**Hazard Ratio (95% CI)**	***P***
Overall survival				
LowA vs. Others†	3.91 (1.88-8.13)	<0.001	3.40 (1.56-7.39)	0.002
Age	1.28 (0.88-1.87)	0.19	1.53 (0.97-2.42)	0.07
Tumor size	1.76 (1.23-2.51)	0.002	1.58 (1.05-2.40)	0.03
ER (negative vs. positive)	5.73 (2.60-12.61)	<0.001	4.36 (1.36-14.01)	0.01
PR (negative vs. positive)	2.66 (1.27-5.58)	0.009	0.68 (0.24-1.92)	0.47
Lymph Nodes (positive vs. negative)	3.18 (1.30-8.00)	0.01	1.59 (0.57-4.39)	0.37
(axillary dissection not performed vs. negative)	3.92 (1.20-12.86)	0.02	3.90 (1.10-13.86)	0.04
Grade (3 vs. 1 and 2)	4.05 (1.93-8.49)	<0.001	2.84 (1.18-6.82)	0.02
Therapy (treated vs. untreated)	0.52 (0.18-1.49)	0.22	0.74 (0.24-2.26)	0.60
Breast Cancer-Specific Survival				
LowA vs. Others	5.17 (2.36-11.35)	<0.001	3.98 (1.74-9.12)	0.001
Age	1.08 (0.73-1.60)	0.70	1.20 (0.74-1.92)	0.46
Tumor size	1.81 (1.24-2.64)	0.002	1.43 (0.91-2.27)	0.12
ER (negative vs. positive)	10.15 (3.80-27.07)	<0.001	5.38 (1.35-21.54)	0.02
PR (negative vs. positive)	3.94 (1.70-9.13)	0.001	0.99 (0.31-3.16)	0.99
Lymph Nodes (positive vs. negative)	6.49 (1.91-22.02)	0.003	3.46 (0.94-12.75)	0.06
(axillary dissection not performed vs. negative)	6.20 (1.39-27.70)	0.02	8.47 (1.71-41.93)	0.009
Grade (3 vs. 1 and 2)	6.03 (2.60-14.00)	<0.001	3.08 (1.17-8.13)	0.02
Therapy (treated vs. untreated)	0.61 (0.18-2.03)	0.42	0.82 (0.22-3.00)	0.76
Disease-Free Survival				
LowA vs. Others	2.47 (1.31-4.69)	0.005	2.32 (1.19-4.53)	0.01
Age	0.97 (0.72-1.31)	0.86	0.97 (0.70-1.33)	0.83
Tumor size	1.70 (1.27-2.28)	<0.001	1.47 (1.06-2.05)	0.02
ER (negative vs. positive)	3.04 (1.66- 5.54)	<0.001	1.40 (0.55-3.55)	0.48
PR (negative vs. positive)	2.46 (1.35-4.49)	0.003	1.41 (0.58-3.42)	0.44
Lymph Nodes (positive vs. negative)	4.23 (1.94-9.22)	<0.001	2.83 (1.24-6.44)	0.01
(axillary dissection not performed vs. negative)	3.06 (1.00-9.37)	0.05	4.02 (1.24-13.04)	0.02
Grade (3 vs. 1 and 2)	3.06 (1.68-5.58)	<0.001	2.28 (1.13-4.59)	0.02
Therapy (treated vs. untreated)	0.66 (0.26-1.68)	0.39	0.76 (0.28-2.04)	0.58
Distant Disease-Free Survival				
LowA vs. Others	2.95 (1.53-5.68)	0.001	2.88 (1.44-5.78)	0.003
Age	0.95 (0.70-1.30)	0.77	0.94 (0.67-1.32)	0.72
Tumor size	1.76 (1.30-2.39)	<0.001	1.48 (1.04-2.10)	0.03
ER (negative vs. positive)	3.38 (1.79-6.38)	<0.001	1.67 (0.62-4.50)	0.31
PR (negative vs. positive)	2.50 (1.33 -4.72)	0.005	1.26 (0.49-3.22)	0.63
Lymph Nodes (positive vs. negative)	4.49 (1.96-10.29)	<0.001	2.96 (1.23-7.10)	0.02
(axillary dissection not performed vs. negative)	2.76 (0.81-9.44)	0.11	3.72 (1.03-13.46)	0.05
Grade (3 vs. 1 and 2)	3.01 (1.61- 5.66)	0.001	2.17 (1.05-4.51)	0.04
Therapy (treated vs. untreated)	0.60 (0.24-1.55)	0.30	0.61 (0.22-1.68)	0.34

NOTE: Cox proportional hazards model was used for unadjusted, univariate and adjusted, multivariate analyses. 50 patients were excluded due to incomplete data regarding tumor characteristics.

*Multivariate analysis included adjustment for age, tumor size, lymph nodes, tumor grade, estrogen receptor, progesterone receptor, and therapy.

†LowA refers to the combination of patients with low plasma SDF-1 levels and SDF-1-3'A polymorphism versus Others (low SDF-1 + GG genotype, or high SDF-1 + AA/AG, or high SDF-1 + GG genotype)

Connecting Text

The last chapter demonstrated the significance of low plasma SDF-1 as the first host-derived blood marker predictive of distant metastasis in breast cancer. In order to determine if tumor expression of CXCR4 can modulate the prognostic effect of this host-derived marker, we constructed a tissue microarray to determine the tumor expression of CXCR4 for the same patients we previously measured the plasma SDF-1 levels. The third chapter discusses the prognostic value of CXCR4 and the activated form of the receptor, phosphorylated-CXCR4, both alone, and in combination with low plasma SDF-1 levels.

CHAPTER 3 – MANUSCRIPT 2

The Influence of Tumor-Host Interactions in the SDF-1/CXCR4 Ligand/Receptor Axis in Determining Metastatic Risk in Breast Cancer

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3.1 Abstract

The stromal cell-derived factor (SDF-1) chemokine may function to home in CXCR4-expressing cancer cells to metastatic organs. We previously demonstrated that low plasma SDF-1, a host-derived marker, increases distant metastatic risk in breast cancer. We hypothesized that tumors over-expressing the CXCR4 receptor had an enhanced ability to metastasize in patients with low plasma SDF-1 levels. We also determined the prognostic significance of activated CXCR4, or phosphorylated (p)-CXCR4 and CXCR7, another receptor for SDF-1. Immunohistochemistry was performed on a tissue microarray built using 237 samples from the same cohort of patients for which we measured plasma SDF-1 levels. We found that the prognostic value of p-CXCR4 expression (hazard ratio (HR) 3.95; P = 0.004) was superior to total CXCR4 expression (HR, 3.20; P = 0.03). The rate of breast cancer-specific mortality was much higher in patients with both high p-CXCR4 expression and low plasma SDF-1 levels (HR, 5.96; P < 0.001), than either low plasma SDF-1 (HR, 3.59; P = 0.01) or high p-CXCR4 expression (HR, 3.83; P = 0.005) alone. The added prognostic value of low plasma SDF-1 was only effective in patients with high p-CXCR4 expression, and as such, provides clinical validation for modulation of the metastatic potential of tumor cells by an inherent host-derived metastatic risk factor.

3.2 Introduction

Breast cancer is the second most common cancer in women and represents a major risk to women's lives because of the life-threatening consequences of metastatic disease[1]. The process of metastasis has often been reported as a cascade of events, with emphasis placed upon the tumor cell and its potential to proliferate, invade into the circulation, exit the bloodstream and grow at the metastatic site[2]. However, little is known about the manner in which the host can modulate tumor progression and the propensity of the tumor to metastasize. Indeed, the role of the host was recognized over a century ago in the "seed and soil" theory, whereby the presence of a "congenial" environment of the host metastatic organ influenced the colonization of tumor cells at specific distant organs[3]. More recently, a chemokine-receptor model was proposed to help explain the manner in which the host influences the homing of cancer cells to specific target organs. Muller et al. proposed that chemokines, such as stromal cell-derived factor (SDF)-1, are normally over-expressed by those target organs to which breast cancer metastasizes, such as lung, liver, and bone, and serve to attract breast cancer cells that express their receptors, such as CXCR4[4]. Various animal studies have subsequently demonstrated the functional role of CXCR4 as the prime chemokine receptor involved in distant metastasis in breast and other types of cancers[5-9].

Several studies, including our own, have since observed an association between CXCR4 expression and distant metastasis in primary breast cancer patients[10-13]. Furthermore, we recently identified circulating levels of SDF-1 as a prognostic blood marker in a series of patients with primary breast cancers. Interestingly, circulating SDF-1 levels were found to be independent from tumor-derived SDF-1, and as such, plasma SDF-1 is the first candidate host-derived blood marker in breast cancer. We found that a low plasma SDF-1 level was predictive of distant metastasis, suggesting that low SDF-1 in the circulation may favor the extravasation of tumor cells from the circulation to the metastatic site[14]. In accordance with Muller's hypothesis, the differential concentration gradient of SDF-1, that is, low blood SDF-1 and high tissue SDF-1 at the metastatic site, may enhance the homing of CXCR4-expressing cancer cells. In this case, it would be expected that tumors with high CXCR4 expression would be especially sensitive to the SDF-1 gradient at metastatic target organs. To test this hypothesis, we determined the

expression of CXCR4 in primary tumors using the same cohort of breast cancer patients in which we previously measured plasma SDF-1 levels. We determined if patients with an innate susceptibility for metastasis, associated with low levels of plasma SDF-1, demonstrated a greater risk of metastasis when their tumors expressed higher levels of CXCR4. In addition to tumor expression of CXCR4, we also measured the levels of the phosphorylated CXCR4 receptor as a means of quantifying CXCR4 activity, and compared its expression in the primary tumor and metastatic lymph nodes. We also measured tumor expression of the two factors which may activate CXCR4: its ligand SDF-1, and another chemokine receptor, CXCR7, which may activate CXCR4 via heterodimerization[15]. In this way, we provide a more detailed picture of metastatic risk associated with the activity of CXCR4 in the primary breast tumor, and relate it with the risk of metastasis associated with low plasma SDF-1 levels.

3.3 Materials and Methods

Patients. We used the same cohort of patients as described in ref.[14]. 305 patients with primary breast cancers of stages I, II, and III were recruited from 2000-2003 with a median follow-up of 3.3 years, with informed consent, as per the Research Ethics Committee of the Centre Hospitalier de l'Université de Montréal (CHUM). 37 patients were excluded due to unavailability of tissue blocks. 31 patients were further excluded due to absence of the prognostic tumor lesion, leaving 237 patients for correlation with clinico-pathological characteristics and survival analysis. Tissue cores from the microarray were damaged for up to 3 other patients, resulting in a minimum of 234 patients. Due to incomplete data available regarding HER2 status from the pathology reports, HER2 was re-stained using our tissue microarray. Nine percent of the patients were HER2 positive by immunohistochemistry (either 2+ or 3+), and 18% were estrogen receptor (ER) negative/progesterone receptor (PR) negative/HER2 negative (ER-/PR-/HER2-), also known as triple negative. Corresponding plasma samples were available for 212 patients.

Western Blot Analysis. Human umbilical vein endothelial cells (HUVECs; Cambrex Bio Science, Walkersville, MD) were serum starved for 3 hours prior to being stimulated with recombinant human SDF-1 (R&D Systems, Minneapolis, MN). Cell lysates were

prepared using lysis buffer consisting of 1% Triton X-100, 25 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, and 5 mmol/L EDTA was supplemented with protease inhibitor cocktail set III (Calbiochem) and 1mM sodium orthovanadate. Cell lysates (30 μg protein) were solubilized in NuPAGE lithium dodecyl sulfate sample buffer, incubated at 37°C for 30 minutes, and run through 10% NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA). After transfer, Immobilon-P membranes (Millipore, Billerica, MA) were incubated overnight with antibody against p-CXCR4 (courtesy of Dr. Joshua Rubin**,** Washington University, St. Louis, MO, 1:1000)[16]. Relative protein expression levels were estimated by membrane rehybridization with anti-mouse CD184 (2B11, 1:250, BD Pharmingen, San Jose, CA). Antibody detection was performed using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

Tissue microarray construction. Formalin-fixed, paraffin embedded tissue blocks were collected from the Department of Pathology from the CHUM. All blocks were re-sectioned and stained for hematoxylin phloxine saffron stain prior to marking of histological lesions. We constructed a tissue microarray (TMA) as described in ref.[17], using a Manual Tissue Arrayer I (Beecher, Sun Prairie, WI). In total, 1619 cores were punched and distributed into four recipient blocks. Lesions were placed in either duplicate or triplicate cores adjacent to one another. Six micron sections were cut using the tape transfer system (Instrumedics, St. Louis, MO).

Immunohistochemistry. Immunohistochemistry was performed via the labeled streptavidin biotin method for p-CXCR4, SDF-1, CXCR7, and Ki67 as we previously described[10]. Primary antibodies and concentrations used were: p-CXCR4, (courtesy of Dr. Joshua Rubin) at a 1/250 dilution; SDF-1, (MAB350, clone 79018, R&D Systems) at 10 μg/mL; CXCR7 (MAB4227, clone 358426, R&D Systems) at 10 μg/mL; and Ki67 at a 1:50 dilution (M7240, clone MIB-1, Dako, Denmark). All primary antibodies were incubated overnight at 4°C. A biotin-labeled secondary antibody was used, either goat anti-mouse at 2.4 μg/mL or 9 μg/mL for Ki67 (Cat. No. 115-065-003, Jackson Immunoresearch Laboratories, West Grove, PA) or goat anti-rabbit at 2.75 μg/mL (Cat. No. 111-065-003, Jackson Immunoresearch Laboratories). Biotin detection was performed with peroxidase-conjugated streptavidin (Cat. No. 016-030-084, Jackson Immunoresearch Laboratories) at 0.2 μg/mL for p-CXCR4, 0.1 μg/mL for SDF-1, 0.08

μg/mL for CXCR7, and 0.25 μg/mL for Ki67. CXCR4 expression was detected using a biotin-labeled CXCR4 antagonist, TN14003, synthesized in the Saragovi laboratory, following reported methods[6, 18]. The staining intensity (0, 1, 2, 3) and percentage of positively-stained cells (0 to 100%) were scored in a blinded manner.

Statistical Analysis. Analysis for each biomarker was carried out using the product score, whereby the product of the staining intensity and percentage of positive cells of the cytoplasm was used to create a continuous score from 0 to 300. The product score of each biomarker was analyzed as both a continuous variable for correlations between biomarkers and with clinico-pathological characteristics, and as a categorical variable, for survival and comparative analysis between primary tumor and lymph nodes. The product score was divided into low, medium, and high expression categories using outcome-derived cutpoints from X-tile (Version 3.6.1, Robert Camp, Yale University of New Haven, CT[19]). For survival analysis, high expression of the biomarker was compared with low expression, whereas the medium and high categories were combined and termed as "high" for all other categorical variable analysis. Correlations between biomarkers and clinico-pathological characteristics were performed using Spearman's rank correlation and with Chi square or Fisher's exact test for categorical variable analysis, as previously described[14]. Clinico-pathological correlations examined include age, tumor size, lymph nodes, stage, tumor grade, ER, PR, HER2 status, triple negative disease. Survival analysis was performed for breast cancer-specific survival and distant disease-free survival as described in ref.[14]. Since this is the first survival analysis of p-CXCR4 in cancer patients, an *a priori* sample size could not be determined. Survival analysis was first performed on X-tile from which cut-points were obtained with subsequent cross-validation. Subsequently, a Cox proportional hazards regression model[20] was used for univariate (n = 237) and multivariate (n = 196) analysis. Covariates included in multivariate analysis were: age, tumor size, lymph node status, tumor grade, ER, PR, and neoadjuvant or adjuvant hormonal therapy or chemotherapy. Correlation analysis between tissue biomarkers and plasma SDF-1 levels was performed using Spearman's rank correlation. Survival analysis for the combination of tissue biomarker and plasma SDF-1 levels was performed as described above for univariate (n = 212) and multivariate (n = 177) analysis. No statistical significance was identified for patients who were

excluded due to unavailability of blood or missing information for multivariate analysis for each endpoint. All reported p-values are two-sided. All statistical analysis was performed using STATA Version 9.2 (College Station, Texas).

3.4 Results

Correlation of p-CXCR4 with CXCR4, SDF-1 and CXCR7. In order to gain a more complete understanding of the role of the CXCR4/SDF-1 receptor/ligand axis in breast cancer, we measured the expression of total CXCR4 receptor together with phosphorylated-CXCR4, the activated form of the receptor, its ligand, SDF-1, as well as the CXCR7 receptor. To verify the specificity of the p-CXCR4 antibody, we treated HUVECs that are known to express CXCR4 endogenously, with recombinant human SDF-1 for 15 minutes. Expression of p-CXCR4 was induced upon SDF-1 stimulation of HUVECs (Fig. 3-1). In order to detect the expression of total CXCR4, we synthesized a biotinylated-anti-CXCR4 peptide, biotinylated-TN14003, as this peptide was previously reported to show greater specificity in immunohistochemistry in comparison with a commercially available antibody[6]. Immunohistochemical analysis of p-CXCR4 and CXCR4 from the tissue microarray revealed cytoplasmic and nuclear expression for both biomarkers (Fig. 3-2A,B,C,D). Cytoplasmic p-CXCR4 was expressed at moderate to high levels in 47% of breast tumors (See Supplemental Figure S1 at *http://ajp.amjpathol.org*). Expression of cytoplasmic p-CXCR4 correlated positively with tumor progression (rho = 0.42, $P < 0.0001$): 9% of normal lesions, 54% of DCIS, 47% of tumors, and 54.8% of lymph nodes demonstrated high expression of p-CXCR4, showing that stage 0, I, II and III breast cancer had much higher levels of p-CXCR4 expression than normal breast tissues (See Supplemental Figure S2 at *http://ajp.amjpathol.org*). Levels of cytoplasmic tumor p-CXCR4 expression correlated strongly with both cytoplasmic CXCR4 (rho = 0.58, $P < 0.0001$) and nuclear CXCR4 (rho = 0.54, $P < 0.0001$) expression. These results are in concordance with our previous results for CXCR4[10]. For the sake of simplicity, from here onwards, we will only refer to the cytoplasmic expression of both markers. To further understand the significance of p-CXCR4, we examined the expression of SDF-1 and CXCR7. SDF-1 expression was found mainly in the cytoplasm of tumor cells (Fig. 3-3A,B). Interestingly, expression of

SDF-1 correlated positively with p-CXCR4 (rho = 0.19, P =0.004), but not with CXCR4 (rho = 0.05, P = 0.41), suggesting that autocrine stimulation of CXCR4 may contribute to CXCR4 phosphorylation in breast tumors. CXCR7 was predominantly expressed in the cytoplasm, and less so in the nucleus, and thus we refer only to cytoplasmic expression (Fig. 3-3C,D). A strong positive correlation was found between CXCR7 and SDF-1 (rho = 0.32, P < 0.0001), CXCR4 (rho = 0.45, P < 0.0001) and, above all, p-CXCR4 (rho = 0.49, P < 0.0001) expression. Therefore it is plausible that the phosphorylation of CXCR4 may be induced by SDF-1 and/or by co-expression of CXCR4 and CXCR7 in breast cancers (Table 3-1).

P-CXCR4 has a better prognostic value than CXCR4. To determine the prognostic significance of CXCR4 and p-CXCR4 expression, survival analysis was performed using the product score. Categories of low, medium, and high expression were obtained using cutpoints derived from the X-tile software. For CXCR4, the population was divided into low (41%, with product score (PS) ranging from 0-53.3), medium (30%; PS, 55.6-130), and high (29%; PS, 131-300). For p-CXCR4, the population was divided into low (53%; PS, 0-150), medium (24%; PS, 152-203) and high (24%; PS, 209-300). High p-CXCR4 expression demonstrated a greater prognostic value than high CXCR4 expression for breast cancer-specific survival and distant disease-free survival in univariate analysis. Patients with high p-CXCR4 expression demonstrated a 4-fold higher rate of death (hazard ratio (HR); 3.95, 95% CI, 1.55-10.03; P = 0.004) due to breast cancer-related causes, which is greater than that for patients with high CXCR4 expression (HR, 3.20; 95% CI, 1.09-9.37; P = 0.03). Furthermore, for the risk of distant-disease-free survival, high p-CXCR4 expression exhibited greater significance (HR, 2.38; 95% CI, 1.13-5.00, P = 0.02) than high total CXCR4 expression (HR, 1.90; 95% CI, 0.85-4.25, P = 0.12). To determine if p-CXCR4 or CXCR4 is an independent marker for survival, multivariate analysis was performed for both endpoints, and no statistical significance was found for either p-CXCR4 or CXCR4 (data not shown). Although this may be due to the small size of our patient cohort, the superiority of p-CXCR4 over CXCR4 in breast cancer-specific survival and distant disease-free survival suggests that p-CXCR4 expression may be a more sensitive marker than CXCR4 expression for metastatic risk.

P-CXCR4 enhances prognostic value of plasma SDF-1 level. In order to better understand the metastatic risk of high p-CXCR4 expression in the context of host-derived risk, we examined the prognostic significance of p-CXCR4 in combination with blood SDF-1 levels. We previously measured SDF-1 blood levels from the same cohort of breast cancer patients and found that plasma SDF-1 is a host-derived marker predictive of distant metastasis[14]. To confirm once again that circulating SDF-1 levels are independent of the tumor, we compared the tumor expression of SDF-1with plasma SDF-1 levels and found no correlation between the two variables (rho = -0.08, P = 0.23). We now investigated the prognosis of patients who expressed both high levels of p-CXCR4 and low plasma SDF-1 levels. Using the median value of plasma SDF-1, as in our previous study, the cohort was again divided into two groups, high and low SDF-1. Patients with both a low plasma SDF-1 level and high p-CXCR4 expression (n=29, or 14% of the entire cohort) (Table 3-2) demonstrated a significant correlation with the development of distant metastasis (rho = 0.25, P = 0.0003), stronger than that of plasma SDF-1 alone (rho = -0.17, P = 0.01). As there were 22 fewer patients for whom both plasma and tissue samples were available (n = 212), the prognostic value for each variable was re-calculated for breast cancer-specific survival and revealed similar values: low plasma SDF-1 (HR, 3.59; 95% CI, 1.33-9.74; P = 0.01) and high p-CXCR4 (HR, 3.83; 95% CI, 1.49-9.90; P = 0.005) (Fig. 3-4A,B). Patients with the combination of both low plasma SDF-1 and high p-CXCR4 showed a very poor prognosis (HR, 5.96; 95% CI, 2.57-13.81; P < 0.001) (Fig. 3-4C), which remained significant after multivariate analysis (adjusted HR, 3.78; 95% CI, 1.31-10.94; P = 0.01). After adjustment for Ki67-LI (Labelling index) (n=142), a marker for cellular proliferation, the combination remained significant for breast cancer-specific survival (HR 3.70; 95% CI, 1.02-11.48; P = 0.005).

A similar enhancing effect was also apparent in distant disease-free survival whereby patients with the combination showed an almost 4-fold greater rate of distant recurrence (HR, 3.75; 95% CI, 1.82-7.76, P <0.001), greater than either biomarker alone: low plasma SDF-1 (HR, 2.15; P = 0.04); high p-CXCR4 (HR, 2.31; P = 0.03). The combination was also significant after multivariate analysis for distant disease-free survival (adjusted HR, 2.80; 95% CI, 1.14-6.83; P = 0.02). On the other hand, patients with both low levels of p-CXCR4 expression and low plasma SDF-1 levels did not

exhibit a significantly poorer prognosis for breast cancer-specific survival (HR, 0.69; 95% CI, 0.23-2.04; P = 0.50) or distant disease-free survival (HR, 0.78; 95% CI, 0.36-1.69, P = 0.52) than the remainder of the entire cohort. Therefore, the poor prognostic value that we observed in patients with low plasma SDF-1 levels is enhanced in patients with tumors showing high expression of p-CXCR4, and not low p-CXCR4. No interaction between plasma SDF-1 and p-CXCR4 expression was observed in univariate or multivariate analysis for both endpoints (data not shown). Therefore, the prognostic value of high tumor p-CXCR4 and low plasma SDF-1 levels are independent from one another, reflecting the independent source of each marker. Thus, we have identified a specific cohort of primary breast cancers that express high levels of p-CXCR4, suggesting a propensity for significant CXCR4 activity, whose later extravasation into metastatic target sites may be especially promoted in the presence of low plasma SDF-1 levels. We also examined the prognostic value of tumor expression of total CXCR4 and plasma SDF-1 levels. We found that patients with high CXCR4 tumor expression and low plasma SDF-1 demonstrated a significantly worse prognosis due to breast cancer-related causes (HR, 3.45; 95% CI, 1.49-7.99; P = 0.004), compared to patients with both low plasma SDF-1 and low CXCR4 expression (HR, 0.96; 95% CI, 0.33-2.83; P = 0.95). Therefore, in patients with low plasma SDF-1 levels, tumor metastasis appears to be promoted particularly in cancer cells which express high levels of p-CXCR4 or CXCR4.

Elevated expression of CXCR4 in lymph nodes. If tumor cells expressing high CXCR4 or p-CXCR4 are more likely to metastasize, we would expect to find more of these cells in the first site of metastasis, regional lymph nodes. Lymph nodes were available for 34 patients with their matched primary tumor also present on the TMA. Although the frequency of elevated p-CXCR4 expression was the same in primary tumor and lymph nodes, 88% of patients demonstrated high total CXCR4 expression in the lymph nodes, in comparison to 64% in the primary tumor, which was statistically significant via McNemar's test (P = 0.02). Paired t-test analysis demonstrated a significant difference in the product score of CXCR4 in lymph nodes compared to primary tumor (P < 0.0001), which was not the case for p-CXCR4. 64% of patients demonstrated a higher product score of CXCR4 in the lymph nodes versus primary tumor. Therefore, these results suggest that tumor cells with higher expression of the CXCR4 receptor are more likely to

undergo regional metastasis. Given the lack of difference in p-CXCR4 expression levels between the matched primary tumor and lymph nodes, it may be that the microenvironment of the lymph nodes does not particularly select for activation of the CXCR4 receptor.

Clinical implications for tumor expression of p-CXCR4, CXCR4, and CXCR7.

