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THE ROLE OF CCL21/CCR7 CHEMOKINE AXIS IN VEGF-C MEDIATED BREAST CANCER INDUCED LYMPHANGIOGENESIS

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By

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ABSTRACT

Although the molecular mechanisms underlying lymphangiogenesis associated with breast cancer continue to remain insufficiently understood, a growing body of evidence suggests that many of the currently unknown answers revolve around the crosstalk between the lymphangiogenic factor VEGF-C and chemokines. The present study proposed the CCL21/CCR7 chemokine axis as a regulatory mechanism of VEGF-C mediated breast cancer-induced lymphangiogenesis. In order to address the hypothesis, the positive correlations between CCR7 signalling and VEGF-C expression/secretion by MDA-MB-231 cells were sought, along with the molecular mechanism underlying their correlation. Furthermore, the direct effect of CCL21/CCR7 interaction on lymphatic endothelial cells (LECs) was tested through a series of *in vitro* lymphangiogenic assays. CCL21/CCR7 axis has been found to regulate lymphangiogenesis in two distinct ways: i) directly, through stimulation of the lymphangiogenic traits of LECs; and ii) indirectly, through the promotion of VEGF-C secretion by breast cancer cells. These results suggest a novel role of the CCL21/CCR7 axis in the promotion of breast cancer-induced lymphangiogenesis.

KEYWORDS: CCL21 chemokine, CCR7 chemokine receptor, vascular endothelial growth factor-C, phosphatidylinositol 3-kinase, protein kinase B, lymphangiogenesis, breast cancer.

To the memory of my father

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
COX-2	Cyclooxygenase-2
CCL21	Chemokine CC Motif Ligand 21
CCL2	Chemokine CC Motif Ligand 2
CCR7	Chemokine CC Motif Receptor 7
CCXCKR	ChemoCentryX Chemokine Receptor
CXCL8	Chemokine CXC Motif Ligand 8
CXCR4	Chemokine CXC Motif Receptor 4
DAPI	4', 6-Diamidino-2-Phenylindole, Dihydrochloride
DARC	Duffy Antigen Receptor for Chemokines
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco's Phosphate-Buffered Saline
EBM	Endothelial Basal Medium
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Estrogen Receptor

ERK1/2	Extracellular Regulated Kinase 1/2
EP2, EP4	Prostanoid Receptor 2, 4
FBS	Fetal Bovine Serum
GAG	Glycosaminoglycan Chain
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GFR	Growth Factor Reduced
GPCR	G Protein-Coupled Receptor
Her2	Human Epidermal Growth Factor Receptor 2
HGF	Hepatocyte Growth Factor
HIF-1α	Hypoxia-Inducible Factor 1α
HMVEC-dLy	Human Dermal Lymphatic Microvascular Endothelial Cells
HUVEC	Human Umbilical Vein Endothelial Cells
IGF-1R	Insulin-Like Growth Factor Type 1 Receptor
IL-6	Interleukin 6
ITLs	Intratumoral Lymphatics
LECs	Lymphatic Endothelial Cells
LMP-1	Latent Membrane Protein-1
LYVE-1	Lymphatic Vessel Endothelial Receptor-1
MAPK	Mitogen-Activated-Protein Kinase

MAb	Monoclonal Antibody
M-PER	Mammalian Protein Extraction Reagent
mRNA	Messenger Ribonucleic Acid
PGE 2	Prostaglandin E2
PI3K	Phosphatidylinositol 3-Kinase
PKB/AKT	Protein Kinase B
PLC	Phospholipase C
PROX-1	Prospero Homebox Protein-1
PVDF	Polyvinyllidene Fluoride
Q Real-Time PCR	Quantitative Real-Time Polymerase Chain Reaction
Real-Time PCR	Real-Time Polymerase Chain Reaction
SAGE	Serial Analysis of Gene Expression
SFM	Serum Free Medium
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TBS	Tris-Buffered Saline
TBS-T	Tris-Buffered Saline with Tween-20
TNF-α	Tumor Necrosis Factor-α
VEGF-C, D	VascularEndothelial Growth Factor-C, D
VEGFR-2, 3	Vascular Endothelial Growth Factor Receptor-2, 3