CXCR4 and p-CXCR4 expression were correlated with clinico-pathological characteristics using Spearman's rank correlation analysis (Table 3-3). Categorical variable analysis of low and high expression of each biomarker is provided in Supplemental Table S1 at *http://ajp.amjpathol.org*. We found that levels of both CXCR4 and p-CXCR4 expression inversely correlate with ER and PR positivity, and positively correlate with tumor grade and ER-/PR-/HER2- (triple negative) status. Triple negative tumors were almost twice as likely to have medium or high expression of p-CXCR4 as all other tumors (77% vs 41%, P<0.001).

Interaction analysis from the multivariate Cox model of p-CXCR4 revealed an interaction between p-CXCR4 expression and ER status (HR, 0.13; 95% CI, 0.02-1.12; P = 0.06) such that ER-positive patients with high p-CXCR4 expression (15% of the total) had a 6.5-fold worse prognosis than all other ER-positive patients (HR, 6.49; 95% CI, 1.08-38.9; P = 0.04). This remained essentially unchanged after multivariate analysis (HR, 6.40; 95% CI, 0.95-42.9; P = 0.06). No such correlation with survival was found with high phosphorylated-CXCR4 expression in ER-negative patients. Thus, despite the otherwise good prognosis of all ER-positive patients, high p-CXCR4 expression has the power to identify a subset of these patients with poor prognosis. Furthermore, subgroup analysis revealed that the prognostic value of the combination was the greatest among patients with Luminal A or B subtype (ER+/PR+/HER2-, or ER+/PR+/HER2+) (n= 133; HR 8.08, 95% CI, 2.28-28.7; P = 0.001) and the least in the triple negative group of breast cancers (n=33, HR 2.24, 95% CI, 0.60-8.37; P = 0.23). Thus the presence of the combination marker appears to have a great effect in ER+ breast cancers, while it may not contribute as much to prognostic information in triple negative breast cancers (although numbers in these subgroup analyses are limited).

Due to the functional significance of CXCR7 previously reported in breast cancer tumorigenesis and metastasis[21, 22], we analysed the clinical relevance of CXCR7. High

expression of CXCR7 was associated with poorer outcome in breast cancer-specific survival (HR, 3.63; 95% CI, 1.35-9.76; P = 0.01), and distant disease-free survival (HR, 2.21; 95% CI, 1.00-4.87; P = 0.05), both of which were not significant after multivariate analysis (data not shown).

3.5 Discussion

To date, much of the cancer literature has interpreted the metastatic process to be largely dependent upon the aggressive potential of the tumor and its ability to invade surrounding tissues and metastasize. However, in addition to the tumor, recent evidence has introduced the significance of the host and its role in predicting metastatic propensity. For example, a genetic influence upon metastatic progression has been observed: a Swedish study reported that mothers and daughters of patients with breast cancer of poor outcome who developed breast cancer themselves demonstrated poor prognosis like their first degree relatives[23]. We previously identified the first host-derived blood marker predictive of distant metastasis in breast cancer, the SDF-1 chemokine[14], and found that low levels of plasma SDF-1 were predictive of distant metastasis, suggesting that the concentration gradient of SDF-1 between metastatic site and plasma may play a critical role in promoting the extravasation of cancer cells. Since we and others have previously shown an association between overexpression of CXCR4 in primary breast tumors and metastatic risk[10-12], we investigated whether the metastatic potential of CXCR4 over-expression could be further augmented in the context of low blood SDF-1 levels. Indeed, we found that patients who showed both low blood SDF-1 levels and high tumor CXCR4 expression demonstrated a significantly worse prognosis in comparison to patients with low plasma SDF-1 levels whose tumors did not express high levels of CXCR4. These results suggest that a low plasma SDF-1 level may favor the extravasation of tumor cells expressing high CXCR4. This hypothesis is further corroborated by the higher levels of CXCR4 expression we observed in lymph nodes compared to matched primary tumors, although the mechanism of lymphatic dissemination may be different from hematogenous spread. Enrichment for CXCR4 expressing tumor cells at the metastatic site has been reported previously[24, 25]. The tumor-derived risk of metastasis (CXCR4) was thus enhanced with an intrinsic host-derived risk (SDF-1). As a result, we present here, for the

first time, evidence for a biologically plausible scenario, providing insight into a dysfunctional relationship between the tumor and its host, which impacts the capacity of breast cancers to form metastasis.

We also found that over-expression of CXCR4 was frequently associated with activation of CXCR4 via phosphorylation. Consistent with a previous report in brain tumors[16], we found that expression of p-CXCR4, and not total CXCR4, highly correlated with SDF-1 expression, suggestive of the presence of autocrine stimulation of CXCR4 in primary breast tumors. Furthermore, p-CXCR4 expression also correlated strongly with the expression of CXCR7, a recently discovered receptor for SDF-1, implying that heterodimerization of CXCR7 with CXCR4[15, 21] may also contribute to activation of CXCR4 patients with primary breast cancers. We then found that high expression of p-CXCR4 is predictive of a 4-fold higher rate of breast cancer-specific mortality, and that the prognostic value of high p-CXCR4 is superior to that of high CXCR4 expression for breast cancer-specific survival and distant disease-free survival. Moreover, patients with both high p-CXCR4 levels and low blood SDF-1 levels had a nearly 6-fold higher rate of mortality due to breast cancer related causes, which was more significant than either p-CXCR4 expression or plasma SDF-1 levels alone, and remained significant after multivariate analysis. Although our immunohistochemical analysis deals with the primary tumor and not the distant metastatic site, the presence of activated CXCR4 receptor in this setting may imply a particular dependence of the tumor cell on its CXCR4 receptor, facilitating the selection of CXCR4 expressing cells during the metastatic process.

Finally, several therapeutic agents have been designed to target the SDF-1/CXCR4 ligand/receptor axis[26-28], one of which is presently being tested in a clinical trial[29]. In pre-clinical models, such treatments have been shown to be effective not only in decreasing metastasis from breast cancer, but also in inhibiting primary tumor growth in breast cancer [5, 6]. As most breast cancers express at least moderate to high levels of CXCR4[10, 11, 30], there is a risk that these therapeutic agents may not be adequately targeted, perhaps impeding their clinical development. Since we found that most (77%) patients with triple negative disease express high levels of p-CXCR4, it is possible that these patients who do not benefit from hormonal or anti-HER2 therapy may potentially

benefit from agents targeting CXCR4 activity. Most interestingly, we also identified a subset of ER+ patients with high p-CXCR4 expression/low plasma SDF-1 that demonstrated an 8-fold higher risk of mortality, who may also potentially benefit from anti-CXCR4 therapy. In these good prognosis patients, the measurement of p-CXCR4 tumor expression and plasma SDF-1 may contribute most to provide novel prognostic and potentially predictive information. Although our findings will require follow-up and validation with independent clinical material, elevated p-CXCR4 together with low plasma SDF-1 levels may provide a new paradigm for breast cancer biomarkers, highlighting the interaction between corresponding host and tumor molecular factors.

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Figures

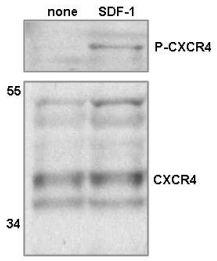


Fig. 3-1. Detection of CXCR4 phosphorylation in primary human endothelial cells. HUVECs were incubated in medium alone or were treated with SDF-1 (100 ng/ml) for 15 minutes. p-CXCR4 expression was evaluated by immunoblotting with an antibody to p-CXCR4 and reblotting with an anti-mouse CXCR4 (CD184) antibody. Results are representative of three independent experiments.

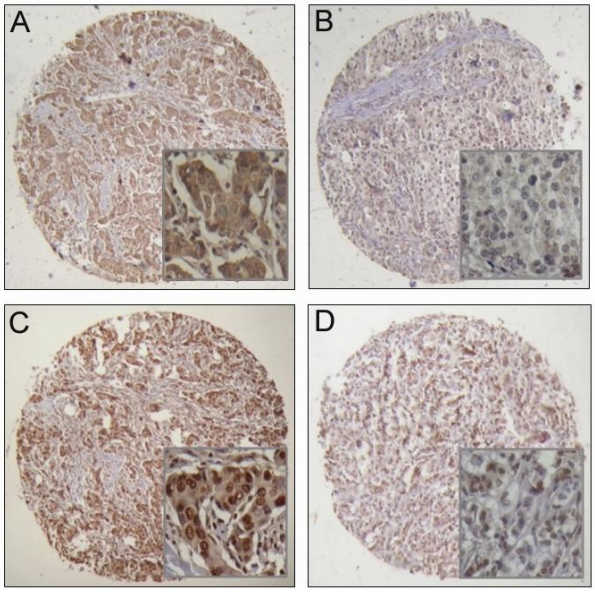


Fig. 3-2. Immunohistochemical analysis of phosphorylated (p)-CXCR4 and CXCR4 using 20x objective lens magnification. *A*, High expression of (p)-CXCR4; *B*, Low expression of p-CXCR4; *C*, High expression of CXCR4; *D*, Low expression of CXCR4.

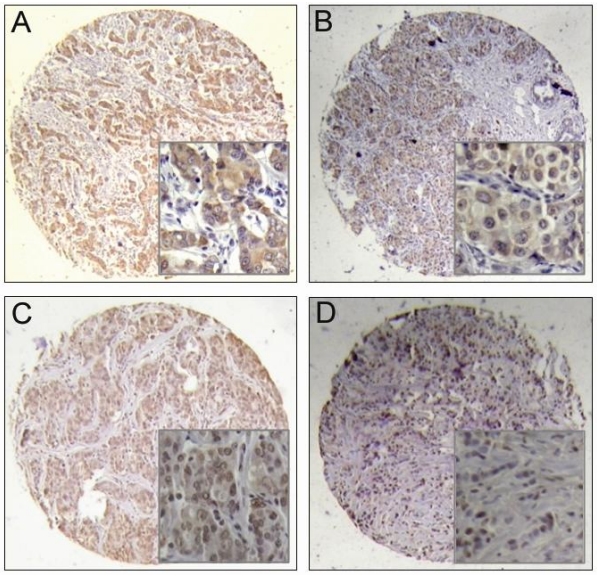


Fig. 3-3. Immunohistochemical analysis of SDF-1 and CXCR7 using 20x objective lens magnification. *A*, High expression of SDF-1; *B*, Low expression of SDF-1; *C*, High expression of CXCR7; *D*, Low expression of CXCR7.

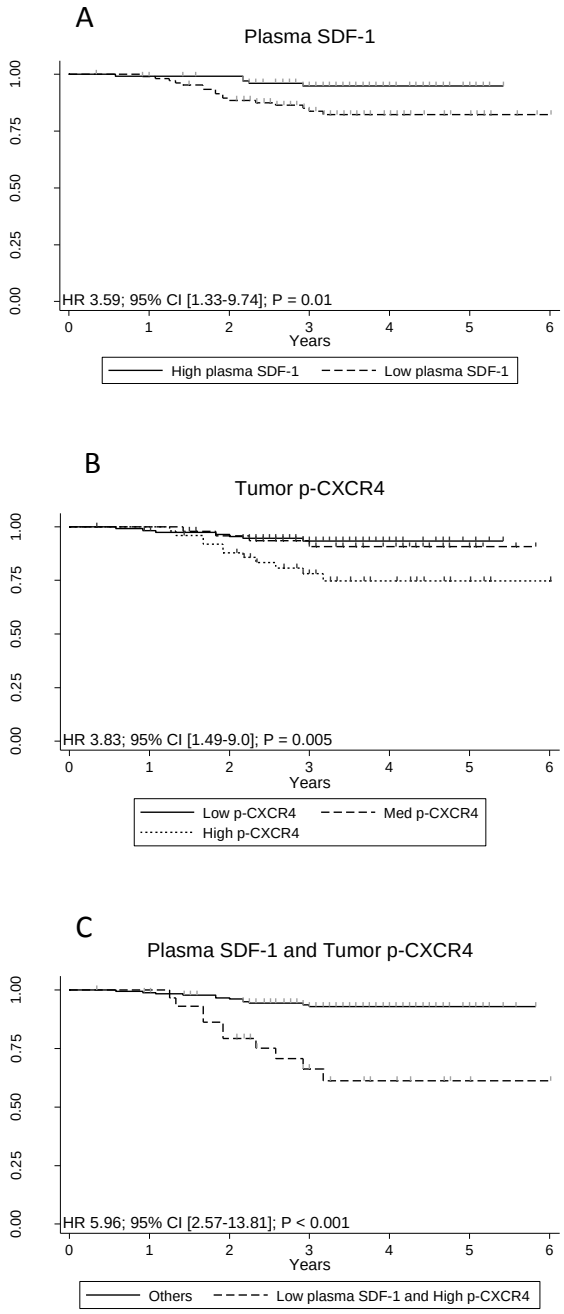


Fig. 3-4. Kaplan-Meier survival curves for breast cancer-specific survival for *A*, Plasma SDF-1; *B*, Tumor phosphorylated (p)-CXCR4; *C*, Combination of low plasma SDF-1 and high p-CXCR4.

Tables

Table 3-1. Correlation analysis between biomarkers

Variable	Rho	P-value
CXCR4 and p-CXCR4	0.58	<0.0001
SDF-1 and CXCR4	0.05	0.41
SDF-1 and p-CXCR4	0.19	0.004
CXCR7 and SDF-1	0.32	<0.0001
CXCR7 and CXCR4	0.45	<0.0001
CXCR7 and p-CXCR4	0.49	<0.0001

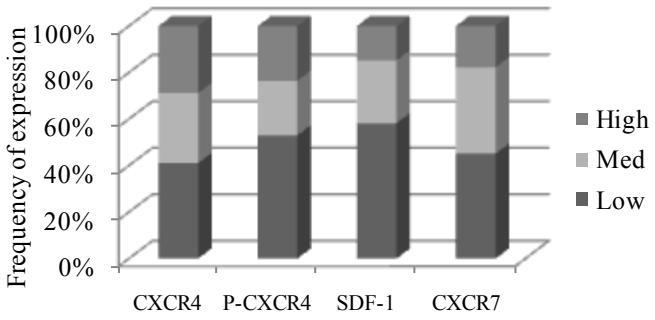
Table 3-2. Clinico-pathological properties of patients who expressed both low plasma SDF-1 and high tumor p-CXCR4

Variable	No. of Patients	(%)
Age (y)		
≤ 50	12	(41.4)
>50	17	(58.6)
Tumor size (cm)		
T1 (≤ 2)	10	(34.5)
T2 (2-5)	14	(48.3)
T3 (>5)	4	(13.8)
T4	1	(3.4)
Nodal status		
N0	13	(44.8)
N1	7	(24.1)
N2	4	(13.8)
N3	2	(6.9)
Lymphadenectomy not performed	3	(10.3)
Stage		
I	7	(24.1)
II	12	(41.4)
III	7	(24.1)
Unavailable	3	(10.3)
Tumor grade		
1	0	(0)
2	9	(31.0)
3	15	(51.7)
Unavailable	5	(17.2)
ER Status		
Negative	17	(58.6)
Positive	12	(41.4)
PR Status		
Negative	20	(69.1)
Positive	9	(31.0)
Triple Negative		
Present	13	(44.8)
Absent	13	(44.8)
Unavailable	3	(10.3)
Luminal A/B		
Present	11	(37.9)
Absent	15	(51.7)
Unavailable	3	(10.3)
HER2 status		
Positive	3	(10.3)
Negative	23	(79.3)
Unavailable	3	(10.3)

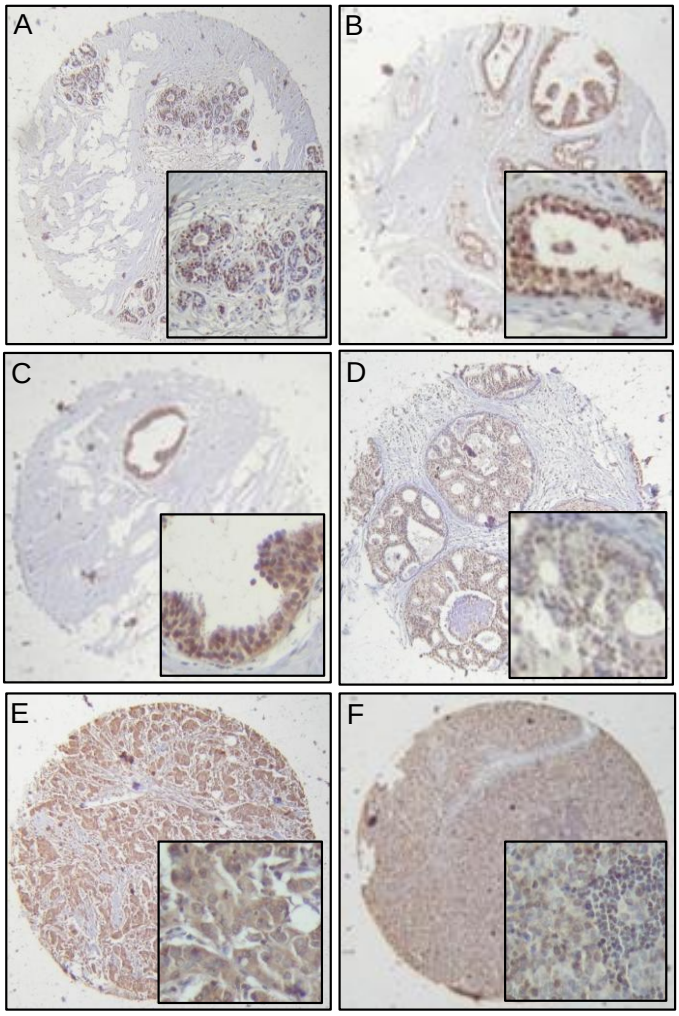
Table 3-3. Clinico-pathological correlations of tumor CXCR4, P-CXCR4, and CXCR7 expression.

Variable	CXCR4		P-CXCR4		CXCR7	
	Rho	*P-Value*	*Rho*	*P-value*	*Rho*	*P-value*
Age	-0.02	0.73	-0.05	0.41	0.08	0.21
Tumor Size	0.11	0.11	0.13	0.05	0.02	0.76
Lymph Nodes	-0.02	0.76	0.01	0.84	0.005	0.94
Stage	-0.002	0.98	0.08	0.25	0.03	0.71
Grade	0.35	<0.0001	0.37	<0.0001	0.14	0.04
ER positivity	-0.27	<0.0001	-0.32	<0.0001	-0.18	0.007
PR positivity	-0.26	<0.0001	-0.31	<0.0001	-0.20	0.002
HER2+	-0.06	0.39	0.09	0.23	0.004	0.95
ER-/PR-/HER2-	0.37	<0.0001	0.33	<0.0001	0.14	0.05

Supplementary Material



Supplemental Fig. S1: Frequency distribution of expression of 4 biomarkers: CXCR4, phosphorylated (p)-CXCR4, SDF-1, and CXCR7.



Supplemental Fig. S2: Expression of phosphorylated (p)-CXCR4 increases with tumor progression. Representative images of p-CXCR4 expression via immunohistochemistry using 20x objective lens magnification in *A*, Normal breast tissue; *B*, Columnar cell change; *C*, Atypical Ductal Hyperplasia; *D*, Ductal Carcinoma In-Situ; *E*, Tumor; *F*; Lymph Node.

Supplementary Table S1. Clinico-pathological correlations of low and high expression of CXCR4, P-CXCR4, and CXCR7.

Variable	CXCR4			P-CXCR4			CXCR7		
	Low	*High*	*P-value*	*Low*	*High*	*P-value*	*Low*	*High*	*P-value*
	N (%)	*N (%)*		*N (%)*	*N (%)*		*N (%)*	*N (%)*	
Age (yrs)									
≤ 40	7 (7.2)	8 (5.8)	0.82	9 (7.3)	6 (5.5)	0.69	9 (8.5)	6 (4.6)	0.56
41-50	24 (24.7)	41 (29.5)		31 (25)	34 (30.9)		31 (29.3)	34 (26.2)	
51-65	28 (28.9)	41 (29.5)		36 (29.0)	33 (30.0)		29 (27.4)	41 (31.5)	
≥ 66	38 (39.2)	49 (35.3)		48 (38.7)	37 (33.6)		37 (34.9)	49 (37.7)	
Tumor Size (cm)									
T1 (≤ 2)	51 (52.6)	59 (42.5)	0.21	66 (53.2)	44 (40.0)	0.07	54 (50.5)	56 (43.1)	0.27
T2 (2-5)	35 (36.1)	63 (45.3)		43 (34.7)	53 (48.2)		41 (38.3)	57 (43.9)	
T3 (> 5)	4 (4.1)	11 (7.9)		6 (4.8)	9 (8.2)		4 (3.7)	11 (8.5)	
T4	7 (7.2)	6 (4.3)		9 (7.3)	4 (3.6)		8 (7.5)	6 (4.6)	
Nodal Status									
N0	45 (53.6)	63 (51.2)	0.86	58 (52.7)	50 (51.6)	0.97	46 (50.6)	62 (52.5)	0.64
N1	22 (26.2)	35 (28.5)		29 (26.4)	27 (27.8)		29 (31.9)	29 (24.6)	
N2	11 (13.1)	19 (15.5)		16 (14.6)	15 (15.5)		12 (13.2)	19 (16.1)	
N3	6 (7.1)	6 (4.9)		7 (6.4)	5 (5.2)		4 (4.4)	8 (6.8)	
Stage									
I	28 (33.3)	35 (28.2)	0.53	39 (35.1)	24 (24.7)	0.15	28 (30.4)	35 (29.7)	0.56
II	34 (40.5)	60 (48.4)		43 (38.7)	50 (51.6)		44 (47.8)	50 (42.4)	
III	22 (26.2)	29 (23.4)		29 (26.1)	23 (23.7)		20 (21.7)	33 (28.0)	
Grade									
1	17 (21.5)	15 (12.5)	<0.001	22 (21.2)	8 (8.8)	<0.001	19 (20.7)	13 (12.2)	0.05
2	52 (65.8)	59 (49.2)		68 (65.4)	41 (45.1)		54 (58.7)	57 (53.3)	
3	10 (12.7)	46 (38.3)		14 (13.5)	42 (46.2)		19 (20.7)	37 (34.6)	
ER status									
Negative	15 (15.6)	51 (37.0)	<0.001	19 (15.5)	46 (42.2)	<0.001	20 (18.7)	46 (35.9)	0.003
Positive	81 (84.4)	87 (63.0)		104 (84.6)	63 (57.8)		87 (81.3)	82 (64.1)	
PR status									
Negative	22 (23.2)	62 (44.9)	0.001	27 (22.1)	57 (52.3)	<0.001	26 (24.5)	59 (46.1)	0.001
Positive	73 (76.8)	76 (55.1)		95 (77.9)	52 (47.7)		80 (75.5)	69 (53.9)	
HER2 status									
Negative	71 (91.0)	108 (91.5)	0.90	97 (93.3)	83 (89.3)	0.32	81 (93.1)	100 (90.1)	0.45
Positive	7 (9.0)	10 (8.5)		7 (6.7)	10 (10.8)		6 (6.9)	11 (9.9)	
ER-/PR-/HER2-									
Yes	4 (5.2)	31 (26.5)	<0.001	8 (7.8)	27 (29.4)	<0.001	9 (10.5)	27 (24.8)	0.01
No	73 (94.8)	86 (73.5)		94 (92.2)	65 (70.7)		77 (89.5)	82 (75.2)	

Connecting Text

Overexpressed in 67% of breast cancers, CXCR4 has been shown to be a significant prognostic marker in breast cancer. The previous chapter demonstrated that p-CXCR4 was a superior prognostic marker to CXCR4. Moreover, the prognostic value of p-CXCR4 was further enhanced amongst patients with already an elevated metastatic risk, that is, with low plasma SDF-1 levels. We thus provided further rationale from patient samples that CXCR4 is a plausible therapeutic target. In Chapter 4, we evaluated the role of a CXCR4 antagonist alone, and in combination with other anti-cancer therapies, in a transgenic mouse model to determine its role upon primary tumor growth and metastasis inhibition.

CHAPTER 4 – MANUSCRIPT 3

Targeting CXCR4 with a peptide antagonist inhibits distant metastasis, primary tumor growth, and enhances the efficacy of anti-VEGF treatment or docetaxel in a transgenic mouse model of breast cancer

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In final preparation, for submission to Breast Cancer Research and Treatment

4.1 Abstract

Purpose: CXCR4 is a chemokine receptor implicated in the homing of cancer cells to target metastatic organs which overexpress its ligand, stromal cell-derived factor (SDF)-1. To determine the efficacy of targeting CXCR4 upon primary tumor growth and metastasis, we used a peptide inhibitor of CXCR4, CTCE-9908, that was administered in a clinically relevant approach using a transgenic mouse model.

Experimental Design: We first performed a dosing trial of CTCE-9908 in the PyMT mouse model, testing 25, 50, and 100 mg/kg versus the scrambled peptide in groups of 8-16 mice. We then combined CTCE-9908 with docetaxel or DC101 (an anti-VEGFR2 monoclonal antibody).

Results: We found that 50 mg/kg of CTCE-9908 (CTCE-9908-50) slowed the rate of tumor growth with a 29% inhibition of tumor volume at necropsy ($P = 0.02$). We found that reductions in levels of phosphorylated-AKT correlated with increasing doses of CTCE-9908. CTCE-9908-50 also inhibited lung metastasis by 40% ($P = 0.07$). In combination with docetaxel, 25 mg/kg of CTCE-9908 (CTCE-9908-25) demonstrated an additive effect, with a 38% decrease in tumor volume ($P = 0.02$), compared to 19% with docetaxel alone. In combination with DC101, CTCE-9908-50 also demonstrated an additive effect, with a 37% decrease in primary tumor volume ($P = 0.02$) and a 75% reduction in distant metastasis ($P = 0.009$).

Conclusion: As a single agent, CTCE-9908 inhibited primary tumor growth and metastasis in a transgenic breast cancer mouse model. In combination with docetaxel or an anti-angiogenic agent, these effects were markedly enhanced.

4.2 Introduction

Although the survival of patients with breast cancer has improved in recent years partly due to therapeutic advances, further progress will require a better understanding of the metastatic process, which is the cause of breast cancer mortality. The process of metastasis has been divided into several stages including the growth of the primary tumor, invasion into the bloodstream, circulation, extravasation, and proliferation at the distant metastatic site (1). A recently proposed model that explains the process of extravasation of tumor cells from the peripheral circulation into target organs of metastasis is the chemokine-receptor model. Muller et al. showed that chemokines are overexpressed by those organs to which cancer metastasizes and serves to attract cancer cells which express their receptor (2), analogous to their function in recruiting inflammatory cells to sites of tissue injury. They identified the chemokine/receptor pair, stromal cell-derived factor (SDF)-1/CXCR4 as a candidate metastasis promoter in breast cancer (2). We have since shown that CXCR4 is overexpressed in 67% of breast cancer samples and that elevated expression of CXCR4 carries a poor prognosis and is strongly associated with HER2 status in these tumors (3). Therefore, it appears plausible that inhibition of CXCR4 activity may hinder the metastatic process. Moreover, the SDF-1/CXCR4 ligand/receptor pair has also been implicated in regulating primary tumor growth. Overexpression of SDF-1 in cancer-associated fibroblasts present in the tumor microenvironment was reported to promote primary tumor growth by inducing tumor neo-angiogenesis via both paracrine (direct stimulation of tumor CXCR4), and endocrine (recruitment of endothelial progenitor cells from the bone marrow) mechanisms (4). Hence, targeting CXCR4 may be effective in inhibiting primary tumor growth as well as the formation of distant metastases. Indeed, blocking CXCR4 activity has been found to inhibit primary tumor growth and metastasis in xenograft models, albeit using treatment strategies that may not translate well to the clinic (5-7).

To target CXCR4, we selected a peptide antagonist that is most advanced in clinical development for solid tumors. CTCE-9908 (Chemokine Therapeutics Corp, Vancouver, BC), is an SDF-1 analog consisting of a dimer of the first 8 amino acids of SDF-1 (Supplementary Fig. S1A), and serves as a competitive inhibitor to SDF-1.

Radioligand binding assays have shown that CTCE-9908 competitively binds to CXCR4. CTCE-9908 has previously been reported to inhibit metastasis in osteosarcoma and melanoma mouse models (8). Unlike other CXCR4 antagonists, evaluation of this CXCR4 antagonist has already begun in patients. Safety of CTCE-9908 has been demonstrated in both a single dose Phase I trial in healthy adults and in a Phase I/II trial in cancer patients (9). To test the efficacy and toxicity of CTCE-9908 in a breast cancer model, as well as in combination with other treatments, we selected a well-known transgenic mouse model for breast cancer, the mouse mammary tumor virus (MMTV)-driven Polyoma Middle T Antigen (PyMT) model. The PyMT mammary tumor has been shown to overexpress HER2/neu, and demonstrates progression from hyperplasia to pre-invasive, invasive, and distant metastasis (10). Thus, this model may be especially appropriate to test both the growth inhibitory and the anti-metastatic potential of anti-CXCR4 therapy. We proposed to examine the efficacy of CTCE-9908 in the PyMT mouse model both alone, and in combination with other anti-cancer therapies such as an anti-angiogenic agent or a cytotoxic agent commonly used in breast cancer patients.

4.3 Materials and Methods

Mice. All experimental procedures were conducted according to McGill University Animal Care Committee. FVB/N TgN (MMTV-PyMT)^{634} male mice were obtained from Dr. William Muller (McGill University, Québec, Canada), and were mated with FVB/N from Taconic Farms (Albany, New York). Female mice heterozygous for the PyMT oncogene were identified by extracting tail DNA as per (11) using PCR to amplify a 540 base pair fragment with the following primers: forward, 5'-GGA AGC AAG TAC TTC ACA AGG G-3'; reverse, 5'-GGA AAG TCA CTA GGA GCA GGG - 3'.

Pharmacological Agents. CTCE-9908 was obtained from Chemokine Therapeutics Corp and reconstituted in sterile water for injection. The control used for CTCE-9908 was either the scrambled peptide, SC-9908 (Supplementary Fig. S1B), reconstituted in sterile water for injection at a dose of 25 mg/kg, or its vehicle, sterile water for injection. Docetaxel (Sanofi-Aventis; Jewish General Hospital Oncology Pharmacy) was diluted in normal saline; normal saline was used as the vehicle control. To target the vascular

endothelial growth factor (VEGF) pathway in this transgenic mouse model, we used DC101, an anti-VEGF receptor 2 (VEGFR2) rat IgG1 monoclonal antibody (courtesy of Imclone Systems Incorporated, New York, NY), with its control purified rat IgG (Chrompure rat IgG, Jackson Immunoresearch, West Grove, PA).

Three major experiments were conducted in total. First, a CTCE-9908 dosing experiment was performed to identify the most effective dose in the PyMT model. CTCE-9908 was administered at 25, 50, and 100 mg/kg subcutaneously, at four alternating sites of injection, starting from 7 weeks of age, 5 days per week for a total of 4.5 weeks (Supplementary Fig. S2A). Second, CTCE-9908 was given at 25 mg/kg in combination with docetaxel (Supplementary Fig. S2B). One dose of docetaxel was administered first, and in the subsequent week, CTCE-9908 was started 5 days per week. A total of 3 doses of docetaxel were administered, 1 dose per week at 35 mg/kg intraperitoneally (I.P.). Third, CTCE-9908 was given at 25 mg/kg concomitantly with DC101 at 1000 ug per dose I.P. twice per week for a total duration of 4.5 weeks (Supplementary Fig. S2C). We also tested CTCE-9908 at 50 mg/kg administered simultaneously with DC101 at 400 ug per dose twice per week for a total duration of 4.5 weeks. All experiments were designed such that the mean age at sacrifice of the mice from each treatment or control group was 82 days, thereby allowing every mouse the same lifespan for tumor growth and metastasis formation (12). An average of 8-16 mice were dosed in each treatment group in each of the three major experiments.

Tumor monitoring. All tumors from each of the mammary fat pads (total number 10) were measured twice a week using calipers. Length and width of each tumor were measured in mm and tumor volume was estimated using the following formula: $(x^2*y)/2$, where x refers to the shortest diameter, and y refers to the larger diameter (13). Tumors from all 10 fat pads were summed up together. After the final dose of treatment was administered, mice were sacrificed within 24 hours. All 10 tumors were harvested and fragments were either snap-frozen or fixed in 10% buffered formalin for paraffin embedding. Lungs were examined, macroscopic nodules counted, weighed and fixed in formalin. Kidneys, liver, spleen, and spine were also grossly examined.

Protein extraction, western blot analysis. Proteins from 5 representative mice from each group (total 20 mice) from the dosing trial were extracted from frozen tumor samples

with Cell Lysis Buffer (Cat. No. 9803, Cell Signalling Technology, Danvers, MA) supplemented with phenylmethylsulphonyl fluoride (PMSF) (Cat No. P7626; Sigma, St Louis, MO). Protein concentrations were measured via spectrophotometry using the bicinchoninic acid (BCA) kit for protein determination (Cat. No. BCA-1, Sigma). For western blot analysis, Hybond ECL nitrocellulose membranes (Cat No. RPN203D, Amersham Biosciences) were used with the enhanced chemiluminescence method (Cat No. RPN2132, Amersham ECL Plus Western Blotting Detection Reagent; GE Healthcare, Buckinghamshire, UK). The following primary antibodies were used: Phospho-Akt (Ser473) (D9E) (Cat No. 4060, Cell Signalling Technology); Akt (pan) (C67E7) (Cat No. 4691, Cell Signalling Technology). Protein detection was carried out as per manufacturer's protocol. Images were acquired on GeneSnap from SynGene software (Version 6.08, Cambridge, England), and quantification of western blot bands were performed on GeneTools from SynGene software (Version 3.06, Cambridge, England).

Immunohistochemistry. Expression of CXCR4 in the PyMT model was verified on a section of a tissue microarray constructed from a total of 33 tumors and in-situ lesions obtained from 11 different mice, using immunohistochemistry via the labeled-streptavidin method. CXCR4 expression was measured using anti-CXCR4 antibody (Cat No. 2074, Abcam, Cambridge MA) at 1/50 dilution overnight at 4°C. The secondary antibody used was a biotin-labeled, goat anti-rabbit (Cat. No. 111-065-003, Jackson Immunoresearch Laboratories), at 2.75 μg/mL. Paraffin embedded tissue sections of mammary tumors from 5 representative mice were stained for Ki67 and TUNEL. Ki-67 was stained with a monoclonal rat anti-mouse antibody (Cat No. M7249, Clone TEC-3, DakoCytomation, Mississauga, ON) at a dilution of 1:25 overnight at 4°C. Secondary antibody conjugation was performed with a biotinylated polyclonal rabbit anti-rat antibody (Cat No. E0468, DakoCytomation) at a dilution of 1:50. TUNEL visualization was performed using the In Situ Cell Death Detection Kit, POD (Cat No. 11 684 817 910, Roche Applied Science, Laval, QC). Positive and negative controls were first verified as per protocol, followed by labeling of paraffin-embedded samples. Images were visualized on a UNICO H602 compound microscope. Images were acquired on a 10x objective lens using a Nikon digital camera.

Statistical Analysis. Kruskal-Wallis tests were used to determine a difference in the median value between the control or treatment groups. Statistical significance between each treatment and control group was determined using Mann Whitney tests. P-values < 0.05 were considered statistically significant. All statistical analysis was performed using Graph Pad Prism 4.

4.4 Results

CXCR4 is expressed in PyMT mouse tumor. We first verified the degree of CXCR4 protein expression in mammary tumors from the PyMT mouse model. Since it was reported that the rate of tumor growth differed between the anterior and posterior tumors (14), we determined the expression of CXCR4 in different tumors from the same mouse. We examined the expression of CXCR4 in 18 tumors from 7 untreated mice. All 18 tumors demonstrated ≥ 50% cells positive nuclear staining, and 17/18 tumors demonstrated ≥ 50% cells positive for cytoplasmic staining. Thus, the expression of CXCR4 is uniform amongst different mice and within different tumors from the same mouse (Supplementary Fig. S3).

CXCR4 antagonist delays primary tumor growth rate. Previous reports that have studied CXCR4 antagonists in xenograft cancer models have either pre-mixed the cancer cell line with the antagonist or administered the antagonist prior to the injection of the cancer cell line (5, 15). To maximize the clinical relevance of our results, we opted for a more rigorous approach. Since breast tumors are diagnosed and treated only when they become clinically evident (often palpable), we first performed a dosing trial in the PyMT mouse with the CXCR4 antagonist administered only when the first tumors became palpable. We tested three doses of CTCE-9908: 25 (CTCE-9908-25), 50 (CTCE-9908-50) and 100 (CTCE-9908-100) mg/kg and compared it to a scrambled peptide. Treatment with CTCE-9908 for 2.5 weeks already resulted in a delay in the growth of the primary tumor ($P_{trend} = 0.01$) (Fig. 1A). A maximal effect upon primary tumor growth inhibition was observed at 3.5 weeks, whereby there was a 10% decrease in tumor volume with CTCE-9908-25 (P = 0.42), a 45% reduction in tumor volume with 50 mg/kg (P = 0.005), and a 56% reduction was observed with 100 mg/kg (P = 0.003). However, after 4 weeks

of treatment, the inhibitory effect of CTCE-9908 at 50 mg/kg and 100 mg/kg was less pronounced (Fig. 1B). At necropsy, a statistically significant trend was identified for tumor inhibition with the three doses of CTCE-9908 ($P_{trend} = 0.02$). No significant effect was observed with CTCE-9908-25 (P = 0.75), however an inhibitory effect was ultimately observed with 50 mg/kg (23% reduction, P = 0.07) and 100 mg/kg (36% reduction, P = 0.05). To summarize, although CTCE-9908-25 did not impact tumor volume, CTCE-9908 administered at 50 and 100 mg/kg slowed tumor growth at 2.5 weeks of treatment, with the maximal inhibitory effect obtained at 3.5 weeks. Thereafter, the rate of tumor growth subsequently increased, such that the reduction of tumor volume was less evident at necropsy.

CXCR4 antagonist decreases cellular proliferation in primary tumor. To determine if the reduction in primary tumor growth was due to a decrease in cellular proliferation or an increase in apoptotic cell death, we measured the immunohistochemical expression of Ki67 and performed the TUNEL assay in tumors obtained at necropsy of five representative mice from each dosing group. The majority of the nuclei staining positively for Ki67 were identified at the proliferating edge of the tumor. Quantification of the positive cells revealed that 40% of the cells treated with the scrambled peptide stained positively for Ki67. Although there was a decrease in the proportion of cells that were positive for Ki67 amongst the mice treated with CTCE-9908 ranging from 8 to 11% compared with scrambled peptide (Fig. 2A and B), this difference was not statistically significant (P = 0.57) (Fig. 2C). To determine the contribution of apoptosis to cell survival secondary to CTCE-9908 treatment, we examined the staining of TUNEL on representative mice from each dosing group. We did not find any statistically significant difference between control and each treatment group (data not shown). This lack of difference may be due to our observation that most tumors had begun to escape CXCR4 inhibition at the time of necropsy.

CXCR4 antagonist inhibits AKT activity in primary tumor. We next examined the activity of AKT, a critical mediator of cell survival and migration downstream of the CXCR4 receptor. To determine if blocking CXCR4 with CTCE-9908 could affect AKT

activity in the mouse tumors, we measured the expression of phosphorylated (p)-AKT in relation to total AKT from protein cell lysates obtained from five representative mice per dosing group. There were no changes in p-AKT/AKT expression with CTCE-9908-25 versus control. Although not statistically significant, a 14% reduction in expression of p-AKT/AKT was observed with CTCE-9908-50 (P = 0.69), while a 30% reduction in expression was seen with CTCE-9908-100 (P = 0.84). Thus, the p-AKT/AKT expression ratio may reflect the effectiveness of CTCE-9908 blockade of the CXCR4 receptor on the primary mammary tumor in our mouse model (Fig. 3A and B).

CTCE-9908 and its effect on mammary tumor metastasis. We tested the effect of each dose of CTCE-9908 on the formation of pulmonary metastasis. The number of visible lung nodules were counted at necropsy, and correlated with the number of microscopic lesions identified in five representative mice, in a blinded manner. A statistically significant correlation was obtained between the number of macroscopic nodules and microscopic metastatic lesions (rho = 1.0; P = 0.02), confirming that the nodules identified grossly could be used as surrogate markers for total metastatic tumor burden in the lungs. We found that mice treated with 25 mg/kg of CTCE-9908 showed a 32% decrease in the number of visible lung nodules in comparison with control, which was not statistically significant (P = 0.38). Administration of 50 and 100 mg/kg of CTCE-9908 resulted in similar effects of approximately 40% inhibition of lung metastasis (P = 0.07; P = 0.23, respectively) (Fig. 4). Thus, lower doses of CTCE-9908 may be more effective in preventing metastatic disease than inhibiting primary tumor growth.

CTCE-9908 combines with docetaxel to further delay primary tumor growth. Since we found a modest effect of CTCE-9908 on primary tumor growth, we combined it with a cytotoxic agent known to have activity in breast cancers, docetaxel. Docetaxel was administered at 35 mg/kg intraperitoneally, a dose that was previously reported to be non-toxic (16), and was initiated one week prior to the start of administration of 25 mg/kg of CTCE-9908. Such a dosing regimen was previously shown to be the most effective in prolonging survival in a prostate cancer xenograft model (17). CTCE-9908 was administered for a total of 4.5 weeks as above and one dose of docetaxel was

administered per week for a total of three doses, as per ref. 14. Over the course of 5.5 weeks from the start of docetaxel treatment, the maximal effect of docetaxel was noted at 3.5 weeks with a 42% decrease in tumor volume (P = 0.02), and the combination of docetaxel and CTCE-9908 resulted in a 47% reduction in tumor volume (P = 0.004) compared to scrambled peptide control (Fig. 5A). At necropsy (Fig. 5B), a 19% decrease in tumor volume was obtained with docetaxel alone (P = 0.28), while the combination of CTCE-9908 and docetaxel resulted in a doubling of this effect, a 37% decrease in tumor volume (P = 0.02). Thus, CTCE-9908 can further enhance and sustain the inhibitory effect of docetaxel upon primary mammary tumor growth.

CTCE-9908 combines with DC101 to inhibit primary tumor volume and distant metastasis. Due to the role of SDF-1 in promoting endothelial cell proliferation and tumor angiogenesis (4, 18, 19), we combined CTCE-9908 with an anti-angiogenic agent. We decided to test the anti-VEGFR2 antibody DC101 (Imclone) in our transgenic mouse model. We first tested DC101 at 1000 ug/dose, since this was the average dose previously tested in breast cancer mouse models (20, 21), in conjunction with CTCE-9908 at 25 mg/kg, a partially effective dose of CTCE-9908. We found a marked suppressive effect upon tumor growth as early as 2.5 weeks, with a 60% reduction with DC101 alone (P = 0.11), and a 65% reduction with the combination of DC101 and CTCE-9908-25 (P = 0.04). At necropsy, DC101 treatment had inhibited tumor growth by 39% (P = 0.05), whereas the combination of DC101 and CTCE-9908-25 resulted in a 48% reduction in primary tumor volume (P = 0.001) compared to treatment with scrambled peptide. No statistically significant reduction in pulmonary metastasis was noted with the combination of DC101 and CTCE-9908-25 compared with DC101 alone (data not shown). To better uncover any effect of CTCE-9908 on the activity of DC101, we increased the dose of CTCE-9908 to 50 mg/kg (maximum efficacy as shown above), and decreased DC101 to 400 ug/dose, a dose that has also been used in mouse models of different cancer types (22, 23). At this dose, our necropsy measurements showed that DC101 treatment alone was much less effective in inhibiting tumor growth (13% decrease in primary tumor volume compared to scrambled peptide P = 0.24). However, the combination of DC101 and CTCE-9908-50 resulted in a 37% decrease in primary tumor

volume (P = 0.02) compared to scrambled peptide control (Fig. 6A). This combination also resulted in a remarkable 75% inhibition in the number of visible lung metastasis (P = 0.009), compared to 58% with DC101 alone (P = 0.09) (Fig. 6B). Thus, we identified an additive effect of CTCE-9908 when combined with DC101 in inhibiting primary tumor growth and lung metastasis.

Toxicity of CTCE-9908 alone and in combination. The toxicity of CTCE-9908 alone and in combination was documented by the animals' weight prior to and 4.5 weeks post administration of the drug. No change in percentage of weight gain was noted amongst the three different doses of CTCE-9908 given in comparison to control. No change in percentage of weight was also found when docetaxel or DC101 was given alone or in combination with CTCE-9908. Furthermore, no macroscopic changes were observed in the other organs such as liver, spleen, kidney and spine.

4.5 Discussion

The SDF-1/CXCR4 ligand/receptor pair has been shown to play a critical role in many aspects of breast tumorigenesis. Although initially implicated as a key regulator of metastasis, and specifically, of the extravasation of circulating tumor cells into target metastatic organs, this ligand/receptor pair also plays a role in primary tumor growth. This may be due to the role of SDF-1/CXCR4 in recruiting endothelial precursor cells for neo-angiogenesis (4) and to its transactivation of the HER2 signaling pathway (24). Various preclinical approaches have been used to inhibit SDF-1/CXCR4 activity in primary breast tumor growth and metastasis. SiRNA knockdown of CXCR4 in implanted breast cancer cells was shown to delay the formation of lung metastases as well as the development of mammary tumors, thereby prolonging survival in mice (6). The administration of a neutralizing anti-CXCR4 mouse monoclonal antibody resulted in a marked suppression of lung metastasis in an experimental or orthotopic lung metastasis xenograft model using MDA-MB-231 breast cancer cells (2). Administration of a polypeptide inhibitor of CXCR4, TN14003, also resulted in a similar reduction in lung metastases in a similar xenograft model (5). Moreover, two recent studies using CTCE-9908, the molecule under study in the present report, in xenograft mouse models of breast

cancer revealed a significant decrease in metastatic tumor burden (15, 25). However, all of the aforementioned studies used a dosing schedule whereby the CXCR4 antagonist was either pre-mixed with the cancer cell line or started in mice prior to the injection of xenografted cancer cells. Such timing of intervention does not correspond to the timing of therapeutic intervention in the clinic, and may be one of the reasons for which preclinical models are not translating well to the clinic (7).

We sought to test the efficacy of CXCR4 blockade in a pre-clinical model that is more similar to human breast tumor progression than xenograft animal models. We selected a transgenic mouse model, the PyMT model, known to overexpress Her2/neu, and undergo tumor progression from hyperplasia to pulmonary metastasis relatively rapidly, from four to sixteen weeks of age (10). With approximately ten tumors growing simultaneously in all mammary fat pads (similar to the multicentricity of human breast cancer), the PyMT model reflects a strong genetic drive for tumor progression, making therapeutic intervention even more challenging. Furthermore, we administered the CXCR4 antagonist only when the tumor was palpable, in an attempt to determine its potential use in a clinical setting similar to that of breast cancer patients. We selected the peptide antagonist CTCE-9908 since it has demonstrated an excellent safety profile in patients (9).

We found that CTCE-9908 slowed the rate of growth of the primary tumor in the PyMT model. Administration of CTCE-9908 at 50 or 100 mg/kg resulted in a delay in tumor growth first observed after 2.5 weeks of treatment, with the maximal effect at 3.5 weeks, whereby both 50 mg/kg and 100 mg/kg of CTCE-9908 inhibited tumor growth by about 50%. Although the effect of these two doses also demonstrated a similar trend at necropsy, it was less pronounced, suggesting that the tumors were beginning to escape the inhibitory effect of the drug. This effect on tumor growth was associated with a modest decrease in cellular proliferation and AKT phosphorylation levels at the time of necropsy. As expected, the administration of CTCE-9908 also resulted in a 40% decrease in lung metastasis at the time of necropsy.

Recent experience has shown that the use of biological agents or targeted therapy alone has not met with great clinical success in the treatment of both primary and metastatic cancers. The clinical utility of these agents is often dependent on the

combination of their use with a cytotoxic agent. Moreover, it may be more effective to target more than one molecular factor involved in the aberrant cellular functions of cancer cells to inhibit tumor growth. For example, recent reports suggest that anti-VEGF treatment may enhance metastatic potential of tumors. Hence, there is rationale for combining anti-angiogenic therapy with anti-metastatic agents (26-28). Furthermore, several groups have reported the presence of a positive feedback loop whereby vascular endothelial growth factor (VEGF) upregulates CXCR4 expression, and CXCR4 stimulation promotes VEGF expression (29, 30). We found that the administration of CTCE-9908 further enhanced the inhibitory effect of both docetaxel, a cytotoxic agent, and DC101, an anti-VEGFR2 antibody. The enhancing effect that we observed with the combination studies of CTCE-9908 is in concordance with two previous reports, which examined the combination of a CXCR4 antagonist and cytotoxic chemotherapeutic agents in leukemia and glioma (31, 32). Interestingly, when CTCE-9908 was given at 50 mg/kg in combination with DC101, we observed a remarkable 75% decrease in lung metastases, underlining the value of combining anti-metastatic and anti-angiogenic approaches to inhibit the metastatic process. Moreover, we were able to demonstrate a maximal inhibitory effect upon metastasis using a lower dose of DC101. This is particularly important since anti-VEGF therapies, such as bevacizumab, are associated with significant toxicity, including mortality, in patients (33, 34). Therefore, our combinatorial drug approach may allow for a decrease in the dosing of an anti-VEGF therapy without compromising overall efficacy. We also observed the greatest sustained tumor inhibition in our transgenic model when CTCE-9908 was combined with DC101 treatment at 1000 ug/dose (48% tumor inhibition at necropsy), suggesting that combining two “biologic” therapies may result in similar efficacy to combining a single biologic with cytotoxic chemotherapy. In summary, we have demonstrated that treatment with CTCE-9908 delays tumor growth and impacts metastasis in the PyMT model using a clinically relevant treatment strategy, and can be combined with either docetaxel or DC101 to reduce tumor burden and inhibit metastasis, showing potential for future clinical trials in breast cancer.

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Figures

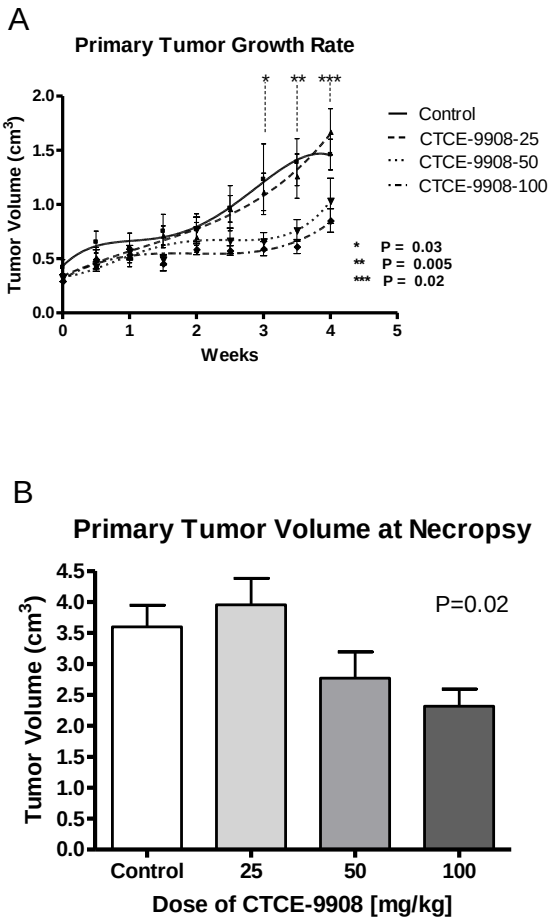


Fig. 4-1. CTCE-9908 inhibits primary tumor growth in MMTV-PyMT transgenic mice. *A,* growth curves of primary mammary tumors, representing the sum of the volume of 10 tumors from the different fat pads of each mouse, measured twice weekly with calipers. A total of 8-16 mice were treated with 3 different doses of CTCE-9908 (25 or 50 or 100 mg/kg s.c. daily for 5 days a week) or with control. The P-values refer to the differences between the control and the 50mg/kg dose of CTCE-9908 at different time points. *B,* histogram of sums of the volumes of 10 primary tumors measured at necropsy of each mouse treated with different doses of CTCE-9908, with a P-value for the trend of increasing inhibitory effect with increasing dosage equal to 0.02. Error bars refer to standard error of the mean.