1.1 Breast Cancer Overview

Breast cancer represents the most common form of malignancy among women worldwide. Recent epidemiologic data indicate that mammary tumors have the highest incidence and mortality rates among females, accounting in 2008, for 23% (1.38 million) of total cancers and 14% (458,400) of total cancer deaths, respectively (Ferlay et al., 2010). While approximately half of the new breast cancer cases and 60% of their related deaths occur in developed countries (Western and Northern Europe, Australia/New Zealand, and North America), the numbers decrease to low levels in sub-Saharan Africa and Asia. However, unlike in the past decade, breast cancer is now regarded as the leading cause of cancer death in both economically developed and developing countries (Jemal et al., 2011).

The large statistical differences between developed and developing countries can be attributed to reproductive and hormonal factors, as well as to early detection and available treatment options. It is important to outline that despite the high breast cancer incidence rates recorded in developed countries, the associated mortality rates seems to have reached a plateau between 1960 and 1990, followed even by notable declines in Northern Europe (WHO, 2008). Due to national mammographic screening programs and advances in therapy, the survival rates in these countries have risen to 85% compared to only 50-60% in the rest of the world, which continues to experience a slow growth in both breast cancer incidence and mortality rates (WHO, 2008; Parkin et al., 2008).

Statistical data show that approximately 80% of breast malignancies develop from ductal epithelium, while only a small proportion begin their development in the

lobular epithelium (WHO, 2008). Despite the prognosis advantages offered by early detection and diagnosis, unfortunately only about 60% of breast cancers are detected at a local stage (Eccles et al., 2007), while the rest are characterized by various degrees of metastatic dissemination.

Traditionally, the metastatic spread of breast carcinomas has been explained on the basis of sequential progression involving the initial invasion of the local blood vasculature followed by subsequent distribution, deposition and development at remote sites (Karnoub and Weinberg, 2006). Similar to many other types of cancers, breast tumors tend to metastasize initially into the regional lymph nodes, a process that typically is followed by dissemination into distant organs. Generally, breast cancers are characterized by increased invasiveness and versatility in metastatic mechanism, which make them able to spread to a wide range of locations within the body.

While colonization of many of the remote sites can be explained – at least in part – by their anatomical proximity and exposure to blood circulatory system, this cannot constitute the basis of the frequency of breast cancer metastasis. Many researchers consider that this represents in fact a strong indication that breast tumors also spread through the alternate systemic pathways that are more or less independent from blood vasculature. One of the most anatomically feasible options in this sense can be constituted by lymphatics.