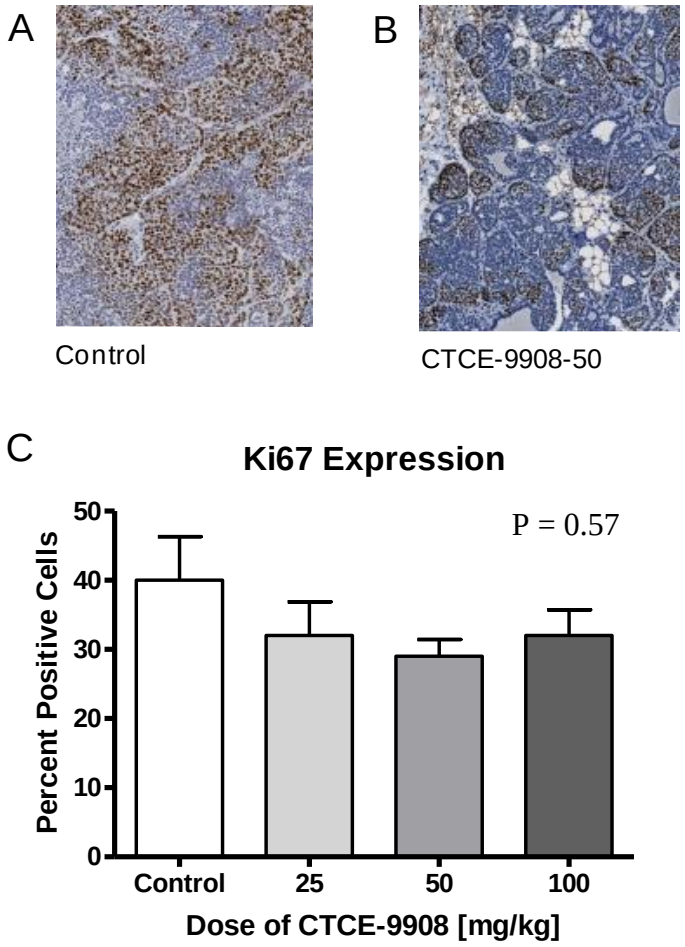


Fig. 4-2. Administration of CTCE-9908 to MMTV-PyMT transgenic mice decreases Ki-67 staining in mammary tumors. *A*, section of mammary tumor immunostained for Ki-67 from mouse treated with control scrambled peptide. *B*, section of mammary tumor immunostained for Ki-67 from mouse treated with CTCE-9908 at 50mg/kg. *C*, histogram of percentage of tumor cells showing nuclear staining with Ki-67 in mammary tumors obtained at necropsy from 5 mice from each treatment group. P – value refers to Kruskal-Wallis test. Error bars refer to standard error of the mean.

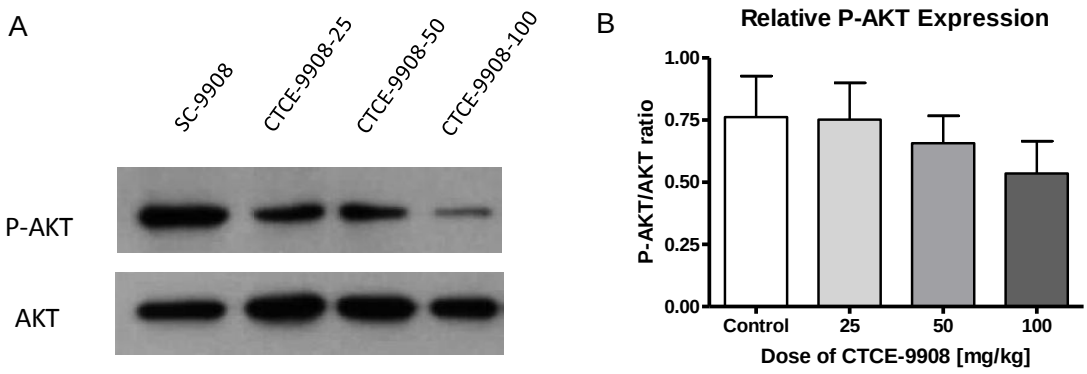


Fig. 4-3. Administration of CTCE-9908 to MMTV-PyMT transgenic mice decreases phospho-AKT expression in mammary tumors. *A*, Western blot of lysates from mammary tumors from mice treated with the 3 doses of CTCE-9908 as well as with scrambled peptide, showing increasing inhibition of phosphorylated AKT expression with increasing dose. Total AKT protein expression is used as control in lower row. *B*, histogram of relative levels of expression of phospho-AKT to total AKT protein in mammary tumors obtained at necropsy from 5 mice from each treatment group. Error bars refer to standard error of the mean.

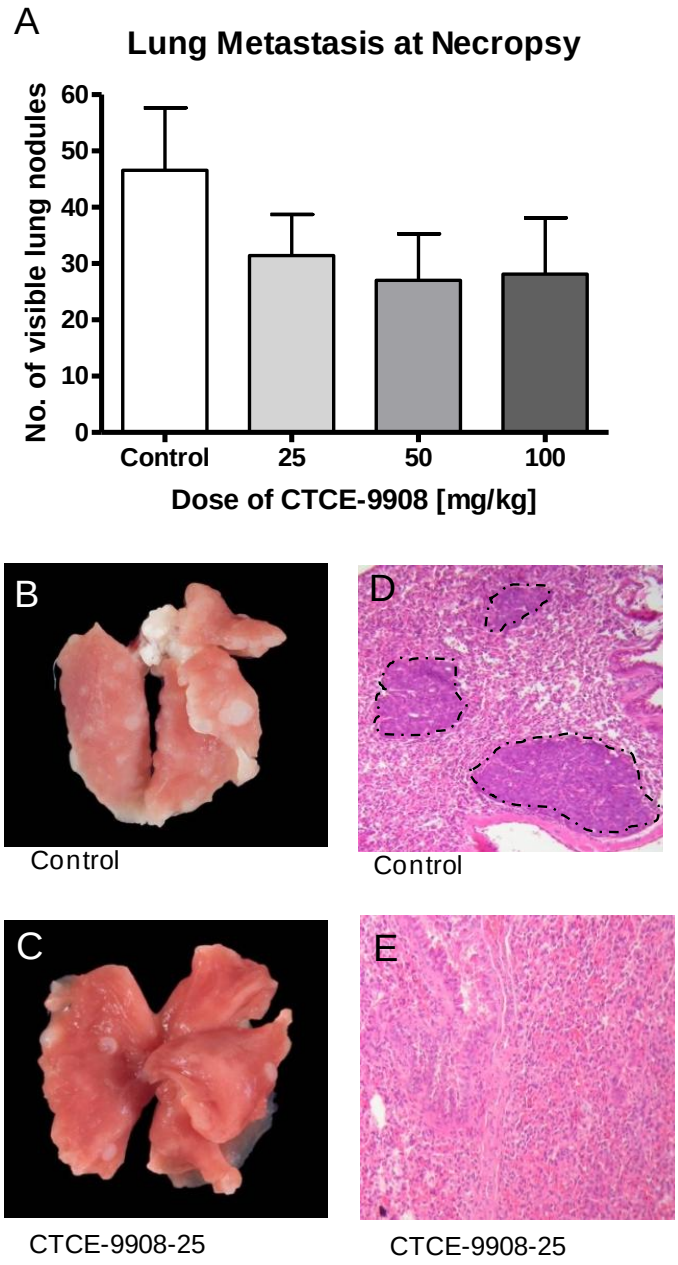


Fig. 4-4. CTCE-9908 inhibits metastatic tumor development in MMTV-PyMT transgenic mice. *A,* average number of visible tumor nodules in lungs obtained at necropsy from 8-16 mice in each treatment group. Error bars refer to standard error of the mean. *B*, whole lung from mouse treated with vehicle control. *C*, whole lung from mouse treated with 25 mg/kg of CTCE-9908. *D*, lung tissue section from control treated mouse stained with hematoxylin and eosin, showing microscopic tumor deposits within the hatched area. *E*, lung tissue section from mouse treated with CTCE-9908, stained with hematoxylin and eosin, without tumor deposits.

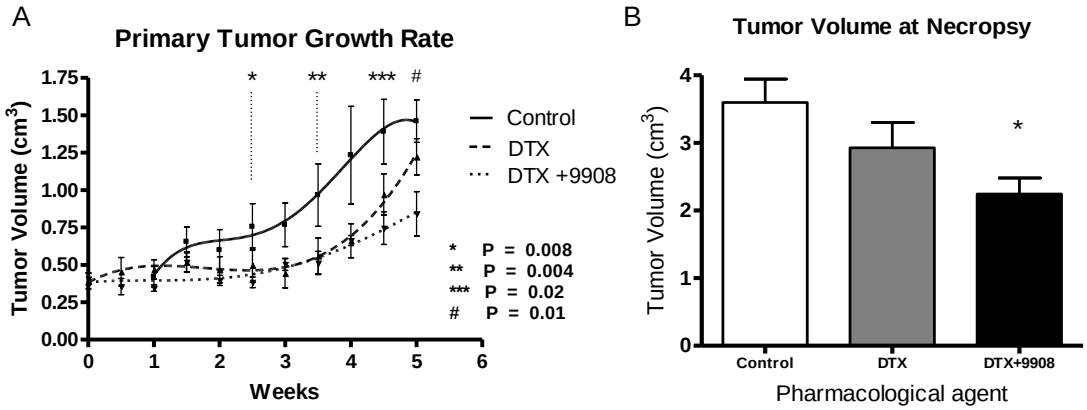


Fig. 4-5. CTCE-9908 combines with docetaxel to further inhibit primary tumor growth. *A*, growth curves of primary mammary tumors, representing the sum of the volume of 10 tumors from the different fat pads of each mouse, measured twice weekly with calipers. A total of 8-16 mice were treated with control scrambled peptide, docetaxel or a combination of docetaxel and CTCE-9908. The P-values refer to the significance of the differences between the control and the combination of docetaxel and CTCE-9908 at various time points (from 2.5 to 5 weeks). *B,* sums of the volume of 10 primary tumors from each mouse measured at necropsy treated with different doses of control scrambled peptide, docetaxel or a combination of docetaxel and CTCE-9908. P-value refers to statistical significance between the control and combination of docetaxel and CTCE-9908. Error bars refer to standard error of the mean.

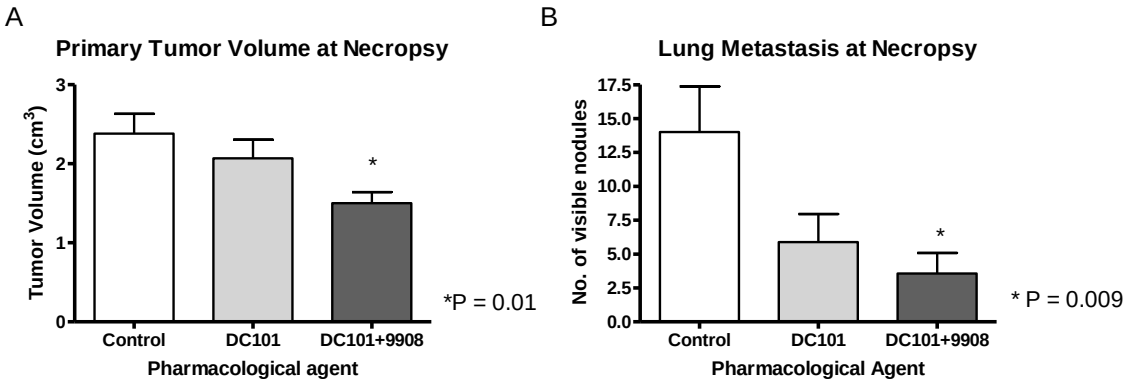
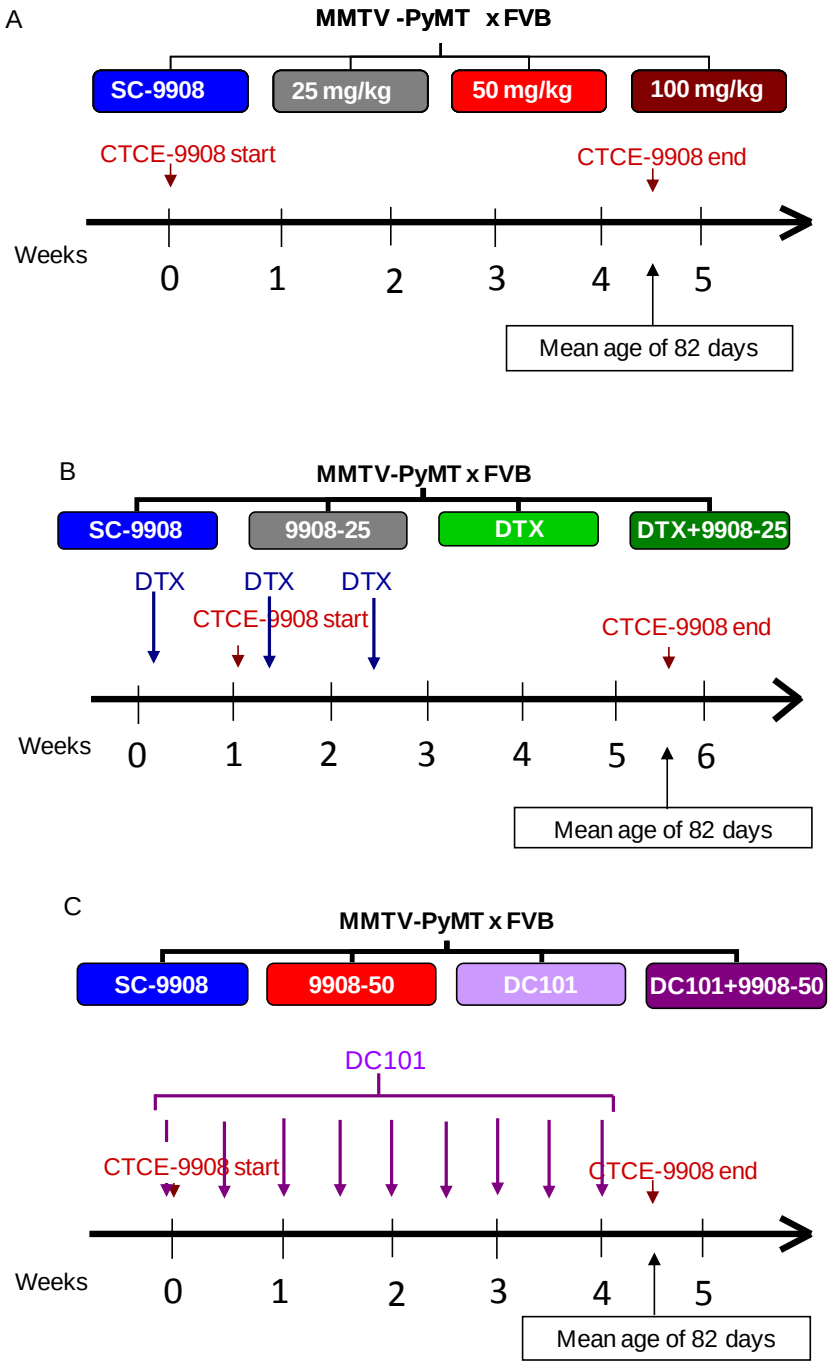


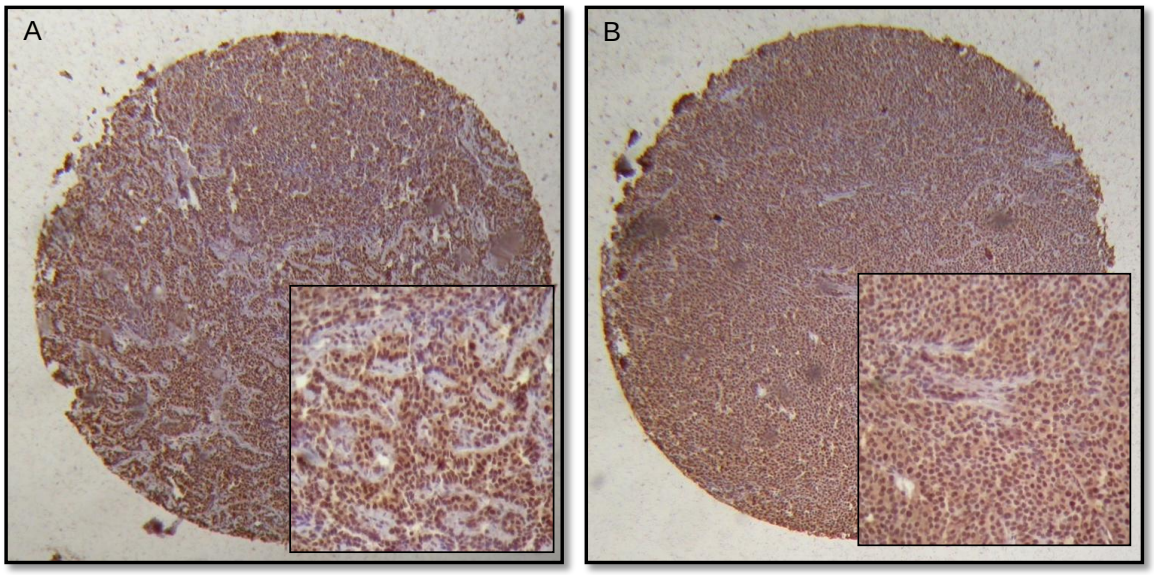
Fig. 4-6. CTCE-9908 combines with the anti-VEGFR2 antibody, DC101, to inhibit primary tumor growth in MMTV-PyMT mice with 7-9 mice per group. *A*, histogram showing the average primary tumor volumes at necropsy in mice treated with control scrambled peptide, DC101 alone and DC101 with CTCE-9908. P-value refers to level of significance of the difference between control and the combination treatment. *B*, bar graph showing the average number of macroscopic lung metastases at necropsy in mice treated with control scrambled peptide, DC101 alone and DC101 with CTCE-9908. P-value refers to level of significance of the difference between control and the combination treatment. Error bars refer to standard error of the mean.

Supplementary Material

Supplementary Fig. S1. Chemical structures of CTCE-9908 and control scrambled peptide. *A*, structure of CTCE-9908. *B*, structure of control scrambled peptide.



Supplementary Fig. S2. Dosing schedules for different treatment cohorts. *A*, dosing schedule for CTCE-9908 alone trial. *B*, dosing schedule for the combination therapy of CTCE-9908 and docetaxel. Note that docetaxel is started prior to initiation of CTCE-9908 therapy. *C*, dosing schedule for the combination therapy of CTCE-9908 and DC101.



Supplementary Fig. S3. CXCR4 immunohistochemical staining of mammary tumors from MMTV-PyMT mice. *A*, *B*, CXCR4 staining of primary mammary tumor.

CHAPTER 5 - DISCUSSION

The focus of this thesis is to provide further insight into the role of the SDF-1/CXCR4 ligand/receptor pair in the process of metastasis in breast cancer. Although much of the literature has addressed cancer metastasis by focusing upon tumor potential in animal models, we provide a new perspective in the physiology of breast cancer metastasis using blood and tissue samples from breast cancer patients. In the second chapter of this thesis, we identified, for the first time, a cohort of breast cancer patients with an innate susceptibility to develop metastatic disease because of plasma levels of a host-derived marker that is implicated in the metastatic process itself: SDF-1. Chapter three further characterizes the manner in which the tumor and the host can interact and modulate metastatic potential. We demonstrated that the rate of mortality was much higher in patients with both low levels of plasma SDF-1 and tumors with high expression of phosphorylated-CXCR4, than in patients with either risk factor alone. Hence, low levels of circulating SDF-1 may facilitate the extravasation of cancer cells that highly express p-CXCR4. Furthermore, the prognostic significance of CXCR4 suggests great potential for this molecule as a therapeutic target. In chapter four, we tested a peptide inhibitor of CXCR4 upon palpable tumors in the PyMT mouse, a transgenic model whose tumors have a strong genetic driver. Using such stringent criteria, we found that targeting CXCR4 alone delays primary tumor growth, and in combination with an anti-angiogenic agent, significantly inhibits the growth of both primary tumors and metastatic lesions. In this last chapter, I intend to highlight the results from these studies and discuss future directions emphasizing potential clinical implications.

5.1 Plasma SDF-1: a host-derived marker predictive of distant metastasis

The chemokine-receptor model postulated that overexpression of SDF-1 in metastatic organs plays an important role in the homing of cancer cells to the metastatic site in breast cancer (1). We found low levels of plasma SDF-1 to be a strong risk factor for distant metastasis, suggesting that low plasma SDF-1 may be important in the extravasation of cancer cells to the metastatic site. We also verified that the tumor was not a major contributor to plasma SDF-1 levels by detecting no significant difference in blood levels of SDF-1 prior to and post-excision of the tumor. We then turned to study a

genetic factor that may modulate SDF-1 plasma levels - a germline polymorphism: *SDF-1-3'A*. A trend, although not statistically significant, was identified, whereby patients with the AA genotype demonstrated lower plasma SDF-1 levels than patients with the wildtype, GG genotype. Patients with the AA or AG genotype also demonstrated a poorer prognosis, and in combination with low plasma SDF-1 levels, demonstrated a much poorer prognosis that ranked comparably to the presence of lymph node metastasis in multivariate analysis. We thus identified plasma SDF-1 as the first host-derived prognostic blood marker, and discovered a cohort of breast cancer patients with an innate susceptibility to develop distant metastasis.

Since the publication of our study, a few studies have reported blood levels of SDF-1 and the *SDF-1-3'A* polymorphism in cancer patients (2, 3). A recent Korean study reported serum levels of SDF-1 in a cohort of patients with gastric cancer which correlated positively with bone metastasis (3). However, the range of serum SDF-1 levels (45.2 to 62.6 ng/mL) in these cancer patients was more than 10-fold higher than the range of plasma levels that we observed in breast cancer patients. There are a few possible explanations for such differences in the range of SDF-1 levels: 1) the biology of gastric and breast cancer metastasis may be different; 2) levels of circulating SDF-1 may be different amongst cohorts of different ethnicities; 3) serum levels of SDF-1 may be different from plasma SDF-1 levels in gastric cancer patients; and 4) the authors did not use a commercially available kit to perform the ELISAs. Although further experimentation is necessary to determine with certainty which of the above reasons is/are responsible for the differences in circulating levels, the third explanation is less probable. Our laboratory has shown in a pilot study of ten healthy volunteers that the levels of serum SDF-1 fall within the same picogram/mL range as those observed in plasma (unpublished data). The fourth option appears to be the most plausible since differences of such magnitude in SDF-1 blood levels are frequently observed between studies which did and did not use a commercially available kit to perform the ELISAs (2, 4-6). This is further corroborated by the finding of much higher levels of serum VEGF in this study compared to other studies of serum VEGF levels in gastric cancer patients (7-9). Thus, it is likely that the differences in the range of SDF-1 levels may be attributed to the technique used to perform the ELISA.

More recently, a study was published discussing the significance of plasma SDF-1 in breast cancer patients (2). The mean plasma SDF-1 level in this cohort was 2448 pg/mL which was comparable to our reported mean of 2661 pg/mL. A weak, but positive correlation was found between plasma SDF-1 levels and tumor grade, and with subtypes of breast cancer associated with poor prognosis (basal, HER2). However, the authors chose to base their findings on a relatively small cohort of 114 consecutive breast cancer patients, thus weakening the statistical power of their conclusions. For instance, HER2-positive patients and the basal phenotype consisted of only 3.5% and 5% of the population, respectively. The authors also described a higher plasma SDF-1 level amongst post-menopausal women in comparison to pre-menopausal women, but no adjustment for age was done in this analysis. In addition, the authors showed that the plasma SDF-1 level in breast cancer patients was higher than in healthy controls. Although an age-adjusted correlation was performed for this analysis, other potentially confounding variables were not taken into consideration. For example, we found that a greater proportion of patients with coronary artery disease had high plasma SDF-1 levels. Nonetheless, further studies with a larger cohort would be necessary to validate these results. Another report was also published describing the role of the *SDF-1-3'A* polymorphism in prostate cancer, with a higher frequency of the AA genotype amongst those patients with lymph node metastasis in comparison to those patients with lymph node negative disease (10). These results are in concordance with our own findings in which patients with the polymorphism had a higher rate of mortality from breast cancer-related causes.

We report the first host-derived blood marker which can predict distant metastasis in breast cancer patients. These results open the door for several questions which remain to be answered: 1) What are some of the factors that can influence the variability in the levels of plasma SDF-1?; 2) How can the role of SDF-1 as a host-derived determinant of metastasis be further confirmed?; 3) How can the role of plasma SDF-1 in the process of metastasis be better understood; and 4) What is the potential role of plasma SDF-1 in the clinic?

Variability in plasma SDF-1 levels may arise either from changes in the production or degradation of SDF-1. Production of SDF-1 can be regulated at a genetic

level and degradation may result from enzyme cleavage at the protein level. Since the amount of mRNA transcript of *SDF-1* was thought to be regulated by the *SDF-1-3'A* polymorphism (11), we correlated plasma SDF-1 levels and the *SDF-1-3'A* polymorphism in breast cancer patients. We found a trend whereby levels of SDF-1 progressively decreased from the GG to AG to AA genotype. As only 4% of our cohort had the AA genotype, a larger cohort would be necessary to confirm the presence of such an association. However, a few studies have reported contradictory results regarding the correlation between the *SDF-1-3'A* polymorphism and plasma SDF-1 levels (12-14). Interestingly, recent genetic analysis in lymphoblastoid cell lines have revealed two more haplotypes (A6201G and C-668G) at the 3'untranslated site, which may be implicated in lowering SDF-1 transcript levels (15). Thus, the role of allelic variants of *SDF-1* in modulating plasma SDF-1 levels is yet to be determined in breast cancer patients. Furthermore, variability in plasma SDF-1 levels can be attributed to variations in SDF-1 degradation in plasma. It may be that the degradation of SDF-1 may be enhanced in breast cancer patients with poor prognosis. As alluded to in Chapter 1, there are several enzymes which have been shown to cleave SDF-1 at its amino and carboxy termini, including matrix metalloproteinases, carboxypeptidases, and dipeptidylpeptidase IV (16, 17). It would be interesting to determine if increased activity of such enzymes in the circulation correlates with lower SDF-1 levels in the plasma, thereby influencing metastatic potential. Therefore, a better understanding of the variability in plasma SDF-1 levels in breast cancer patients may be obtained by correlating the plasma SDF-1 levels with *SDF-1* allelic variants and circulating enzymatic activity.