1.2 The Lymphatic Vascular System

1.2.1 Anatomy and Physiology of the Lymphatics

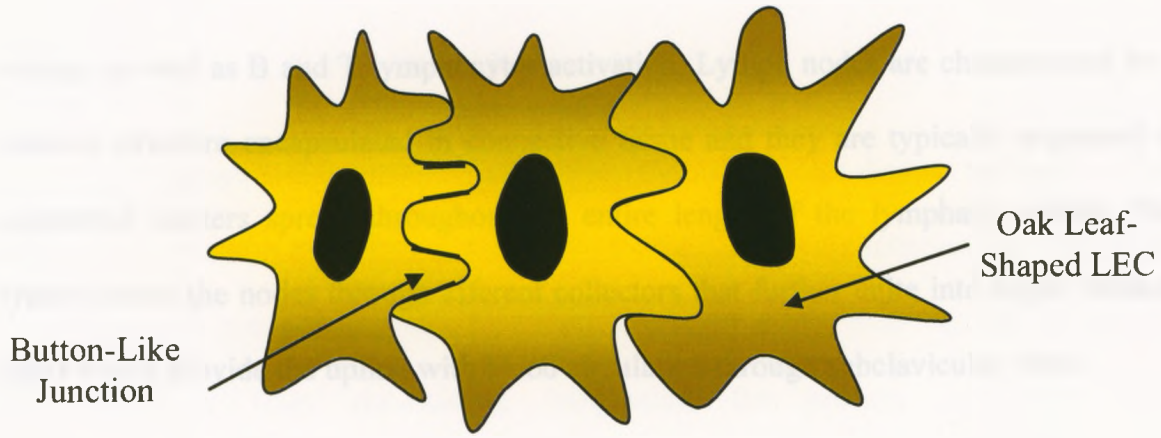
The lymphatic system constitutes a hierarchically-organized network of vessels that unidirectionally transports a protein-rich fluid extravasated from the cardiovascular system (Tammela et al., 2005). Lymphatic vessels have a universal distribution in all vascularized tissues with the exception of the central nervous system, bone marrow, retina, and placenta. The absence of lymphatics in the body is practically incompatible with life, and their dysfunctionality often translates into chronic edema and/or impaired immune responses (Tammela and Alitalo, 2010). Unlike the blood vascular system, the lymphatic network is characterized by an open ended circulatory structure that starts peripherally with blind-end capillaries and increases gradually to large vessel diameters that eventually interlink with the venous system (Sundar and Ganesan, 2007). Besides capillaries, the taxonomy of lymphatic architecture includes pre-collecting vessels that provide a link between capillaries and larger collecting vessels, lymph nodes, trunks, and ducts (Liersch et al., 2010).

Finger shaped lymphatic capillaries are characterized by relatively small diameters (30 – 80 microns) and are lined by a single layer of thin-walled lymphatic endothelial cells that lack pericytes or smooth muscle cells, have incomplete or no basement membrane, and display distinct gene expression patterns (Alitalo et al., 2005; Maby-El Hajjami and Petrova, 2008; Wick et al., 2007). In the absence of basal membrane, smooth muscle cells, or tight cell-cell junctions, lymphatic capillaries connect and stabilize their shape through very thin fibrillin-containing filaments anchored in the

neighboring extracellular matrix (Alitalo et al., 2005). While the lumen of the lymphatic capillaries is closed during physiological conditions, the aforementioned filaments become activated under the increased pressure conditions associated with tissue swelling/inflammation and thus favor interstitial fluid drainage by preventing the lymphatic capillary from collapsing. The interconnections between lymphatic endothelial cells (LECs) are present in the form of highly specialized discontinuous "button-like" connections comprised of specialized adherens and tight junctions (Witte et al., 2011) that facilitate interstitial protein drainage/uptake and immune cells transmigration (Baluk et al., 2007; Tammela et al., 2007; Dejana et al., 2009). These "button-like" connections provide lateral anchoring for the overlapping flap borders of oak leaf shaped LECs (Dejana et al., 2009). This particular type of overlapping LEC junction also fulfils a lymph flow regulatory role due to its dual valve functionality that prevents both intralymphatic and interstitial backflows. Unlike capillaries, the walls of collecting lymphatic vessels are characterized by continuous "zipper-like" interendothelial junctions that are commonly found in blood vessels. The cross sectional aspect of the collecting vessels is regulated by the action of smooth muscle and basal membrane. Collecting vessels are organized into contractile formations called lymphangions, which are separated by bileaflet valves that have role in lymph backflow prevention (Bazigou et al., 2009). The unidirectional lymph propulsion through precollecting and collecting lymphatics is caused by the concurrent action of the intrinsic contractility of smooth muscle cells, as well as surrounding skeletal muscles and arterial pulsations (Figure 1).

The lymph accumulated in the afferent collectors is further drained into subcapsular sinuses of the lymph nodes, fulfilling complex roles in lymph filtration and

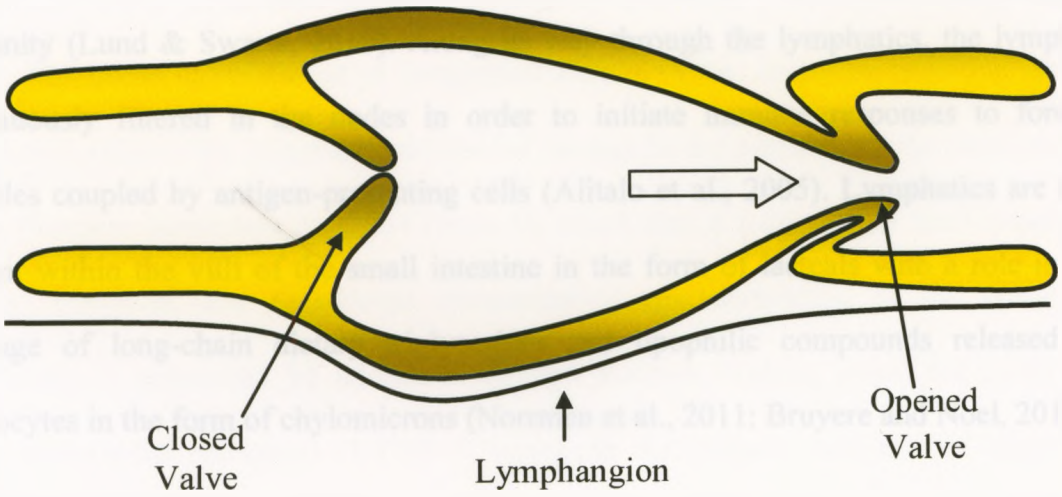
Figure 1. Junctional Organization of LECs. A) Lymphatic capillaries have discontinuous "button-like" junctions which are characteristic of the oak leaf-shaped lymphatic endothelial cells. These junctions serve as primary valves to prevent the backflow of the interstitial fluid from the lymphatic vessels into the tissue. **B)** Endothelial cells in collecting lymphatic vessels are elongated and connected by continuous "zipper-like" junctions. C) Functionally, collecting lymphatic vessels are organized in a series of units, named lymphangions, separated by intraluminal valves. High fluid pressure opens a valve whereas reverse flow closes the valve ensuring a unidirectional lymph flow.


Oak Leaf-
Shaped LEC
Button-Like
Junction

A)


Elongated
LEC
Zipper-Like
Junction

B)


Closed
Valve
Lymphangion
Opened
Valve

C)

storage as well as B and T lymphocytes activation. Lymph nodes are characterized by a discrete structure encapsulated in connective tissue and they are typically organized in sequential clusters spread throughout the entire length of the lymphatic system. The lymph leaves the nodes through efferent collectors that further unite into larger thoracic ducts which provide the uplink with blood circulation through subclavicular veins.

The lymphatic system simultaneously fulfils a number of key physiological roles related to the homeostasis of the tissue fluid, immune cell trafficking, and absorption of dietary fats (Tammela and Alitalo, 2010). To maintain the required tissue-fluid homeostatic balance, the terminal vessels of the lymphatic network are involved in a permanent interstitial absorption of extravasated fluids, macromolecules, lymphocytes and antigen-presenting cells. The interstitial soluble uptake collected at the capillary periphery that forms the lymph returns to the bloodstream via larger lymphatics and the thoracic duct. However, in addition to this conventional passive conduit function, recent evidence suggests that lymphatic vessels also play a strong and active role in the modulation of immunity by adjusting the balance between peripheral tolerance and immunity (Lund & Swartz, 2010). Along its way through the lymphatics, the lymph is continuously filtered in the nodes in order to initiate immune responses to foreign particles coupled by antigen-presenting cells (Alitalo et al., 2005). Lymphatics are also present within the villi of the small intestine in the form of lacteals with a role in the drainage of long-chain dietary triglycerides and lipophilic compounds released by enterocytes in the form of chylomicrons (Norrmen et al., 2011; Bruyere and Noel, 2010).