The host has only recently begun to receive recognition as a determinant of metastasis. Studies in transgenic mice and population genetic studies have identified a genetic link with the development of metastasis (18-20). We showed that circulating SDF-1 is a host-derived marker. In order to further confirm its role as a predictive marker for distant metastasis, validation studies in independent cohorts of patients demonstrating a genetic origin of plasma SDF-1 and a correlation with distant metastasis may be necessary. However, the significance of SDF-1 as a host-derived genetic determinant of metastasis can also be evaluated using a transgenic mouse model. A transgenic mouse which overexpresses SDF-1 in all or most tissues can be created as

described in (21), which can then be cross-bred with the PyMT mouse. Alternatively, SDF-1 function can be downregulated by creating a conditional knockout mouse which also can be cross-bred with the PyMT mouse. Thus, the overexpression and knockdown of SDF-1 would be a means of determining if genetic modulation of SDF-1 can alter metastatic potential. Although one must be cautious since modulation of SDF-1 in all or most tissues may lead to undesired consequences in angiogenesis, inflammatory or immune responses, it is possible that minor changes in SDF-1 transgene levels may be sufficient to demonstrate an effect. We could measure the levels of plasma SDF-1 in these animals to verify the host origin of circulating SDF-1. Furthermore, these mice would serve as a useful model to determine the efficacy of anti-CXCR4 therapy in a specific context of low or high host SDF-1.

The significance of low plasma SDF-1 levels also provides a new perspective into the physiology of breast cancer metastasis. In concordance with Muller's chemokine-receptor model (1), the overexpression of SDF-1 at the target metastatic organ suggests that the concentration gradient of high SDF-1 at the target site and low SDF-1 in the circulation plays an important role in the extravasation of cancer cells to the site of metastasis. In order to verify the significance of such a concentration gradient, one could measure both SDF-1 expression from the metastatic organs and SDF-1 levels in the plasma. However, in order to confirm the predictive value of this concentration gradient, SDF-1 expression from metastatic organs and circulating SDF-1 levels would need to be measured when patients present for surgery, prior to the development of metastasis. Furthermore, the protective effect of a high level of circulating SDF-1 suggests that SDF-1 may retain cancer cells within the circulation and out of the metastatic site. This hypothesis can be validated by correlating plasma SDF-1 with the amount of circulating tumor cells (CTCs). Recent advances in technology have enabled the identification and enumeration of circulating cancer cells in a reproducible manner. The prognostic significance of more than five of these CTCs has been shown in metastatic breast cancer patients (22, 23). Although CTCs have been detected in early breast cancer, the clinical significance of these cells is not clear (24-26). Therefore, correlating plasma SDF-1 and CTCs in the adjuvant setting may offer greater insight into the metastatic process.

The clinical implications of a prognostic host-derived blood marker are multifold. However, first, the significance of plasma SDF-1 levels needs to be validated in blood samples from an independent cohort with a longer follow-up than 3.3 years. Further validation of these results in a prospective, randomized-controlled trial will also be necessary. Validation studies can help in proving the clinical significance of a biomarker in several ways such as by overcoming inherent biases that may be present in a particular cohort of patients from a single institution, or by controlling for potential confounding factors which, in this case, may influence plasma SDF-1 levels. Upon validation, it will be important to understand the clinical context in which the measurement of plasma SDF-1 can be used. Indeed, plasma SDF-1 offers the great advantage of being accessible via a simple blood test. Although important as a strong prognostic marker, it is even more important that such a factor be able to guide therapeutic decisions. Circulating SDF-1 levels may be a valuable tool in selecting patients who may benefit from adjuvant therapy. However, which therapy would be most beneficial in these patients? It appears likely that patients with low plasma SDF-1 may benefit most from CXCR4-targeted therapy (see below). Moreover, as a host-derived marker, it is plausible that SDF-1 may be useful as a prognostic marker in a broader clinical context than tumor-derived markers. It is plausible that patients who are identified with such an inherent risk for metastasis may benefit from anti-CXCR4 therapy. For example, plasma SDF-1 may have greater predictive potential in tumors that are small and are otherwise less aggressive. Patients may also benefit from anti-CXCR4 therapy that can be administered upon diagnosis, as patients wait for surgery. Plasma SDF-1 may also play a role in identifying metastatic risk amongst patients with pre-invasive lesions, such as DCIS. Furthermore, the prognostic value of plasma SDF-1 may play a role amongst those patients who already have a high inherent genetic risk for developing breast cancer. Therefore, the measurement of plasma SDF-1 as a diagnostic tool for distant metastasis may play a role in altering the therapeutic strategy of breast cancer patients.

5. 2 Plasma SDF-1 and tumor CXCR4: an example of a dysfunctional host/tumor relationship influencing metastatic potential

The prognostic significance of low plasma SDF-1 levels suggested that the differential concentration gradient of SDF-1, that is, low blood SDF-1 and high tissue SDF-1 at the metastatic site, may enhance the homing of CXCR4-expressing cancer cells to the metastatic organ. In chapter three, we report that tumors with high and not low CXCR4 expression are especially sensitive to low levels of plasma SDF-1. In addition to tumor expression of CXCR4, we also measured the levels of phosphorylated-CXCR4 receptor as a means of quantifying CXCR4 activity, and found p-CXCR4 to be a superior prognostic marker than total CXCR4. We found that patients with both high tumor p-CXCR4 and low plasma SDF-1 levels demonstrated a much poorer prognosis than either variable alone. Hence, distant metastasis was favoured in those patients at high risk from both host-derived and tumor-derived biomarkers: that is, a low plasma SDF-1 level, and a high expression of tumor p-CXCR4, respectively (Fig. 5-1). We also measured tumor expression of two factors which may activate CXCR4: its ligand SDF-1, and another chemokine receptor, CXCR7, which may activate CXCR4 via heterodimerization (27, 28). We found that the expression of both SDF-1 and CXCR7 correlated positively with p-CXCR4 expression, which may suggest the significance of CXCR7 for CXCR4 activity. Therefore, we were able to provide a more complete picture of metastatic risk associated with the activity of CXCR4 in the primary tumor.

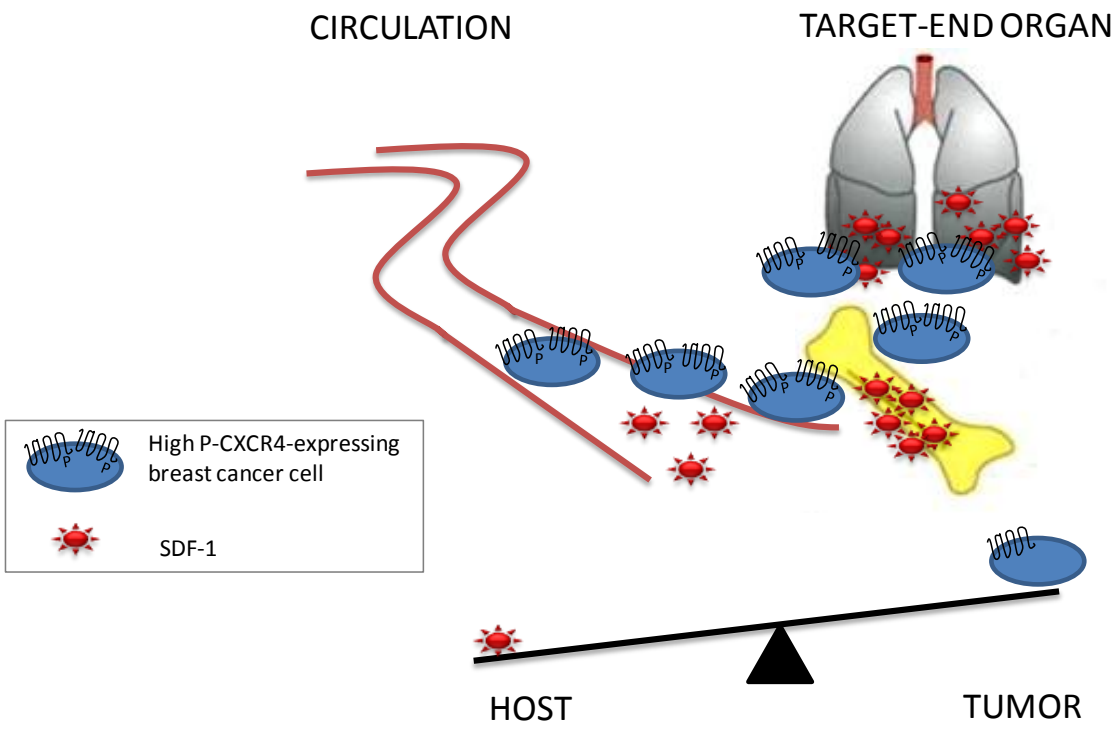


Fig. 5-1. Dysfunctional relationship between host (plasma SDF-1) and tumor (p-CXCR4 expression). Modified from Weinberg, ref. (29).

We report for the first time, the expression and prognostic significance of p-CXCR4 in breast cancer patients. However, there are many gaps of knowledge that need to be filled in order to have a better understanding of the biological, physiological, and potential clinical role of p-CXCR4. We found that p-CXCR4 is a more sensitive marker than CXCR4 as a predictor of metastatic potential, but the biological role of p-CXCR4 in breast cancer has not been studied to date. Although expression of CXCR4 was previously shown to play a role in cell proliferation, invasion, and migration, protein expression of the receptor alone may not be sufficient to predict invasive potential *in-vivo*. In a panel of breast cancer cell lines which uniformly expressed CXCR4, only those cell lines which expressed functional CXCR4 also demonstrated invasive potential (30). Therefore, it would be worthwhile to specifically examine p-CXCR4 as a more sensitive marker of invasive potential in breast cancer, by examining the expression of p-CXCR4 in a panel of CXCR4-expressing breast cancer cell lines in assays of proliferation, invasion, and migration. Further experimentation is also necessary to

acquire greater insight into the regulation of p-CXCR4 expression. For example, although our immunohistochemical analysis suggested the presence of an autocrine feedback loop, such that a positive correlation between SDF-1 expression and p-CXCR4 (but not total CXCR4) expression was observed in primary breast tumors, confirmation of this mechanism requires functional studies. Overexpression or blockade of SDF-1 in tumor cells can determine the impact of modulating SDF-1 expression upon p-CXCR4 expression. Interestingly, Rubin's group demonstrated that there are other factors which can induce the expression of p-CXCR4 to an even greater extent than SDF-1 itself: epidermal growth factor (EGF), and the activator of protein kinase C, PMA (31). Therefore, there may be other signaling pathways involved in the activation of CXCR4. For instance, blocking HER2 activity has been shown to inhibit SDF-1 mediated cell migration (32). Alternatively, overexpression of HER2 has been shown to be an important mediator of SDF-1 induced invasion, adhesion, migration, and formation of metastasis *in-vivo* (33). Thus, it is likely that HER2 activation may also lead to the trans-phosphorylation of CXCR4. Hence, the identification of the molecules which may influence CXCR4 phosphorylation may be important to take into consideration when targeting CXCR4 activity.

The prognostic significance of p-CXCR4 in patients with low levels of plasma SDF-1 provided an original perspective into the dysfunctional relationship between the tumor and the host which can increase metastatic risk. Further validation of this concept can be made by examining the expression of p-CXCR4 at the distant metastatic site, either after the development of metastasis or earlier in the metastatic process in disseminated tumor cells. Disseminated tumor cells have previously been isolated from the bone marrow and were found to have prognostic significance as well (34). We can also determine the presence of p-CXCR4 in circulating tumor cells, to find out if, in the context of a low plasma SDF-1 level, such cancer cells will be favoured out of the circulation into the metastatic site.

Tumor expression of p-CXCR4 may also play an important role in the clinic. First, validation of the prognostic significance of p-CXCR4 is necessary in a larger and independent cohort of breast cancer patients. As CXCR4 antagonists are being developed and entering clinical trials, there is a need to select patients that would most

benefit from this therapy. Since 67% of primary breast tumors overexpress CXCR4, CXCR4 expression itself may not be a very selective marker for such therapy. We found that p-CXCR4 expression is a more sensitive prognostic marker than CXCR4. Positively correlated with triple-negative disease, p-CXCR4 may be a much needed marker for anti-CXCR4 therapy in these patients who are otherwise not responsive to anti-HER2 or anti-hormonal therapy. Furthermore, within ER-positive patients, we identified a small cohort of patients who expressed p-CXCR4 highly and demonstrated a much poorer prognosis than other ER-positive patients; such patients with tumors that overexpress p-CXCR4 could also potentially benefit from anti-CXCR4 therapy as well.

We also showed the prognostic significance of CXCR7, a recently discovered second receptor of SDF-1, in breast cancer patients. We found that overexpression of CXCR7 correlated with breast cancer-specific survival in univariate analysis (HR 3.63, P = 0.01), but did not remain significant in multivariate analysis. This means that further validation of the prognostic significance of CXCR7 would be necessary in a larger cohort. We found a positive correlation between CXCR7, CXCR4 and p-CXCR4 expression. Since CXCR7 and CXCR4 may heterodimerize to stimulate CXCR4 activity (27, 28), it is plausible that an elevation in p-CXCR4 expression may in fact be occurring secondary to CXCR7 and CXCR4 heterodimerization in breast cancer cells, but this would have to be proven mechanistically. Although the functional role of CXCR7 has been demonstrated *in-vivo* (35), the dependence of CXCR4 upon CXCR7 is not yet known. The question which thus arises is whether or not targeting CXCR7 in combination with CXCR4 will further optimize blocking of CXCR4 activity. Since a small molecule inhibitor of CXCR7 is available, combination studies of targeting CXCR4 with CXCR7 are feasible, and can be carried out in order to determine their effect on primary tumor growth and metastasis.

5.3 CXCR4: a therapeutic target

We investigated the role of CTCE-9908, a peptide inhibitor of CXCR4, upon primary tumor growth and metastasis inhibition in the PyMT mouse model. We found that CTCE-9908 slowed the rate of tumor growth. This was first observed after 2.5 weeks of treatment, with the maximal effect observed at 3.5 weeks, whereby CTCE-9908-50

inhibited tumor growth by 45% (P = 0.005), and CTCE-9908-100 inhibited tumor growth by 56% (P = 0.003). We identified a trend with increasing doses of CTCE-9908 resulting in a decrease in cellular proliferation. We also found a molecular marker of CXCR4 activity: phosphorylated – AKT. Increases in levels of activity of AKT correlated well with increasing doses of CTCE-9908. In addition, we found that administration of CTCE-9908-50 resulted in a 40% inhibition in lung metastasis at the time of necropsy. In combination with docetaxel, CTCE-9908-25 demonstrated an additive effect, with a 38% decrease in tumor volume (P = 0.02). In combination with DC101, an anti-VEGFR2 antibody, CTCE-9908-50 also demonstrated an additive effect, with a 37% decrease in primary tumor volume (P = 0.02), and a 75% reduction in distant metastasis (P = 0.009). Thus, we showed efficacy of CTCE-9908 as a single agent in inhibiting primary tumor and metastasis in a transgenic breast cancer mouse model. We showed similar efficacy in primary tumor inhibition with a CXCR4 antagonist in combination with docetaxel or an anti-angiogenic agent. We also showed that in combination CTCE-9908 can further enhance the anti-metastatic effect of anti-angiogenic treatment.

Although CTCE-9908 underwent a Phase I/II clinical trial without demonstrating any untoward toxic effects except for phlebitis in humans (36), there is still much to be understood about this compound before its clinical development can continue. The current dosing regimen in humans consists of a daily, five times per week intravenous injection for a four-week cycle, which requires a significant commitment from patients and housestaff for administration. Further studies are required to determine if similar efficacy can be obtained when the compound is administered less frequently, such as twice or thrice a week. Although the half-life of CTCE-9908 is three hours, the presence of ^{125}I-radiolabelled CTCE-9908 was still detected up to 48 hours after subcutaneous or intravenous injection in rats, and so a bi- or tri-weekly dosing regimen is plausible. Other investigations with regards to optimal dosing regimens are also needed. For example, we noted a maximal anti-tumor effect after 3.5 weeks of administration. It is possible that increasing the dose at this time or prolonging therapy for a few more weeks, may produce a more sustained response. Conversely, it is possible that the tumor cells in this model may be acquiring resistance to CTCE-9908 at this point, and therefore we see less inhibition in primary tumor growth at four weeks and at necropsy.

Our animal studies showed the efficacy of a small molecule inhibitor of CXCR4 activity in breast cancer. Additional comparative studies are required to determine the most efficient and least toxic anti-CXCR4 antagonist. A head-on study comparing the efficacy of CTCE-9908, TN14003, and AMD3100 is necessary in both *in-vitro* and *in-vivo* models. Using a few cell lines of different molecular characteristics such as MDA-MB-231 (ER-negative), MCF-7 (ER-positive), and SKBR3 (HER2-positive), it would be important to determine which antagonist works best at inhibiting tumor cell proliferation and migration *in-vitro*, and tumor growth and metastasis *in-vivo*. Such a study will thus identify which CXCR4 antagonist has the greatest potential for efficacy in the clinic and against which type of tumors would anti-CXCR4 therapy demonstrate the greatest efficiency.

Although we demonstrated a marked response with CTCE-9908 in combination with either docetaxel or DC101, the mechanism underlying the efficacy of this combination needs to be better dissected. The therapeutic agents may be acting together on a common pathway/process or via independent mechanisms. For example, we showed that CTCE-9908 may inhibit cellular proliferation, and so it is plausible that in combination with docetaxel that proliferation may be further inhibited or that another process such as apoptosis may also be promoted. We originally hypothesized that since both of these agents function at the level of the cytoskeleton; mitosis may be further affected, making it worthwhile to also examine cell cycle changes. Indeed, a recent study demonstrated mitotic catastrophe when CTCE-9908 was administered to ovarian cancer cells (37). Analogously, there may also be a few mechanisms by which CTCE-9908 and DC101 may function together to inhibit primary tumor volume and metastasis. The VEGF signaling pathway may be compromised due to CTCE-9908 inhibiting VEGF expression and DC101 blocking its receptor, VEGFR-2. Alternatively, CTCE-9908 may inhibit the recruitment of endothelial progenitor cells, while DC101 may inhibit the maturation of endothelial progenitor cells, together inhibiting endothelial cell function, thereby inhibiting angiogenesis. Therefore, further molecular experimentation may provide us with a mechanism by which the enhancing effect was observed with the combination of anti-CXCR4 therapy and docetaxel or DC101. Furthermore, the precise manner in which these agents function in combination, either via an additive or

synergistic effect, needs to be determined. This effect can be obtained by performing *in-vitro* and *in-vivo* dosing trials with subsequent identification of Chou and Talalay's combination index through statistical analysis (38, 39).

There are other therapeutic agents that should cooperate with anti-CXCR4 therapy to enhance tumor growth and metastasis inhibition. Since SDF-1 was shown to transactivate HER2 and high HER2 expression was shown to be important for SDF-1 induced metastasis (32, 33), we also selected trastuzumab to study in combination with CTCE-9908. We used a xenograft mouse model of a human breast cancer cell line, since trastuzumab is specific for human HER2 activity. We performed a pilot study. From a panel of five breast cancer cell lines, we determined the cell surface expression of CXCR4 using flow cytometry and found that MDA-MB-361 cancer cells highly expressed CXCR4 (Fig. 5-2). In a preliminary study, we found that tumors grew in 5/5 CB-17 SCID (Charles River, Pointe Claire, Canada) mice orthotopically implanted with MDA-MB-361, and macro- and micro-metastasis developed in the lung in 3/5 and 4/5 mice, respectively. No metastasis to the spine or brain was found. We compared CTCE-9908-50 alone and in combination with trastuzumab, administered at 0.3 mg/kg. Since trastuzumab was not tested previously in an orthotopic model using the MDA-MB-361 breast cancer cell line, a dose that was previously shown to inhibit primary tumor volume by 50% in mice with a high HER2-expressing breast cancer cell line was used (40).

To our surprise, mice from the control group developed smaller tumors and fewer metastases than those mice who received CTCE-9908, trastuzumab, or the combination. Although the difference in primary tumor volumes between the control versus CTCE-9908, and control versus the combination groups were statistically significant ($P = 0.008$; $P = 0.008$, respectively), there was no significant difference observed between these groups for the number of metastatic lesions formed. Nonetheless, it is interesting to note that there is a trend, although not statistically significant, which suggested a greater inhibition of distant metastasis when trastuzumab and CTCE-9908 were used in combination in comparison to either agent alone (Fig. 5-3). One possible explanation for the above results is that there may have been a quality control issue with the batch of the control peptide and/or antibody used. However, these results may also demonstrate effectively no difference between the control and therapeutic agents. Since neither

CTCE-9908 nor trastuzumab have been previously tested in such an orthotopic model of MDA-MB-361 where tumors develop slowly over four months, testing of more doses and perhaps sequential administration may be necessary to determine the greatest efficacy in inhibition of primary tumor and distant metastasis.

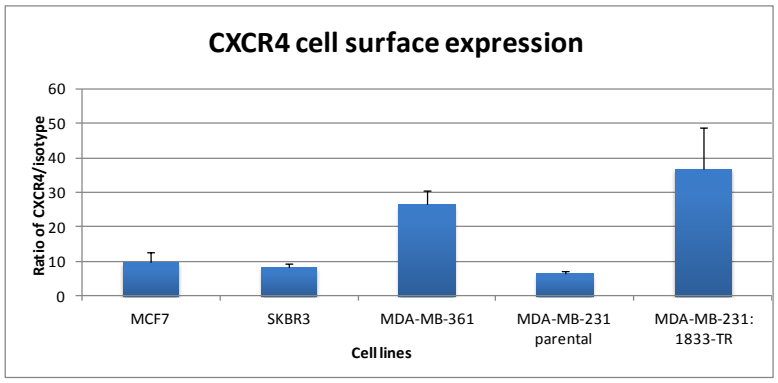
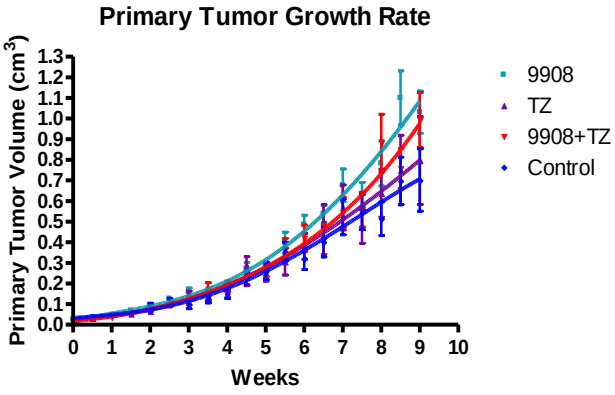


Fig. 5-2. Cell surface expression of CXCR4 in different breast cancer cell lines.

A



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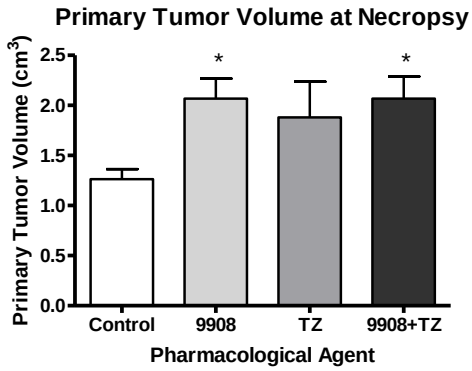

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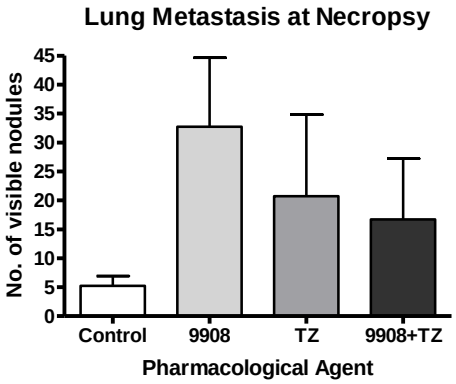

Fig. 5-3: Results of CTCE-9908-50 in combination with trastuzumab on *A*, primary tumor growth rate over 4.5 weeks; *B*, primary tumor volume at necropsy; and *C*, metastasis at necropsy. (TZ = trastuzumab)

There are other interesting therapeutic agents that can be combined with CTCE-9908, such as lapatinib (a dual tryrosine kinase inhibitor of EGFR and HER2) – FDA approved in metastatic breast cancer, cetuximab (an anti-EGFR monoclonal antibody), and rapamycin, an mTOR inhibitor. HER2 phosphorylation has been shown to be significantly enhanced when stimulated with the combination of EGF and SDF-1, over either ligand alone. Interestingly, SDF-1 induced phosphorylation of HER2 or SDF-1 induced cell migration was found to be inhibited by PKI 166, an epidermal growth factor/HER2 tyrosine kinase inhibitor (32), suggesting a plausible role for a combination study with a CXCR4 antagonist and lapatinib. In addition, in an ovarian cancer cell line, *in-vitro* studies have demonstrated cross-talk between the SDF-1/CXCR4 axis and EGFR. SDF-1 was shown to induce phosphorylation of ERK 1/2, leading to transactivation of EGFR. Indeed, an EGF receptor kinase inhibitor (AG1478), was shown to inhibit both SDF-1-induced proliferation and ERK 1/2 phosphorylation (41). Finally, there is also evidence to suggest that the combination of a CXCR4 antagonist and an mTOR inhibitor may have an enhancing anti-tumor effect. In gastric carcinoma cells, rapamycin inhibited SDF-1 induced phosphorylation of p70 ribosomal protein 6 kinase (p70S6K) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1), in addition to tumor cell proliferation and migration *in-vitro* (42). This suggests that targeting mTOR in addition to CXCR4 may also be a promising combination.

In the PyMT and xenograft mouse models, we examined the impact of targeting CXCR4 upon lung metastasis. However, studies have shown that CXCR4 metastasis may occur preferentially to the bone (43, 44). In order to better determine if targeting CXCR4 impacts the process of extravasation, we started to look at CTCE-9908 in an experimental bone metastasis mouse model. We used a clonal variant of the MDA-MB-231 cells, called 1833-TR, which was shown to metastasize to the bone within five weeks of injection into the left ventricle of the heart and was also shown to highly express CXCR4 (Fig. 5-2). This cell line was transfected with firefly luciferase, enabling *in-vivo* imaging through detection and measurement of bioluminescence. In collaboration with Dr. Peter Siegel's laboratory (McGill University), we conducted a pilot study to examine the effect of CTCE-9908-25 in this model. We found that there was a modest inhibition (50% decrease, $P = 0.43$) in bone metastasis in those animals treated for four weeks with

CTCE-9908 versus control (Fig. 5-4). Interestingly, Huang et al. reported the use of CTCE-9908 in a similar bone metastasis model and demonstrated a significant reduction in metastasis after 5 to 6 weeks of treatment (44). Since in our study, some mice started to suffer from morbidity due to bone metastasis after four weeks, we had to stop treatment at four weeks. Furthermore, since we showed that CTCE-9908-50 was more effective than CTCE-9908-25 in inhibiting primary tumor growth and metastasis in our transgenic model, it would be important to look at the efficacy of CTCE-9908-50 in this xenograft model as well.

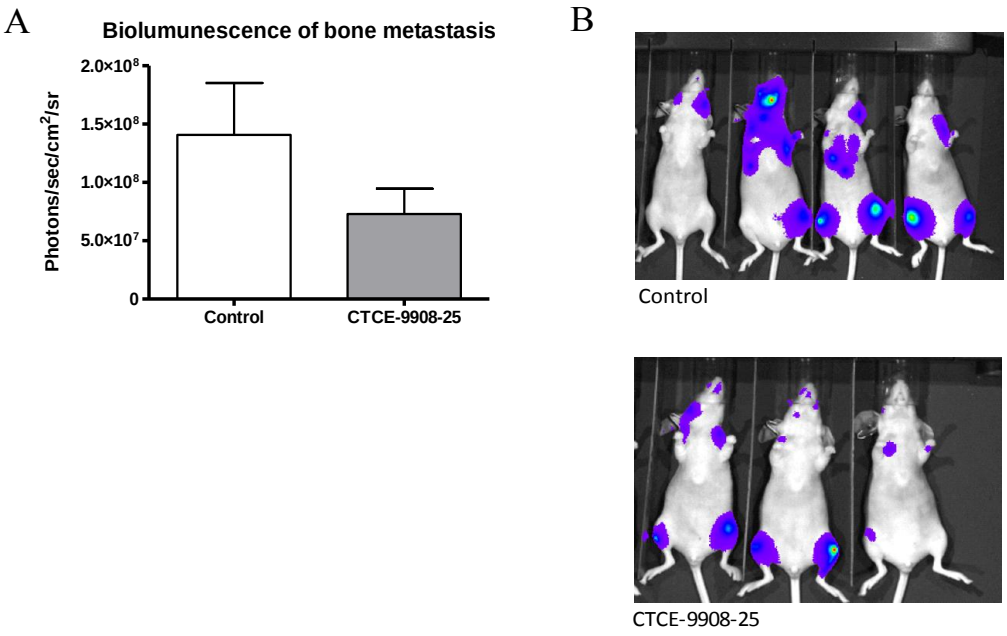

Fig. 5-4. Targeting CXCR4 in a bone metastasis model. Bone metastases formed after MDA-MB-231 (#1833-TR) injected in left cardiac ventricle of athymic mice. *A*, Tumor burden measured at necropsy after four weeks of 5x/week daily injections of CTCE-9908-25 or SC-9908. Tumor burden visualized using bioluminescence imaging and quantified as the normalized photon flux (p/s/cm^2/sr). *B*, Representative images of mice from each group at the time of necropsy.

In addition to identifying the overall efficacy of targeting CXCR4, either alone or in combination, upon inhibition of metastasis, it is also important to determine the tumor properties that may be most responsive to anti-CXCR4 therapy and at which stage of the metastatic process anti-CXCR4 therapy may function best. In order to answer the former question, one approach may be to determine the expression of CXCR4 in the primary

tumor and metastatic site both prior to, and after treatment. Since we previously showed the prognostic significance of p-CXCR4 and high expression of CXCR4 in lymph nodes, it is likely that cancer cells which highly express CXCR4 are being selected and are disseminating both locally and distantly. Thus, it is plausible that anti-CXCR4 therapy, such as CTCE-9908, may be targeting those cancer cells that highly express CXCR4. Furthermore, gene expression profiling of those tumors that were treated with CTCE-9908 could help characterize the tumors that may respond best to anti-CXCR4 therapy.

In order to better understand at which stage of the metastatic process anti-CXCR4 therapy may work best, a better understanding of which steps of the metastatic process that CXCR4 is largely implicated in is necessary. Starting in reverse order, at the target metastatic site, one would need to determine if CXCR4 is involved in the development of micrometastatic to macrometastatic lesions. If so, it would be important to know if targeting CXCR4 can alter the formation or latency in the development of such lesions. Our preliminary results from the bone metastasis model, and one study which found a decrease in the size and not the incidence of metastasis is suggestive that CXCR4 may play a role in the outgrowth of breast cancer micrometastasis (45). This hypothesis can be confirmed with experiments involving intratibial injections of CXCR4-expressing breast cancer cell lines into the bones of mice with the administration of a CXCR4 antagonist. Intracardiac or tail vein injections of cancer cells in mice can assist in determining if extravasation itself implicates CXCR4, as illustrated above. To assess if CXCR4 plays a role in cancer cell intravasation, we can determine if CXCR4 stimulates epithelial to mesenchymal transition by examining the traditional markers of this phenotype such as N-cadherin, vimentin, alpha-smooth muscle actin, twist, and fibronectin (29). In addition, the role of CXCR4 in tumor growth needs to be better defined. For example, it could be useful to determine if targeting CXCR4 plays an important role in the earlier or later stages of primary tumor growth. This may serve as an indication if anti-CXCR4 therapy may be more beneficial in treating smaller versus larger tumors or perhaps in the neoadjuvant (prior to surgery) or adjuvant (post tumor excision) setting. However, the process of metastasis may need to be tackled even before women present with breast cancer because more than one third of women already have either locoregional or distant metastasis at the time of initial presentation (46). Thus,

there is a need to identify women that are at high risk of developing metastasis earlier, and one may even consider treating such patients earlier, such as in the chemopreventive setting. Although one of the short-term endpoints of prevention studies is the incidence of breast cancer, it is still important to determine if a chemopreventive agent can impact survival. There is potential that an anti-CXCR4 agent can have such an effect. As CXCR4 and p-CXCR4 both demonstrate an increase in expression from normal tissue to hyperplasia, to ductal carcinoma-in-situ to florid carcinoma (47), it is likely that targeting CXCR4 may play a role in inhibiting tumor progression. Since tumor progression from hyperplasia to pre-invasive to invasive carcinoma has been well characterized in the PyMT model (48), this model may also be suitable to study anti-CXCR4 therapy in the context of chemoprevention. Therefore, greater insight into the stage of CXCR4 involvement in the metastatic process can help in determining the most appropriate setting for therapeutic intervention.

5.4 The SDF-1/CXCR4 axis: diagnostic and therapeutic implications in breast cancer

In chapter 4, we demonstrated an effect of anti-CXCR4 therapy upon primary tumor growth and metastasis in a transgenic mouse model of breast cancer. However, as a single agent-therapeutic, the results were modest in comparison to when administered in combination. Combination of CTCE-9908 with either a cytotoxic agent or an anti-angiogenic agent revealed a similar effect upon primary tumor volume inhibition. Future studies require identification of the best CXCR4 antagonist with further pre-clinical testing before launching of anti-CXCR4 therapy into clinical trials. Furthermore, it is plausible that improvements in the selection of the patient cohort receiving therapy may improve the efficacy of such agents. Although selecting patients based on overexpression of CXCR4 may be adequate; a more sensitive marker such as p-CXCR4 may better serve this purpose. In addition to the significance of CXCR4 as a therapeutic target, we also found the SDF-1/CXCR4 axis to play an important role in diagnostics, as a strong predictive marker of distant metastasis (Fig. 5-5). Patients with an elevated expression of p-CXCR4 in conjunction with a low plasma SDF-1 level were found to have a very poor prognosis; the identification of such a cohort may help in selecting

patients who may benefit from adjuvant therapy. Plasma SDF-1, as a host-derived marker, may especially play an important role as a tumor-independent prognostic marker that may assist in the selection of therapy for breast cancer patients.

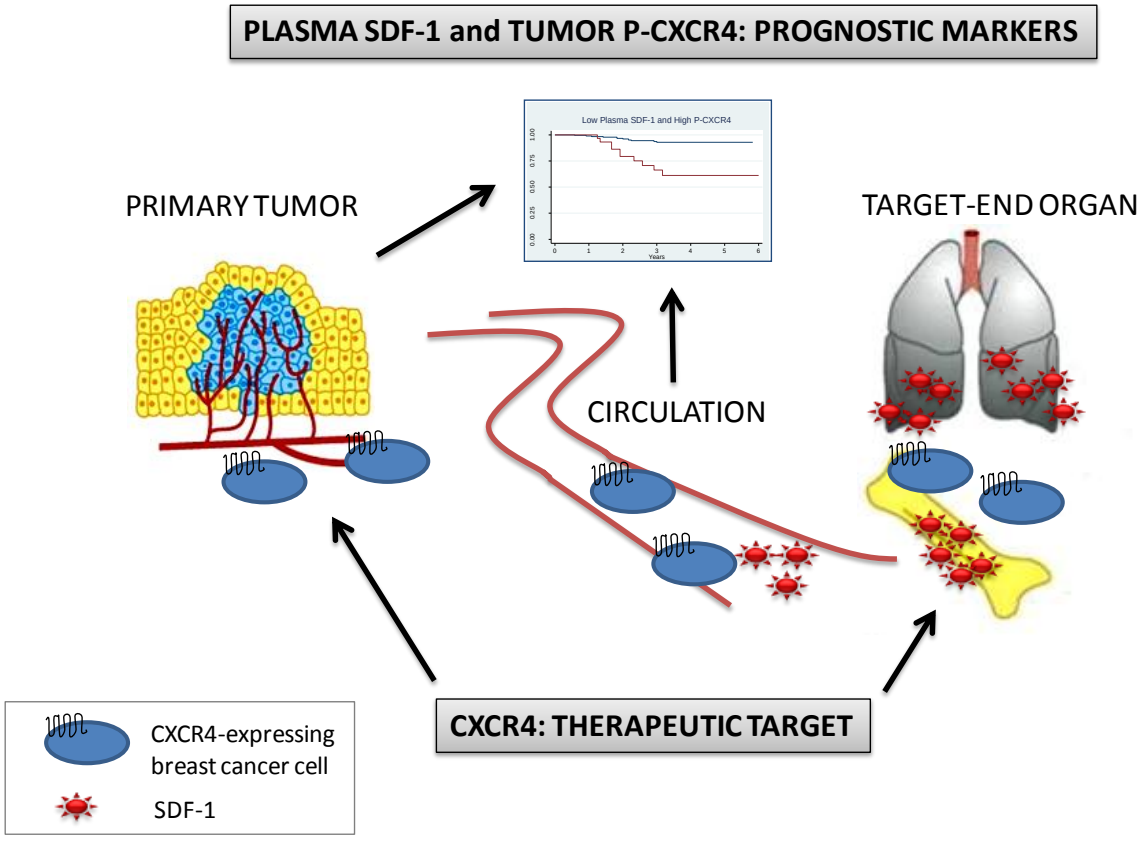

Fig. 5-5: Clinical paradigm of plasma SDF-1 and CXCR4. Adapted from Weinberg (29).

List of Original Contributions

1. Low plasma SDF-1 level is an independent predictive marker for distant metastasis in breast cancer.

2. The prognostic significance of low plasma SDF-1 offers a new perspective into the physiology of distant metastasis, whereby a) the concentration gradient of SDF-1 between the target metastatic organ (high) and plasma SDF-1 (low) may play an important role in driving cancer cells from the circulation to the metastatic site and b) a novel marker which can directly measure distant metastatic potential has been identified.

3. Plasma SDF-1 levels is the first host-derived blood marker predictive of distant metastasis in breast cancer.

4. SDF-1-3'A polymorphism is a poor prognostic marker in breast cancer.

5. The combination of plasma SDF-1 levels and SDF-1-3'A polymorphism has a poorer prognostic value than either variable alone.

6. Phosphorylated-CXCR4 is a stronger prognostic marker than CXCR4.

7. Elevated tumor expression of p-CXCR4 enhances the prognostic value of low plasma SDF-1 levels, illustrating how a dysfunctional relationship between the tumor and its host can be predictive of distant metastasis.

8. High expression of CXCR4 or p-CXCR4 is required for promoting tumor metastasis in patients with low plasma SDF-1 levels.

9. Amongst patients with ER+ tumors, patients who overexpressed p-CXCR4 demonstrated a 6.5-fold worse prognosis compared to other ER+ patients.

10. Expression of CXCR7 correlated positively with CXCR4 and p-CXCR4 in breast cancer patients. CXCR7 is also a poor prognostic marker.

11. Targeting CXCR4 with a peptide antagonist after the tumor became palpable in a transgenic mouse model slowed primary tumor growth and inhibited distant metastasis modestly.

12. Targeting CXCR4 in combination with an anti-angiogenesis agent in a transgenic mouse model inhibited primary tumor volume with the same efficacy as a cytotoxic chemotherapeutic agent (docetaxel).

13. Targeting CXCR4 in combination with an anti-angiogenesis agent resulted in marked inhibition of primary tumor volume and metastasis.

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APPENDIX

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Biomarkers, Genomics, Proteomics, and Gene Regulation

The Influence of Tumor-Host Interactions in the Stromal Cell-Derived Factor-1/CXCR4 Ligand/Receptor Axis in Determining Metastatic Risk in Breast Cancer

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The chemokine stromal cell-derived factor-1 (SDF-1) may function to attract CXCR4-expressing cancer cells to metastatic organs. We have previously demonstrated that low plasma SDF-1, a host-derived marker, increases distant metastatic risk in breast cancer. We therefore hypothesized that tumors overexpressing the SDF-1 receptor CXCR4 have an enhanced ability to metastasize in patients with low plasma SDF-1 levels. In this study, we determined the prognostic significance of activated CXCR4, or phosphorylated CXCR4 (p-CXCR4), and CXCR7, another receptor for SDF-1. Immunohistochemistry was performed on a tissue microarray built using 237 samples from the same cohort of patients for which we measured plasma SDF-1 levels. We found that the prognostic value of p-CXCR4 expression (hazard ratio or HR, 3.95; $P = 0.004$) was superior to total CXCR4 expression (HR, 3.20; $P = 0.03$). The rate of breast cancer-specific mortality was much higher in patients with both high p-CXCR4 expression and low plasma SDF-1 levels (HR, 5.96; $P < 0.001$) than either low plasma SDF-1 (HR, 3.59; $P = 0.01$) or high p-CXCR4 expression (HR, 3.83; $P = 0.005$) alone. The added prognostic value of low plasma SDF-1 was only effective in patients with high p-CXCR4 expression, and as such, provides clinical validation for modulation of the metastatic potential of tumor cells by an inherent host-derived metastatic risk factor. ***(Am J Pathol 2009, 175:66–73; DOI: 10.2353/ajpath.2009.080948)***

Breast cancer is the second most common cancer in women and represents a major risk to women's lives because of the life-threatening consequences of metastatic disease (SEER Cancer Statistics Review, *http://seer.cancer.gov/csr/1975_2005/*; accessed September 1, 2008).[1] The process of metastasis has often been reported as a cascade of events, with emphasis placed on the tumor cell and its potential to proliferate, invade into the circulation, exit the bloodstream, and grow at the metastatic site.[2] However, little is known about the manner in which the host can modulate tumor progression and the propensity of the tumor to metastasize. Indeed, the role of the host was recognized over a century ago in the "seed and soil" theory, whereby the presence of a "congenial" environment of the host metastatic organ influenced the colonization of tumor cells at specific distant organs.[3] More recently, a chemokine-receptor model was proposed to help explain the manner in which the host influences the homing of cancer cells to specific target organs. Muller et al. proposed that chemokines, such as stromal cell-derived factor-1 (SDF)-1, are nor-

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mally overexpressed by those target organs to which breast cancer metastasizes, such as lung, liver, and bone, and serve to attract breast cancer cells that express their receptors, such as CXCR4.[4] Various animal studies have subsequently demonstrated the functional role of CXCR4 as the prime chemokine receptor involved in distant metastasis in breast and other types of cancers.[5–9]

Several studies, including our own, have since observed an association between CXCR4 expression and distant metastasis in primary breast cancer patients.[10–13] Furthermore, we recently identified circulating levels of SDF-1 as a prognostic blood marker in a series of patients with primary breast cancers. Interestingly, circulating SDF-1 levels were found to be independent from tumor-derived SDF-1, and as such, plasma SDF-1 is the first candidate host-derived blood marker in breast cancer. We found that a low plasma SDF-1 level was predictive of distant metastasis, suggesting that low SDF-1 in the circulation may favor the extravasation of tumor cells from the circulation to the metastatic site.[14] In accordance with Muller's hypothesis, the differential concentration gradient of SDF-1, that is, low blood SDF-1 and high tissue SDF-1 at the metastatic site, may enhance the homing of CXCR4-expressing cancer cells. In this case, it would be expected that tumors with high CXCR4 expression would be especially sensitive to the SDF-1 gradient at metastatic target organs. To test this hypothesis, we determined the expression of CXCR4 in primary tumors using the same cohort of breast cancer patients in which we previously measured plasma SDF-1 levels. We determined if patients with an innate susceptibility for metastasis, associated with low levels of plasma SDF-1, demonstrated a greater risk of metastasis when their tumors expressed higher levels of CXCR4. In addition to tumor expression of CXCR4, we also measured the levels of the phosphorylated CXCR4 receptor as a means of quantifying CXCR4 activity and compared its expression in the primary tumor and metastatic lymph nodes. We also measured tumor expression of the two factors that may activate CXCR4: its ligand SDF-1, and another chemokine receptor, CXCR7, which may activate CXCR4 via heterodimerization.[15] In this way, we provide a more detailed picture of metastatic risk associated with the activity of CXCR4 in the primary breast tumor and relate it with the risk of metastasis associated with low plasma SDF-1 levels.

Materials and Methods

Patients

We used the same cohort of patients as described previously.[14] Three hundred five patients with primary breast cancers of stages I, II, and III were recruited from 2000 to 2003 with a median follow-up of 3.3 years, with informed consent, as per the Research Ethics Committee of the Centre Hospitalier de l'Université de Montréal. Thirty-seven patients were excluded due to unavailability of tissue blocks. Thirty-one patients were further excluded due to absence of the prognostic tumor lesion, leaving 237 patients for correlation with clinicopathological characteristics and survival analysis. Tissue cores from the microarray were damaged for up to three other patients, resulting in a minimum of 234 patients. Due to incomplete data available regarding HER2 status from the pathology reports, HER2 was re-stained using our tissue microarray. Nine percent of the patients were HER2 positive by immunohistochemistry (either 2+ or 3+), and 18% were estrogen receptor (ER) negative/progesterone receptor (PR) negative/HER2 negative (ER−/PR−/HER2−), also known as triple negative. Corresponding plasma samples were available for 212 patients.

Western Blot Analysis

Human umbilical vein endothelial cells (Cambrex Bio-Science, Walkersville, MD) were serum starved for 3 hours before being stimulated with recombinant human SDF-1 (R&D Systems, Minneapolis, MN). Cell lysates were prepared using lysis buffer consisting of 1% Triton X-100, 25 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, and 5 mmol/L EDTA was supplemented with protease inhibitor cocktail set III (Calbiochem, Gibbstown, NJ) and 1 mmol/L sodium orthovanadate. Cell lysates (30 μg protein) were solubilized in NuPAGE lithium dodecyl sulfate sample buffer, incubated at 37°C for 30 minutes, and run through 10% NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA). After transfer, Immobilon-P membranes (Millipore, Billerica, MA) were incubated overnight with antibody against p-CXCR4 (courtesy of Dr. Joshua Rubin, Washington University, St. Louis, MO, 1:1000).[16] Relative protein expression levels were estimated by membrane rehybridization with anti-mouse CD184 (2B11, 1:250, BD PharMingen, San Jose, CA). Antibody detection was performed using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

Tissue Microarray Construction

Formalin-fixed, paraffin-embedded tissue blocks were collected from the Department of Pathology from the Centre Hospitalier de l'Université de Montréal. All blocks were re-sectioned and stained for hematoxylin phloxine saffron stain before marking of histological lesions. We constructed a tissue microarray as described previously[17] using a Manual Tissue Arrayer I (Beecher, Sun Prairie, WI). In total, 1619 cores were punched and distributed into four recipient blocks. Lesions were placed in either duplicate or triplicate cores adjacent to one another. Six-micrometer sections were cut using the tape transfer system (Instrumedics, St. Louis, MO).

Immunohistochemistry

Immunohistochemistry was performed via the labeled streptavidin biotin method for phosphorylated CXCR4 (p-CXCR4), SDF-1, CXCR7, and Ki-67 as previously described.[10] Primary antibodies and concentrations used were: p-CXCR4, (courtesy of Dr. Joshua Rubin) at a 1:250

dilution; SDF-1 (MAB350, clone 79018, R&D Systems) at 10 μg/ml; CXCR7 (MAB4227, clone 358426, R&D Systems) at 10 μg/ml; and Ki-67 at a 1:50 dilution (M7240, clone MIB-1, Dako, Denmark). All primary antibodies were incubated overnight at 4°C. A biotin-labeled secondary antibody was used, either goat anti-mouse at 2.4 μg/ml or 9 μg/ml for Ki-67 (catalog no. 115-065-003, Jackson ImmunoResearch Laboratories, West Grove, PA) or goat anti-rabbit at 2.75 μg/ml (catalog no. 111-065-003, Jackson ImmunoResearch Laboratories). Biotin detection was performed with peroxidase-conjugated streptavidin (catalog no. 016-030-084, Jackson ImmunoResearch Laboratories) at 0.2 μg/ml for p-CXCR4, 0.1 μg/ml for SDF-1, 0.08 μg/ml for CXCR7, and 0.25 μg/ml for Ki-67. CXCR4 expression was detected using a biotin-labeled CXCR4 antagonist, TN14003, synthesized in the Saragovi laboratory, following reported methods.[6,18] The staining intensity (0, 1, 2, 3) and percentage of positively stained cells (0 to 100%) were scored in a blinded manner.

Statistical Analysis

Analysis for each biomarker was performed using the product score, whereby the product of the staining intensity and percentage of positive cells of the cytoplasm was used to create a continuous score from 0 to 300. The product score of each biomarker was analyzed as both a continuous variable for correlations between biomarkers and with clinicopathological characteristics, and as a categorical variable, for survival and comparative analysis between primary tumor and lymph nodes. The product score was divided into low, medium, and high expression categories using outcome-derived cut points from X-tile (version 3.6.1, Robert Camp, Yale University, New Haven, CT[19]). For survival analysis, high expression of the biomarker was compared with low expression, whereas the medium and high categories were combined and termed as "high" for all other categorical variable analysis. Correlations between biomarkers and clinicopathological characteristics were performed using Spearman's rank correlation and with χ^2 or Fisher's exact test for categorical variable analysis, as previously described.[14] Clinicopathological correlations examined include age, tumor size, lymph nodes, stage, tumor grade, ER, PR, HER2 status, triple negative disease. Survival analysis was performed for breast cancer-specific survival and distant disease-free survival as described previously.[14] Since this is the first survival analysis of p-CXCR4 in cancer patients, an *a priori* sample size could not be determined. Survival analysis was first performed on X-tile from which cut-points were obtained with subsequent cross-validation. Subsequently, a Cox proportional hazards regression model[20] was used for univariate (n = 237) and multivariate (n = 196) analysis. Covariates included in multivariate analysis were: age, tumor size, lymph node status, tumor grade, ER, PR, and neoadjuvant or adjuvant hormonal therapy or chemotherapy. Correlation analysis between tissue biomarkers and plasma SDF-1 levels was performed using Spearman's rank correlation. Survival analysis for the combination of tissue biomarker and plasma SDF-1 levels was performed as described above for univariate (n = 212) and multivariate (n = 177) analysis. No statistical significance was identified for patients who were excluded due to unavailability of blood or missing information for multivariate analysis for each endpoint. All reported P values are two-sided. All statistical analysis was performed using STATA version 9.2 (College Station, TX).

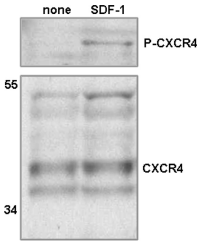

Figure 1. Detection of CXCR4 phosphorylation in primary human endothelial cells. Human umbilical vein endothelial cells were incubated in medium alone or treated with SDF-1 (100 ng/ml) for 15 minutes. p-CXCR4 expression was evaluated by immunoblotting with an antibody to p-CXCR4 and reblotting with an anti-mouse CXCR4 (CD184) antibody. Results are representative of three independent experiments.