1.2.2 Lymphatic Neogenesis and Associated Markers

Although still a matter of open scientific debate, two distinct terms have been advanced in the past in conjunction with the formation of lymphatic vessels. Depending on whether the generation of new lymphatic structures occurs from vascular endothelial progenitors or from pre-existing lymphatics, the process is called either lymphvasculogenesis or lymphangiogenesis, respectively. By contrast to lymphvasculogenesis, which is limited to early embryogenesis, lymphangiogenesis occurs both during intrauterine organ development and in postnatal life. Lymphangiogenesis manifests during both normal and healthy processes, as well as under pathological conditions including malignancies and metastatic dissemination. Nevertheless, physiological lymphangiogenesis in adults – although rare – exists especially in context of ovarian growth and wound healing (Paavonen et al., 2000; Saaristo et al., 2006).

The embryology of lymphatics is tightly interconnected to that of the blood vasculature, since lymphatics begin to develop by sprouting from jugular veins (Sabin, 1902). The initial development of lymphatics starts with the commitment of the venous endothelial cells to the lymphatic endothelial lineage in the presence of the transcription factor Prospero homeobox protein-1 (Prox-1), which also is a commonly used lymphatic marker. However, the subsequent differentiation and maturation of the lymphatic vasculature is controlled by additional lineage markers. Among them, the lymphatic vessel endothelial receptor-1 (LYVE-1) represents the primary indicator of lymphatic endothelial competence and it is generally regarded as a highly specific marker used to differentiate between lymphatic and blood vascular endothelia (Jurisic and Detmar, 2009;

Maby-El Hajjami and Petrova, 2008). Podoplanin is a mucin transmembrane glyco-protein expressed on lymphatic vessel endothelium and with role on its correct function and formation (Breiteneder-Geleff et al., 1999; Schacht et al., 2003). Finally, positioned at the core of *de novo* lymphatics generation is the vascular endothelial growth factor receptor-3(VEGFR-3) with an active role in the regulation of LEC proliferation, migration, and sprouting (Jeltsch et al., 1997; Veikkola et al., 2001).

1.2.3 Role of the Lymphatic System in Cancer

1.2.3.1 Lymphovascular Invasion

In contrast to blood vessels, lymphatic vasculature offers a convenient conduit for invasion and transportation of metastatic cells, since it is natively equipped with cell-transport capabilities that support survival and proper activity of the transported cells. As a result, lymphatics represent a common dissemination pathway for many types of epithelial tumors, including breast cancer.

Although the actual cellular mechanisms responsible for fluid and cell transvasation into lymphatics it is still insufficiently understood (Witte et al., 2011), it is very likely that the increased physiological permeability of the lymphatic to the interstitial products – caused by the "button-like" junctions – also make them easily penetrable by tumor cells that have already detached from the neighbouring tumoral mass. Lymphatic vessels are not only easy to invade, but they also provide ideal routes for migratory tumor cells due to their larger diameters, since even the smallest lymphatic vessels are larger than blood capillaries. Thus, the combined effect of: i) low intralymphatic shear stress caused by the reduced flow rate, ii) the absence of mechanic

deformations, and iii) a high lymph concentration of hyaluronic acid, a molecule with cell protecting properties (Laurent and Fraser, 1992) all tend to significantly enhance the survival rates of metastatic tumor cells that have managed to escape into lymphatics. As such, lymphatics are generally regarded as convenient and preferential tumor dissemination pathways to lymph nodes and beyond (Azzali, 2007).

1.2.3.2 Tumor-Associated Lymphangiogenesis

For an extended period of time, the existence of tumor-induced lymphangiogenesis was denied by a large majority of the researchers in the field. However, after the discovery of lymphatic endothelial markers, this hypothesis began to be questioned by several studies demonstrating that tumor-induced growth of lymphatic vessels constitutes in fact one of the active promoters of metastatic dissemination of primary breast tumors to regional lymph nodes (Padera et al., 2002; Dadras et al., 2003).