Results

Correlation of p-CXCR4 with CXCR4, SDF-1, and CXCR7

To gain a more complete understanding of the role of the CXCR4/SDF-1 receptor/ligand axis in breast cancer, we measured the expression of total CXCR4 receptor together with phosphorylated-CXCR4, the activated form of the receptor, its ligand, SDF-1, as well as the CXCR7 receptor. To verify the specificity of the p-CXCR4 antibody, we treated human umbilical vein endothelial cells that are known to express CXCR4 endogenously, with recombinant human SDF-1 for 15 minutes. Expression of p-CXCR4 was induced on SDF-1 stimulation of human umbilical vein endothelial cells (Figure 1). To detect the expression of total CXCR4, we synthesized a biotinylated anti-CXCR4 peptide, biotinylated-TN14003, as this peptide was previously reported to show greater specificity in immunohistochemistry in comparison with a commercially available antibody.[6] Immunohistochemical analysis of p-CXCR4 and CXCR4 from the tissue microarray revealed cytoplasmic and nuclear expression for both biomarkers (Figure 2, A–D). Cytoplasmic p-CXCR4 was expressed at moderate to high levels in 47% of breast tumors (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). Expression of p-CXCR4 correlated positively with tumor progression (rho = 0.42, $P < 0.0001$): 9% of normal lesions, 54% of ductal carcinoma *in situ*, 47% of tumors, and 54.8% of lymph nodes demonstrated high expression of p-CXCR4, showing that stage 0, I, II and III breast cancer had much higher levels of p-CXCR4 expression than normal breast tissues (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). Levels of cytoplasmic tumor p-CXCR4 expression correlated strongly with

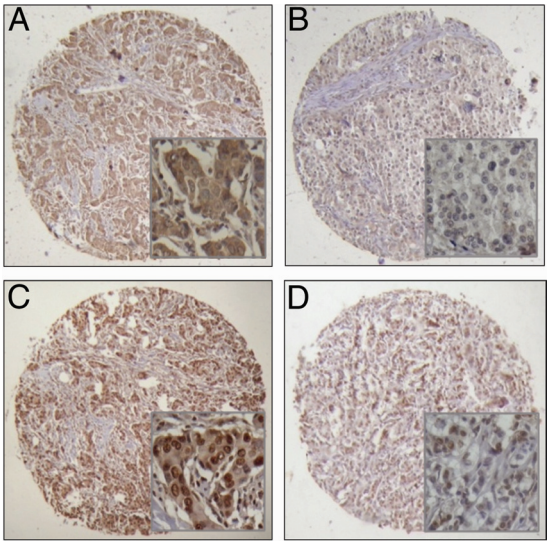

Figure 2. Immunohistochemical analysis of p-CXCR4 and CXCR4 using 20× objective lens magnification. **A:** High expression of p-CXCR4. **B:** Low expression of p-CXCR4. **C:** High expression of CXCR4. **D:** Low expression of CXCR4.

both cytoplasmic CXCR4 (rho = 0.58, $P < 0.0001$) and nuclear CXCR4 (rho = 0.54, $P < 0.0001$) expression. These results are in concordance with our previous results for CXCR4.[10] For the sake of simplicity, from here on, we will only refer to the cytoplasmic expression of both markers. To further understand the significance of p-CXCR4, we examined the expression of SDF-1 and CXCR7. SDF-1 expression was found mainly in the cytoplasm of tumor cells (Figure 3, A and B). Interestingly, expression of SDF-1 correlated positively with p-CXCR4 (rho = 0.19, $P = 0.004$), but not with CXCR4 (rho = 0.05, $P = 0.41$), suggesting that autocrine stimulation of CXCR4 may contribute to CXCR4 phosphorylation in breast tumors. CXCR7 was predominantly expressed in the cytoplasm, and less so in the nucleus, and thus we refer only to cytoplasmic expression (Figure 3, C and D). A strong positive correlation was found between CXCR7 and SDF-1 (rho = 0.32, $P < 0.0001$), CXCR4 (rho = 0.45, $P < 0.0001$) and, above all, p-CXCR4 (rho = 0.49, $P < 0.0001$) expression. Therefore it is plausible that the phosphorylation of CXCR4 may be induced by SDF-1 and/or by co-expression of CXCR4 and CXCR7 in breast cancers (Table 1).

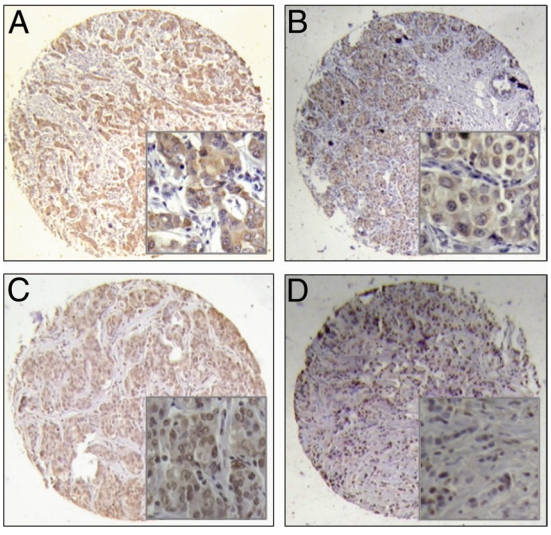

Figure 3. Immunohistochemical analysis of SDF-1 and CXCR7 using 20× objective lens magnification. **A:** High expression of SDF-1. **B:** Low expression of SDF-1. **C:** High expression of CXCR7. **D:** Low expression of CXCR7.

Table 1. Correlation Analysis between Biomarkers

Variable	Rho	*P* value
CXCR4 and p-CXCR4	0.58	<0.0001
SDF-1 and CXCR4	0.05	0.41
SDF-1 and p-CXCR4	0.19	0.004
CXCR7 and SDF-1	0.32	<0.0001
CXCR7 and CXCR4	0.45	<0.0001
CXCR7 and p-CXCR4	0.49	<0.0001

P-CXCR4 Has a Better Prognostic Value than CXCR4

To determine the prognostic significance of CXCR4 and p-CXCR4 expression, survival analysis was performed using the product score. Categories of low, medium, and high expression were obtained using cut points derived from the X-tile software. For CXCR4, the population was divided into low (41%, with product score or PS, ranging from 0 to 53.3), medium (30%; PS, 55.6–130), and high (29%; PS, 131–300). For p-CXCR4, the population was divided into low (53%; PS, 0–150), medium (24%; PS, 152–203), and high (24%; PS, 209–300). High p-CXCR4 expression demonstrated a greater prognostic value than high CXCR4 expression for breast cancer-specific survival and distant disease-free survival in univariate analysis. Patients with high p-CXCR4 expression demonstrated a fourfold higher rate of death (hazard ratio or HR, 3.95; 95% confidence interval or CI, 1.55–10.03; $P = 0.004$) due to breast cancer-related causes, which is greater than that for patients with high CXCR4 expression (HR, 3.20; 95% CI, 1.09–9.37; $P = 0.03$). Furthermore, for the risk of distant disease-free survival, high p-CXCR4 expression exhibited greater significance (HR, 2.38; 95% CI, 1.13–5.00; $P = 0.02$) than high total CXCR4 expression (HR, 1.90; 95% CI, 0.85–4.25; $P = 0.12$). To determine whether p-CXCR4 or CXCR4 is an independent marker for survival, multivariate analysis was performed for both endpoints, and no statistical significance was found for either p-CXCR4 or CXCR4 (data not shown). Although this may be due to the small size of our patient cohort, the superiority of p-CXCR4 over CXCR4 in breast cancer-specific survival and distant disease-free survival suggests that p-CXCR4 expression may be a more sensitive marker than CXCR4 expression for metastatic risk.

P-CXCR4 Enhances Prognostic Value of Plasma SDF-1 level

To better understand the metastatic risk of high p-CXCR4 expression in the context of host-derived risk, we exam-

Table 2. Clinicopathological Properties of Patients Who Expressed Both Low Plasma SDF-1 and High Tumor p-CXCR4

Variable	Number of patients	%
Age (yr)		
≤50	12	(41.4)
>50	17	(58.6)
Tumor size (cm)		
T1 (≤2)	10	(34.5)
T2 (2–5)	14	(48.3)
T3 (>5)	4	(13.8)
T4	1	(3.4)
Nodal status		
N0	13	(44.8)
N1	7	(24.1)
N2	4	(13.8)
N3	2	(6.9)
Lymphadenectomy not performed	3	(10.3)
Stage		
I	7	(24.1)
II	12	(41.4)
III	7	(24.1)
Unavailable	3	(10.3)
Tumor grade		
1	0	(0)
2	9	(31.0)
3	15	(51.7)
Unavailable	5	(17.2)
ER Status		
Negative	17	(58.6)
Positive	12	(41.4)
PR status		
Negative	20	(69.1)
Positive	9	(31.0)
Triple negative		
Present	13	(44.8)
Absent	13	(44.8)
Unavailable	3	(10.3)
Luminal A/B		
Present	11	(37.9)
Absent	15	(51.7)
Unavailable	3	(10.3)
HER2 status		
Positive	3	(10.3)
Negative	23	(79.3)
Unavailable	3	(10.3)

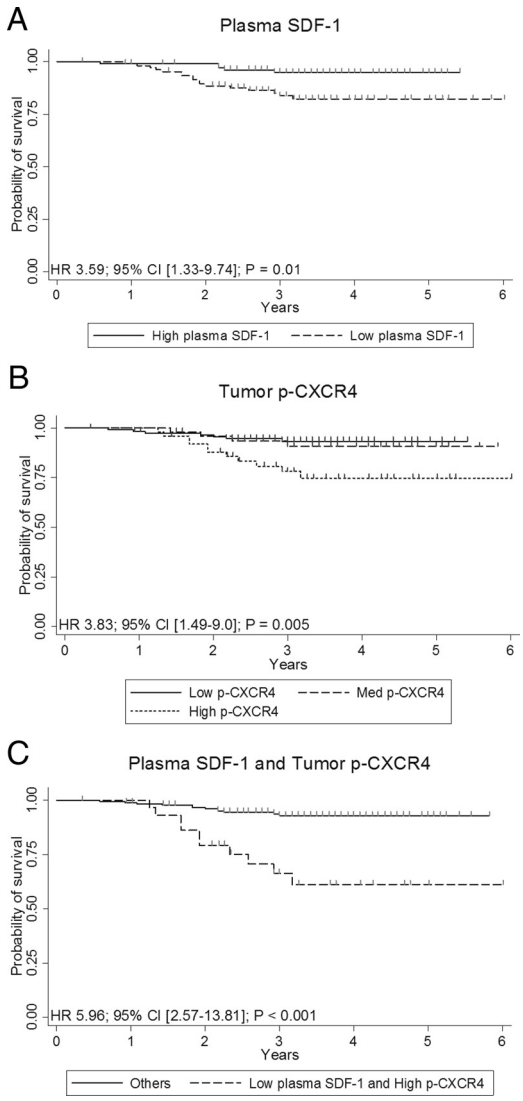

Figure 4. Kaplan-Meier survival curves for breast cancer-specific survival for plasma SDF-1 (**A**), tumor p-CXCR4 (**B**), and combination of low plasma SDF-1 and high p-CXCR4 (**C**).

ined the prognostic significance of p-CXCR4 in combination with blood SDF-1 levels. We previously measured SDF-1 blood levels from the same cohort of breast cancer patients and found that plasma SDF-1 is a host-derived marker predictive of distant metastasis.[14] To confirm once again that circulating SDF-1 levels are independent of the tumor, we compared the tumor expression of SDF-1 with plasma SDF-1 levels and found no correlation between the two variables (rho = −0.08, $P = 0.23$). We now investigated the prognosis of patients who expressed both high levels of p-CXCR4 and low plasma SDF-1 levels. Using the median value of plasma SDF-1, as in our previous study, the cohort was again divided into two groups, high and low SDF-1. Patients with both a low plasma SDF-1 level and high p-CXCR4 expression ($n = 29$, or 14% of the entire cohort) (Table 2) demonstrated a significant correlation with the development of distant metastasis (rho = 0.25, $P = 0.0003$), stronger than that of plasma SDF-1 alone (rho = −0.17, $P = 0.01$). As there were 22 fewer patients for whom both plasma and tissue samples were available ($n = 212$), the prognostic value for each variable was recalculated for breast cancer-specific survival and revealed similar values: low plasma SDF-1 (HR, 3.59; 95% CI, 1.33–9.74; $P = 0.01$) and high p-CXCR4 (HR, 3.83; 95% CI, 1.49–9.90; $P = 0.005$) (Figure 4, A and B). Patients with the combination of both low plasma SDF-1 and high p-CXCR4 showed a very poor prognosis (HR, 5.96; 95% CI, 2.57–13.81; $P < 0.001$) (Figure 4C), which remained significant after multivariate analysis (adjusted HR, 3.78; 95% CI, 1.31–10.94; $P = 0.01$). After adjustment for Ki-67 labeling index ($n = 142$), a marker for cellular proliferation, the combination remained significant for breast cancer-specific survival (HR 3.70; 95% CI, 1.02–11.48; $P = 0.005$).

A similar enhancing effect was also apparent in distant disease-free survival, whereby patients with the combination showed an almost fourfold greater rate of distant recurrence (HR, 3.75; 95% CI, 1.82–7.76, $P < 0.001$),

Table 3. Clinicopathological Correlations of Tumor CXCR4, P-CXCR4, and CXCR7 Expression

Variable	CXCR4		P-CXCR4		CXCR7	
	Rho	*P* value	Rho	*P* value	Rho	*P* value
Age	−0.02	0.73	−0.05	0.41	0.08	0.21
Tumor size	0.11	0.11	0.13	0.05	0.02	0.76
Lymph nodes	−0.02	0.76	0.01	0.84	0.005	0.94
Stage	−0.002	0.98	0.08	0.25	0.03	0.71
Grade	0.35	<0.0001	0.37	<0.0001	0.14	0.04
ER positivity	−0.27	<0.0001	−0.32	<0.0001	−0.18	0.007
PR positivity	−0.26	<0.0001	−0.31	<0.0001	−0.20	0.002
HER2+	−0.06	0.39	0.09	0.23	0.004	0.95
ER−/PR−/HER2−	0.37	<0.0001	0.33	<0.0001	0.14	0.05

greater than either biomarker alone: low plasma SDF-1 (HR, 2.15; $P = 0.04$) and high p-CXCR4 (HR, 2.31; $P = 0.03$). The combination was also significant after multivariate analysis for distant disease-free survival (adjusted HR, 2.80; 95% CI, 1.14–6.83; $P = 0.02$). On the other hand, patients with both low levels of p-CXCR4 expression and low plasma SDF-1 levels did not exhibit a significantly poorer prognosis for breast cancer-specific survival (HR, 0.69; 95% CI, 0.23–2.04; $P = 0.50$) or distant disease-free survival (HR, 0.78; 95% CI, 0.36–1.69, $P = 0.52$) than the remainder of the entire cohort. Therefore, the poor prognostic value that we observed in patients with low plasma SDF-1 levels is enhanced in patients with tumors showing high expression of p-CXCR4, and not low p-CXCR4. No interaction between plasma SDF-1 and p-CXCR4 expression was observed in univariate or multivariate analysis for both endpoints (data not shown). Therefore, the prognostic value of high tumor p-CXCR4 and low plasma SDF-1 levels are independent from one another, reflecting the independent source of each marker. Thus, we have identified a specific cohort of primary breast cancers that express high levels of p-CXCR4, suggesting a propensity for significant CXCR4 activity, whose later extravasation into metastatic target sites may be especially promoted in the presence of low plasma SDF-1 levels. We also examined the prognostic value of tumor expression of total CXCR4 and plasma SDF-1 levels. We found that patients with high CXCR4 tumor expression and low plasma SDF-1 demonstrated a significantly worse prognosis due to breast cancer-related causes (HR, 3.45; 95% CI, 1.49–7.99; $P = 0.004$), compared with patients with both low plasma SDF-1 and low CXCR4 expression (HR, 0.96; 95% CI, 0.33–2.83; $P = 0.95$). Therefore, in patients with low plasma SDF-1 levels, tumor metastasis appears to be promoted particularly in cancer cells that express high levels of p-CXCR4 or CXCR4.

Elevated Expression of CXCR4 in Lymph Nodes

If tumor cells expressing high CXCR4 or p-CXCR4 are more likely to metastasize, we would expect to find more of these cells in the first site of metastasis, regional lymph nodes. Lymph nodes were available for 34 patients with their matched primary tumor also present on the tissue microarray. Although the frequency of elevated p-CXCR4 expression was the same in primary tumor and lymph nodes, 88% of patients demonstrated high total CXCR4 expression in the lymph nodes, in comparison with 64% in the primary tumor, which was statistically significant via McNemar's test ($P = 0.02$). Paired *t*-test analysis demonstrated a significant difference in the product score of CXCR4 in lymph nodes compared with primary tumor ($P < 0.0001$), which was not the case for p-CXCR4. 64% of patients demonstrated a higher product score of CXCR4 in the lymph nodes versus primary tumor. Therefore, these results suggest that tumor cells with higher expression of the CXCR4 receptor are more likely to undergo regional metastasis. Given the lack of difference in p-CXCR4 expression levels between the matched primary tumor and lymph nodes, it may be that the microenvironment of the lymph nodes does not particularly select for activation of the CXCR4 receptor.

Clinical Implications for Tumor Expression of p-CXCR4, CXCR4, and CXCR7

CXCR4 and p-CXCR4 expression were correlated with clinicopathological characteristics using Spearman's rank correlation analysis (Table 3). Categorical variable analysis of low and high expression of each biomarker is provided in Supplemental Table S1 at *http://ajp.amjpathol.org*. We found that levels of both CXCR4 and p-CXCR4 expression inversely correlate with ER and PR positivity, and positively correlate with tumor grade and ER-/PR-/HER2- (triple negative) status. Triple negative tumors were almost twice as likely to have medium or high expression of p-CXCR4 as all other tumors (77% versus 41%, $P < 0.001$).

Interaction analysis from the multivariate Cox model of p-CXCR4 revealed an interaction between p-CXCR4 expression and ER status (HR, 0.13; 95% CI, 0.02–1.12; $P = 0.06$) such that ER-positive patients with high p-CXCR4 expression (15% of the total) had a 6.5-fold worse prognosis than all other ER-positive patients (HR, 6.49; 95% CI, 1.08–38.9; $P = 0.04$). This remained essentially unchanged after multivariate analysis (HR, 6.40; 95% CI, 0.95–42.9; $P = 0.06$). No such correlation with survival was found with high phosphorylated CXCR4 expression in ER-negative patients. Thus, despite the otherwise good prognosis of all ER-positive patients, high p-CXCR4 expression has the power to identify a subset of these patients with poor prognosis. Furthermore, subgroup

analysis revealed that the prognostic value of the combination was the greatest among patients with Luminal A or B subtype (ER+/PR+/HER2-, or ER+/PR+/HER2+) (n = 133; HR 8.08, 95% CI, 2.28–28.7; P = 0.001) and the least in the triple negative group of breast cancers (n = 33, HR 2.24, 95% CI, 0.60–8.37; P = 0.23). Thus the presence of the combination marker appears to have a great effect in ER+ breast cancers, while it may not contribute as much to prognostic information in triple negative breast cancers (although numbers in these subgroup analyses are limited).

Due to the functional significance of CXCR7 previously reported in breast cancer tumorigenesis and metastasis,[21,22] we analyzed the clinical relevance of CXCR7. High expression of CXCR7 was associated with poorer outcome in breast cancer-specific survival (HR, 3.63; 95% CI, 1.35–9.76; P = 0.01), and distant disease-free survival (HR, 2.21; 95% CI, 1.00–4.87; P = 0.05), both of which were not significant after multivariate analysis (data not shown).

Discussion

To date, much of the cancer literature has interpreted the metastatic process to be largely dependent on the aggressive potential of the tumor and its ability to invade surrounding tissues and metastasize. However, in addition to the tumor, recent evidence has introduced the significance of the host and its role in predicting metastatic propensity. For example, a genetic influence on metastatic progression has been observed: a Swedish study reported that mothers and daughters of patients with breast cancer of poor outcome who developed breast cancer themselves demonstrated poor prognosis like their first-degree relatives.[23] We previously identified the first host-derived blood marker predictive of distant metastasis in breast cancer, the SDF-1 chemokine,[14] and found that low levels of plasma SDF-1 were predictive of distant metastasis, suggesting that the concentration gradient of SDF-1 between metastatic site and plasma may play a critical role in promoting the extravasation of cancer cells. Since we and others have previously shown an association between overexpression of CXCR4 in primary breast tumors and metastatic risk,[10–12] we investigated whether the metastatic potential of CXCR4 overexpression could be further augmented in the context of low blood SDF-1 levels. Indeed, we found that patients who showed both low blood SDF-1 levels and high tumor CXCR4 expression demonstrated a significantly worse prognosis in comparison with patients with low plasma SDF-1 levels whose tumors did not express high levels of CXCR4. These results suggest that a low plasma SDF-1 level may favor the extravasation of tumor cells expressing high CXCR4. This hypothesis is further corroborated by the higher levels of CXCR4 expression we observed in lymph nodes compared with matched primary tumors, although the mechanism of lymphatic dissemination may be different from hematogenous spread. Enrichment for CXCR4-expressing tumor cells at the metastatic site has been reported previously.[24,25] The tumor-derived risk of metastasis (CXCR4) was thus enhanced with an intrinsic host-derived risk (SDF-1). As a result, we present here, for the first time, evidence for a biologically plausible scenario, providing insight into a dysfunctional relationship between the tumor and its host, which impacts the capacity of breast cancers to form metastasis.

We also found that overexpression of CXCR4 was frequently associated with activation of CXCR4 via phosphorylation. Consistent with a previous report in brain tumors,[16] we found that expression of p-CXCR4, and not total CXCR4, highly correlated with SDF-1 expression, suggestive of the presence of autocrine stimulation of CXCR4 in primary breast tumors. Furthermore, p-CXCR4 expression also correlated strongly with the expression of CXCR7, a recently discovered receptor for SDF-1, implying that heterodimerization of CXCR7 with CXCR4[15,21] may also contribute to activation of CXCR4 patients with primary breast cancers. We then found that high expression of p-CXCR4 is predictive of a fourfold higher rate of breast cancer-specific mortality, and that the prognostic value of high p-CXCR4 is superior to that of high CXCR4 expression for breast cancer-specific survival and distant disease-free survival. Moreover, patients with both high p-CXCR4 levels and low blood SDF-1 levels had a nearly sixfold higher rate of mortality due to breast cancer-related causes, which was more significant than either p-CXCR4 expression or plasma SDF-1 levels alone, and remained significant after multivariate analysis. Although our immunohistochemical analysis deals with the primary tumor and not the distant metastatic site, the presence of activated CXCR4 receptor in this setting may imply a particular dependence of the tumor cell on its CXCR4 receptor, facilitating the selection of CXCR4 expressing cells during the metastatic process.

Finally, several therapeutic agents have been designed to target the SDF-1/CXCR4 ligand/receptor axis,[26–28] one of which is presently being tested in a clinical trial.[29] In preclinical models, such treatments have been shown to be effective not only in decreasing metastasis from breast cancer, but also in inhibiting primary tumor growth in breast cancer.[5,6] As most breast cancers express at least moderate to high levels of CXCR4,[10,11,30] there is a risk that these therapeutic agents may not be adequately targeted, perhaps impeding their clinical development. Since we found that most (77%) patients with triple negative disease express high levels of p-CXCR4, it is possible that these patients who do not benefit from hormonal or anti-HER2 therapy may potentially benefit from agents targeting CXCR4 activity. Most interestingly, we also identified a subset of ER+ patients with high p-CXCR4 expression/low plasma SDF-1 that demonstrated an eightfold higher risk of mortality, who may also potentially benefit from anti-CXCR4 therapy. In these patients with a good prognosis, the measurement of p-CXCR4 tumor expression and plasma SDF-1 may contribute most to provide novel prognostic and potentially predictive information. Although our findings will require follow-up and validation with independent clinical material, elevated p-CXCR4 expression together with low plasma SDF-1 levels may provide a new paradigm for

breast cancer biomarkers, highlighting the interaction between corresponding host and tumor molecular factors.

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Tissue microarrays: emerging standard for biomarker validation

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With the widespread use of DNA microarrays, hundreds of biomarkers are in need of validation in cohorts of well-annotated clinical samples. Tissue microarrays are emerging as the tool par excellence to rapidly perform DNA, RNA, and especially protein expression analyses on large numbers of clinical samples. Although still somewhat limited by the subjectivity of scoring methods and tissue sample representativeness, TMAs represent an increasingly validated means of understanding the clinical impact of diagnostic-related, prognostic-related, and therapy-related markers. Automated methods are being developed for TMA analysis and cell microarrays and frozen tissue TMAs have been better optimized. More and more biomarker studies are availing themselves of the high-throughput nature of TMAs, recognizing that they are becoming indispensable for rapid translation of laboratory data to the clinic.

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Introduction

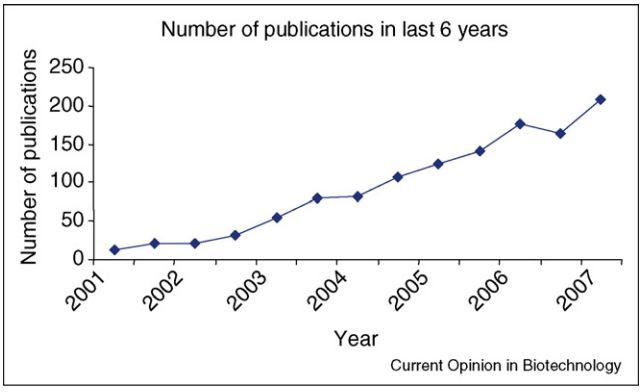
The screening of thousands of genes for changes in either DNA copy number or RNA expression using DNA microarrays has resulted in the discovery of numerous candidate biomarkers that require clinical validation. In fact, the large number of putative molecular targets in preclinical models is sharply contrasted with the small number of markers that have made it to the clinic. In recent years, the process of validating biomarker expression has been facilitated with the increasing use of tissue microarrays (TMAs). TMAs consist of arrays of miniature core biopsies from hundreds of paraffin-embedded tissue samples arrayed in an organized fashion on a microscope slide, and are used as multi-sample platforms to quantify protein levels, RNA expression and DNA copy number [1]. This technique has the advantages of high-throughput analysis as well as minimal variability upon repeated analysis for different markers. Using clinically annotated samples, TMAs serve as a multipurpose scaffold: not only can protein expression and subcellular localization be identified, but defining the relationship of biomarker expression with clinico-pathological features, different stages of disease, other protein biomarkers, and disease prognosis provides a clinical context for putative biomarkers [2]. Using TMAs leads to savings of time, costs, and above all, tissues. As can be seen in Figure 1, more and more research laboratories are publishing results of TMA analyses, especially in oncology research. Invaluable TMAs are being built from samples from controlled clinical trial populations, enabling rapid biomarker validation in homogeneous patient populations associated with treatment outcome data. This review will briefly describe recent trends in TMA applications and analysis, marking the emergence of TMAs as a standard for biomarker validation in clinical samples (Figure 2). We will not detail here the construction of TMAs, except to describe innovative uses recently reported in the literature.

TMA applications: diagnostic and prognostic biomarkers

The most popular use of TMAs remains in basic/translational research, for the validation of diagnostic biomarkers in annotated clinical samples. Diagnostic biomarkers are tissue biomarkers not necessarily linked to therapy that can serve as surrogate endpoint biomarkers for clinical studies, as screening tools for certain diseases, as indicators of prognosis or for the identification of specific subclasses of disease. For instance, in oncology, biomarkers expressed during tumor progression can be monitored on a 'progression TMA' that includes samples from patients with different stages of a specific tumor type, from preneoplastic to metastatic cancer [3]. The major difficulty in building and using a 'progression TMA' lies in the relatively small size or histological limits of pre-invasive lesions, which may prevent the use of deeper sections of the TMA block.

TMAs are becoming the standard for the validation of prognostic biomarkers. As tumor banks and clinical sample cohorts are maturing with regard to clinical follow-up times, it is becoming possible to perform correlations of biomarker expression with clinical endpoints such as disease-free or overall survival [4]. Moreover, the prognostic strength of different biomarkers can be compared using serial sections from the same TMA. When the overexpression of a protein is shown to correlate with prognosis, it strengthens the case for its further development as a therapeutic target [5], though it must be remembered that prognosis is not equivalent to prediction of therapeutic efficacy.

Figure 1

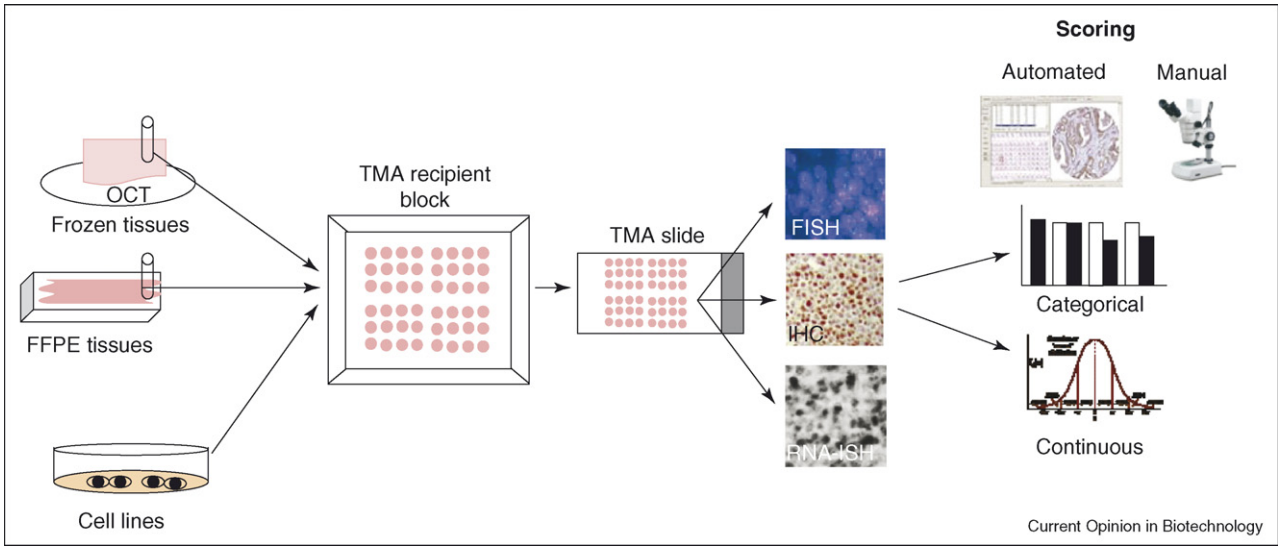


The explosion in TMA use. Trend of increased number of studies that have been using tissue microarrays over the past six years.

An increasing number of studies are applying analytical techniques learned from DNA microarrays to a smaller set of protein markers, in order to perform patient risk stratification or disease classification. As DNA microarrays have shown so well, clusters of biomarkers usually outperform individual markers because of the molecular complexity of disease. Reflecting this complexity, serial TMA sections have been used not only to validate individual markers in the same tissues but also to discover novel clinically relevant biomarker clusters. Unsupervised analysis including hierarchical clustering, random forest clustering, and K-means clustering have all been applied on 8–35 protein signatures to classify patient subgroups [6–10]. Diallo-Danebrock *et al.* 'converted' the distinct gene expression profiles of the molecular subgroups of breast cancer [11] into 'protein expression profiles' using TMAs [9]. Using hierarchical clustering, a set of 11 markers was found to have similar prognostic value as the presence of metastatic cancer in lymph nodes in breast cancer [6]. Other groups have used supervised analysis as well as training and validation sets of clinical samples to identify and then validate biomarker clusters for stratifying patients [12,13]. For example, supervised analysis was used to generate a panel of prognostic biomarkers in estrogen receptor-positive breast cancer patients [13]. Genetic algorithms suited to a smaller number of protein biomarkers have been shown to prospectively assign a score from a continuous variable which can be used to stratify patients [14•]. Models based on these biomarker panels were of comparable significance to prognostic indices currently used in the clinic [7]. Although TMA studies have a selection bias in choosing proteins with available antibodies and with known or suspected links to the disease of interest, the TMA platform can facilitate the identification and validation of protein signatures in clinical studies.

TMAs can also be used to validate DNA-based biomarkers, such as, for instance, genes with increased DNA copies (gene amplification) in cancer tissues. Such applications have used either fluorescent *in situ* hybridization (FISH) or, more recently, chromogenic *in situ* hybridization (CISH). In CISH, hybridization of probes

Figure 2



An updated flow chart of the tissue microarray process, illustrating its present versatility. Core biopsies from frozen tissues embedded in OCT medium or formalin-fixed paraffin-embedded (FFPE) tissues, or cell-agarose gels obtained from cells in culture are placed into a TMA recipient block. TMA blocks are sectioned to provide TMA slides, which can be used for DNA copy number analysis (FISH), protein expression measurement (immunohistochemistry, IHC) or RNA level quantification (RNA-*in situ* hybridization). Scoring can be performed in an automated fashion or manually. IHC scoring can be categorical or continuous.

is analogous to FISH, but the chromogenic signal can be detected with the ordinary light microscope and it costs one-quarter as much as FISH [15]. FISH on TMAs has been frequently used to validate findings of gene amplifications discovered by genome-wide screening of DNA from tumor tissues. Recently, Holst *et al.* [16•] showed that the estrogen receptor α gene (ESR1) is amplified in 21% of breast cancer samples using FISH on TMA. This is the first reported case in which a TMA was used for the discovery of a novel amplification of a gene in cancer tissues. FISH on TMAs may also be used to detect chromosomal deletions and translocations [6]. In both cases (DNA copy loss or gain), because of the discrete 'dot'-like signals obtained with FISH, the scoring of copy number on microscope slides is dependent on the thickness of the TMA sections, which may truncate nuclei, and the care with which multiple focal planes are visualized [17].

TMA applications: therapy-related biomarkers

TMAs are also of increasing utility in defining the feasibility of developing particular therapeutic targets. Functional *in vitro* or animal studies have led to the discovery of candidate therapeutic targets, whose expression characteristics (e.g. frequency in clinical samples, association with other clinico-pathologic features) need to be evaluated in human samples. TMAs containing matching normal tissues can be used to assess expression of putative targets in these tissues, as an initial predictor of the toxicity of approaches targeting the candidate marker. Finally, immunohistochemistry allows *in vivo* protein localization, which can aid in selecting the type of pharmaceutical approach most appropriate for specific targets (e.g. antibodies versus small molecules) [18].

TMAs are also being constructed from collections of samples from patients receiving specific treatments, providing a unique tool for validating biomarkers predictive of response to these treatments. This approach is becoming increasingly popular in translational cancer research, not only for standard chemotherapy agents [19], but also for new biological agents, whose astronomical costs provide a compelling financial reason to really 'target' them. Many clinical trials are now routinely planning sample collection for correlative studies, and the construction of TMAs of tissue biopsies. The ideal setting is that of randomized controlled trials [20], wherein it is feasible to prospectively correlate candidate biomarkers with more complete clinical endpoints, like survival advantage in a more specific and homogeneous population. For example, candidate biomarkers were subsequently analyzed on TMAs built from tumor samples from a randomized controlled trial that demonstrated the addition of bevacizumab to chemotherapy significantly increased survival in patients with metastatic colon cancer [21]. In the future, new candidate predictive markers for bevacizumab could be quickly tested on new sections of that same TMA. The potential for TMAs to enable predictive biomarker research is enormous.

Clinical applications of TMAs

TMAs are also being used for clinical applications in Pathology Departments. For instance, the routine tedious work of testing new antibodies or determining optimal staining conditions can be done more efficiently through small 'test' TMAs, collecting biopsies from samples representative of diverse or specific pathological lesions. In our laboratory, we maintain a supply of test TMAs for the testing of every new antibody destined for immunohistochemical studies. Furthermore, staining sections from larger clinically annotated TMAs with different antibodies for the same protein makes it possible to compare these antibodies, with regards to the clinical outcome for which the reagents were developed [22].

The creation of TMAs sent out to different laboratories can be used as an efficient means of quality control between laboratories. Recently, the College of American Pathologists Immunohistochemistry Committee created TMAs of 80 breast carcinomas, each containing ten 3 mm cores of individual tumors and sent them to more than 100 laboratories for HER2 testing. Over 90% of tested laboratories correctly scored at least 90% of the graded cores [23••]. These TMAs now enable individual pathology laboratories to perform their own quality control against a validated benchmark.

Novel TMA-based platforms

The recent development of cell microarrays (CMAs) will expand the applicability of TMAs in the laboratory. Several groups [24,25•,26] have described a technique wherein both suspension-grown and adherent-grown cells are placed in agarose gels, fixed and then embedded in paraffin. These paraffin-embedded cell-agarose gels can be used to create regular TMAs for the high-throughput analysis of the expression of biomarkers in a wide variety of different cell lines, or in the same cell line subjected to different culture conditions.

Although not a novel idea, there is progress in the development of TMAs of frozen tissues. To date, few studies have reported RNA-*in situ* hybridization (RNA-ISH) on TMAs using paraffin-embedded samples because fixatives affect the quality of RNA, thereby limiting the use of TMAs for this purpose [27–29]. One alternative is to construct TMAs from unfixed frozen tissues embedded in frozen optima cutting temperature (OCT) media by transferring donor samples into frozen recipient OCT blocks [30]. However, the brittleness of frozen OCT renders coring for biopsies as well as for array cavities difficult. Furthermore, smaller numbers of samples can be placed on each TMA because the OCT compound may bend and crack when samples are placed at less than 1 mm apart. Recently, Zhou *et al.* [31] described the use of a novel

gelatin–sucrose-based recipient block in which 96 cores can be prepared for frozen tissue samples. Apparently, RNA and protein integrity is well maintained. It is likely that, as in all frozen tissue-based histological examinations, one may observe some distortion of cell morphology and tissue architecture compared to formalin-fixed tissue sections. Nevertheless, this approach may provide a viable platform for the measurement of mRNA levels in multiple tissue samples on TMAs.

Innovation in TMA scoring and data analysis

Immunohistochemical analyses have been justly criticized for their subjective and semiquantitative means of determining the level of protein expression. Subjectivity is not eliminated even when more than one reader of the TMA is solicited. Moreover, methods of quantifying qualitative IHC readings can vary. Semiquantitative methods include intensity scores, which categorize data into three or four groups (typically 0 to 3+), percentage of highly stained cells, and also multiplicative scores whereby the product of the percentage of cells and intensity scores are calculated to generate a score from 0 to 300 [10,32,33]. The recent use of automated image analysis improves the objectivity of IHC reading and allows for the creation of a continuous variable, enabling more robust comparisons of readings across different institutions. There are at least eight different software programs available that have been reviewed elsewhere [34], which enable the user to select a particular tissue compartment of interest on the TMA. In some cases, the automated approach has been shown to increase the sensitivity of detection, identifying markers of prognostic significance or therapeutic response not previously shown with manual reading [35,36].

Because manual scoring of FISH slides is laborious and difficult, automated scoring software has also been developed to ensure rapid detection of gene amplifications on TMAs. As described by Brown and Huntsman, the use of the Metacyte system (Metasystems, Altlussheim, Germany) can correctly classify 83–95% of ovarian tumor cases with respect to the presence of a gene amplification on chromosome 11. Such systems are not yet reliable enough to obviate user definition of regions for analysis. However, they allow the user to store a digital data set of the spot counts as well as an image gallery of each analyzed cell, which is important in the case of FISH, as fluorescent signals weaken in time and re-evaluation of a particular case may not be feasible several months later [17].

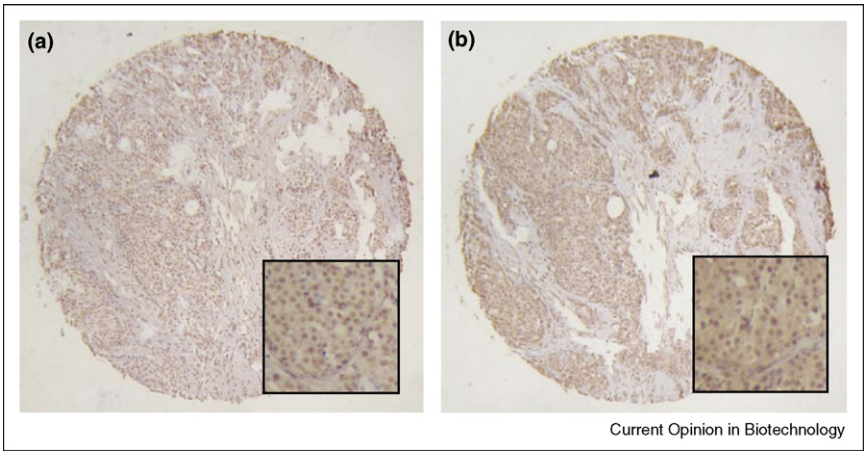
The use of continuous scoring counts requires more sophisticated biostatistical data analysis. Although the best way to categorize data would be a biological one, such information is often lacking when a novel biomarker is being characterized. As a result, either arbitrary divisions are made, or 'optimal' cutpoints are sought based on the categorization that yields the minimum p-value [37]. The high rate of false positivity inherent in the latter method may contribute to the poor reproducibility of such studies for prognostic markers [38]. This can be overcome with the use of a training and validation set or other cross-validation techniques. For example, X-tile (Robert Camp, Yale University) is a software that uses the prognosis of patients to elicit the 'optimal' cutpoint of continuous data, that is, the cutpoint is derived from the most significant Chi-square value and minimum p-value, which is validated with either Monte Carlo simulations or training/validation sets. Interestingly, this software also shows the frequency distribution of the marker in a way that may provide further insights into underlying biology [39].

Limitations of TMAs

Despite the increasing widespread use of TMAs, old questions and limitations remain. The foremost limitation of TMAs is because of their dependence on the uneven reliability or poor availability of antibodies functioning in immunohistochemistry. Thanks to international efforts such as the HUPO Antibody Initiative [40], we can expect that the increased availability of validated antibodies will expand TMA applications. Moreover, increasing commercial production of reliable, specific phospho-protein antibodies now allows users to supplement the quantification of protein expression with measurements of activity in the same tissues in serial TMA sections (Figure 3).

Since the first report in 1998 [1], a criticism of TMAs has been the possibility that small cores might not adequately represent the whole tissue section, particularly in the case of tumors, because of intratumoral heterogeneity of protein expression. Several groups have assessed the representativeness of TMAs by comparing results obtained from small cores with corresponding whole tissue sections. Most validation studies focused on IHC analysis of protein expression in several cancer types, while only a few studies have validated FISH analysis on TMAs [41]. To increase the reliability of TMA assays, the most representative areas should be selected for sampling by careful examination of sections of the original donor blocks. The two usual ways to ensure TMA representativeness are to increase the number of cores collected from each sample and/or to increase the size of the single cores (e.g. to 1.0 mm or even 3.0 mm) [42,43]. For example, a recent study reported that concordance in positive staining for the melanoma cell adhesion molecule, MCAM, in TMA cores versus whole sections ranges from 57% for one core, to 90% and 97% for three and four cores, respectively [44]. In fact, most published validation studies comparing IHC on large tumor sections to TMAs containing two to four cores per sample indicated that intratumoral heterogeneity for many antigens is not an issue [45]. However, the concordance of biomarkers between TMA cores and whole sections appears to be marker-specific and dependent on the tissue type, as tissue heterogeneity of marker expres-

Figure 3



Phospho-protein staining on TMAs: (**a**) immunohistochemistry staining of CXCR4 using MAB172 (R&D Systems) and (**b**) immunohistochemistry staining of Phospho-CXCR4 (antibody courtesy of Dr Joshua Rubin, Washington University).

sion may vary in different contexts. For example, the expression of Ki67 in glioblastomas [46] and S-100 in gastrointestinal stroma tumors [45] demonstrated relatively poor concordance between TMAs and whole tissue sections. In one study, four identical TMAs with single 0.6 mm spots were created from 553 cases of breast cancer [47]. A single core was found to correctly represent the expression of ER, PR, and p53 in 72–95% of cases, depending on the marker. However, if a sufficient number of cases are present in the TMA, lower rates of representativeness may not hamper the usefulness of the TMA. In the above example of the TMA with 553 breast cancers, the prognostic value of each of the three markers was maintained when analyzed on each of the four TMAs alone compared to large section analysis, suggesting that if a sufficient number of cases are present on a TMA, imperfect representativeness may not hamper the usefulness of the TMA. Finally, the ERBB2 gene is the only amplified gene for which validation has been performed for the use of FISH on TMAs. As with IHC validation studies, these reports showed a 91–96% concordance between TMAs using a single 0.6 mm biopsy per sample and whole section analysis [48,49].

Most studies using TMAs lack any specific 'validation' (i.e. comparison to whole tissue sections) for their particular biomarkers. It could be reasonably assumed that a significant association between biomarker over-expression and clinical outcome probably reflects a real correlation because one usually scores the highest expressing zone of the tissue section. In the case of a nonsignificant association between over-expression and outcome, the possibility that a TMA-based strategy may be confounded by intratissue marker heterogeneity should be considered, especially for single-core TMAs. In general, in our opinion, it is safer to use TMAs with at least two cores per sample. This also diminishes the number of cases not evaluable because of loss of samples during block sectioning. Moreover, in our experience, the placing of duplicate core biopsies side-by-side increases the confidence of sample reading on TMAs.

Conclusions

In summary, more and more research laboratories are availing themselves of the high-throughput advantage of TMAs in biomarker validation. TMAs are the perfect complement to clinically annotated tissue banks, as they enable the preservation of these precious clinical materials. Their construction is not difficult, and their analysis rapid. Sophisticated tools are being developed to automate TMA scoring and facilitate data analysis, which will further accelerate the TMA process. More validated and versatile antibodies can be used in immunohistochemistry, while TMAs can now more reliably be applied for biomarker validation in cultured cell lines and frozen tissues. Although routine clinical applications are not feasible, TMAs are becoming an indispensable tool to translate laboratory findings to the clinic. As more individual biomarkers and biomarker clusters are validated on TMAs, we expect their use to continue to increase. Academic pathologists are becoming more familiar with them, recognizing the enormous time savings they offer, which is enabling their participation in translational research. During the decade since their discovery, we can observe that the potential of TMAs to accelerate the translation of laboratory discoveries to the clinic is already being fulfilled.

Conflicts of interest

We would like to disclose that none of the authors has any financial interest in or arrangement with a company whose product was used in this study, or with a competing company.

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- •• of outstanding interest

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October 2, 2003

Dr. Mark Basik
Surgery & Oncology
SMBD-Jewish General Hospital

SUBJECT: **Protocol #03-085 entitled** "Stromal cell-derived factor 1 and its receptor as markers of breast cancer metastasis"

Dear Dr. Basik:

Thank you for submitting the above-mentioned research project to the Research Ethics Office for review.

The Research Ethics Committee of the SMBD-Jewish General Hospital (Federalwide Assurance Number: 0796) is designated by the province (MSSS) and follows the published guidelines of the Tri-Council Policy Statement (1998), in compliance with the "Plan d'action ministériel en éthique de la recherche et en intégrité scientifique" (MSSS, 1998), and the Food and Drugs Act (17 June, 2001); and acts in conformity with standards set forth in the (US) code of Federal Regulations governing human subjects research, and functions in a manner consistent with internationally accepted principles of good clinical practice.

As this study involves no more than minimal risk in accordance with Article 1.6 of the Canadian Tri-Council Policy Statement of Ethical Conduct for Research Involving Humans, we are pleased to inform you that the above-mentioned protocol is granted expedited approval. It is our understanding that this protocol the use of sera and lymphocyte DNA from patients with breast cancer who consented to the collection of their specimens for the purpose of tumor banking at the Centre Hospitalier de l'Université de Montréal (CHUM) and its use in research involving breast cancer within the Reseau de Cancer of the FRSQ. Please be informed that this study proposal will be presented for corroborative approval at the next meeting of the Committee, October 17, 2003.

Expedited Approval Date: **October 2, 2003**
Expiration date of Expedited Approval: **October 1, 2004**

...2

A "Continuing Review Package" must be received by the Research Ethics Office **one month** prior to the expiration date mentioned-above in order to ensure timely review (otherwise the study will be terminated). If any modification to the study occurs over the next twelve months (including changes to the consent), please advise the REO promptly. Should this study be completed before this time, you must submit a "Completion Form" to the Research Ethics Office, A-702.

Sincerely,

Jack Mendelson, MD
Chairman, Research Ethics Committee

JM/fc
03-085ExpApp.doc

Guidelines for completing the form are available at www.mcgill.ca/rgo/animal/

McGill University
Animal Use Protocol – Research

Protocol #: 5168
Investigator #: —
Approval End Date: June 30, 2007
Facility Committee: 3GH

Title: Anti-CXCR4 therapy in breast cancer
(must match the title of the funding source application)

☒ New Application ☐ Renewal of Protocol # ____ ☐ Pilot **Category** *(see section 11)*: D

1. Investigator Data:

Principal Investigator: Dr. Mark Basik **Phone #:** (514) 340-8222; ext 4930
Unit/Department: Oncology **Fax#:** (514) 340-8716
Address: 3755 Cote Ste. Catherine, Jewish General Hospital, Montreal, H3T 1E2 **Email:** mark.basik@mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: Saima Hassan **Work #:** 340-8222 ext 2782 **Emergency #:** (514) 306-4250
Name: Mark Basik **Work #:** 340-8222 ext 4930 **Emergency #:** (514) 413-0993

3. Funding Source:

External ☒
Source (s): Chemokine Therapeutics
Peer Reviewed: ☐ YES ☒ NO**
Status : ☐ Awarded ☒ Pending
Funding period: ____

Internal ☐
Source (s): ____
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period: ____

For Office Use Only:

ACTION	✓	DATE
CC	✓	" 17
DB	✓	July 13/06
APPROVED		

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/rgo/animal

Proposed Start Date of Animal Use (d/m/y): 1/02/2006 **or ongoing** ☐
Expected Date of Completion of Animal Use (d/m/y): 1/01/2007 **or ongoing** ☐

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature: MBasik **Date:** 30/3/06

Approved by:

Chair, Facility Animal Care Committee:	Louise Chabot	**Date:** 30 March 06
University Veterinarian:		**Date:** May 17 2006
Chair, Ethics Subcommittee (as per UACC policy):		**Date:** 6/8/06
Approved Animal Use	**Beginning:** July 1, 2006	**Ending:** June 30, 2007

☐ This protocol has been approved with the modifications noted in Section 13.

ACTION		
CCs		
DB	✓	
APPROVED		

www.mcgill.ca/research/compliance/animal/forms/

For Office Use Only:
Protocol #: 5168
Approval end date: June 30, 2008
Facility Committee: SCH
Renewal#: (1st) 2nd

McGill University Animal Care Committee
RENEWAL of Animal Use Protocol

For: Research ☒ Teaching ☐ project

Principal Investigator: Dr. Mark Basik **Protocol #** 5168

Protocol Title: Anti-CXCR4 therapy in breast cancer **Category:** (D)

Unit, Dept. & Address: Oncology

Email: mark.basik@mcgill.ca **Phone:** 340-8222 ext. 4930 **Fax:** 340-8716

Funding source: Chemokine Therapeutics

Start of Funding: August 2006 **End of Funding:**

Emergency contact #1 + work AND home phone #s: Saima Hassan Work: 340-8222, ext. 2782 Pager: (514) 306-4250

Emergency contact #2 + work AND home phone #s: Mark Basik Work: 340-8222, ext. 4930 Pager: (514) 413-0993

1. Personnel and Qualifications

List the names of the Principal Investigator and of all individuals who will be in contact with animals in this study and their employment classification (investigator, technician, research assistant, undergraduate/ graduate student, fellow). Indicate if the Principal Investigator is not handling animals. If an undergraduate student is involved, the role of the student and the supervision received must be described. Training is mandatory for all personnel listed here. Refer to *www.animalcare.mcgill.ca* for details. Each person listed in this section must sign. ***(Space will expand as needed)***

Name	Classification	Animal Related Training Information		Occupational Health Program *	Signature *"Has read the original full protocol"*
		UACC on-line Theory course	Workshops + others		
Mark Basik	Principal Investigator	UACC Theory on-line course taken, will not be handling animals.			
Saima Hassan	surgery resident/ graduate student	UACC Theory and worskhop taken		Yes	
Orlando Angulo	Volunteer, Foreign MD	UACC Theory and workshop taken		Yes	

*** Indicate for each person, if participating in the local Occupational Health Program, see http://www.mcgill.ca/research/compliance/animal/occupational/ for details.**

Approved by:

2. Approval Signatures

Principal Investigator/ Course Director		**Date:** July 5, 2007
Chair, Facility Animal Care Committee		**Date:** July 17, 2007
RESEARCH ETHICS OFF.		**Date:** Aug 10 '07
Chairperson, Ethics Subcommittee NOT *(D level or Teaching Protocols Only)*	—	**Date:** —
Approved Animal Use Period	**Start:** July 1, 2007	**End:** June 30, 2008

☐ **Renewal requires submission of full Animal Use Protocol form**

25 JUIL. 2007

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Stacey Taylor

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