Despite these findings, the presence of tumor-induced lymphangiogenesis in breast carcinomas continues to remain uncertain since the strength of somewhat indirect proofs based on the association of pro-lymphangiogenic markers expression and high incidence of lymph node metastasis can always be regarded as insufficient (Agarwal, et al., 2005; Williams et al., 2003; Vleugel et al., 2004; van der Auwera et al., 2004; van der Schaft et al., 2007). Beyond this, it is still unclear whether the lymphatic spread of mammary tumors occurs through pre-existing or newly-formed lymphatic vasculature (Ran et al., 2009).

One of the possible explanations for these seemingly contradictory results resides in the partially reduced or even totally absent functionality that was often noticed

for intratumoral lymphatics (ITL). Indeed, while lymphatics can easily penetrate tumor stroma and provide a minimal resistance/maximum contact dissemination pathway for detached cancer cells, their thin-walled conformation makes their lumen more prone to collapse than blood vessels (Ji, 2006). The increased compression forces exerted on the walls of ITLs are caused either by the growing tumors (Helmlinger, 1997) or by the elevated interstitial pressure. Moreover, in addition to the increased compressive mechanical stress applied on the ITLs by the surrounding environment, several researchers have linked their reduced/absent functionality to their inherent physiological abnormal features (Padera et al., 2002; Leu et al., 2000).

In reality, it seems that tumors might be capable of altering the functions of the neighbouring pre-existing lymphatic network that will eventually be co-opted into tumor cell migration with little involvement from endothelial progenitors (He et al., 2004).The lymphangiogenic process is known to be controlled by the action of endothelial extracellular matrix and growth factors (Banerji et al., 1999; Jackson, 2003). Among them, tumor-expressed exogenous VEGF-C has been proven to play a decisive role on LECs development and tumor cell movement, therefore its effect on tumor-induced lymphangiogenesis and lymph node metastasis is deemed of paramount importance (Karpenen et al., 2001; Skobe et al., 2001; Ji, 2005; Ji, 2006; Timoshenko et al., 2006; Matsui et al., 2008). According to more recent evidence, LECs are capable of extending filopodia towards the VEGF-C-expressing tumoral mass. These VEGF-driven extensions will essentially form the future lymphatic vascular sprouts (Alitalo et al., 2005) that tend to develop either within or at the periphery of the tumors, as demonstrated in several experimental tumor models (Saharinen et al., 2004; Stacker et al., 2001; Karpanen et al.,

2001). In addition to the aforementioned pro-lymphagiogenic factors, macrophages are also regarded as active players of pathological lymphangiogenesis due to their dual involvement with both transdifferentiation/direct endothelial incorporation and with the promotion of pre-existing LECs growth (Kerjaschki, 2005).

However, breast carcinomas might also trigger the lymphangiogenic processes within the draining nodes. Lymph node metastasis is promoted by a multitude of intricate factors like: the increased permeability of lymphatics for tumor cells along with their diminished flow rates, the inherent process of tumor-induced lymphangiogenesis, and the permanent flow of the peripherally collected lymph into the sentinel lymph nodes (Das and Skobe, 2008). Based on these findings, more research studies involving both animal models and clinical observations have started to acknowledge that lymph node lymphangiogenesis could be one of the key promoters of the metastatic dissemination of breast tumor cells at remote sites and/or organs (Kaplan et al., 2006; Tobler and Detmar, 2006; Hirakawa et al., 2007; Sleeman et al., 2009). Although it is still unclear how breast tumors manage to enhance their spreading precisely by the means of lymph nodes characterized by extremely well defined roles in the immune response, it is believed that tumor-induced sentinel lymph node lymphangiogenesis could in fact "prepare the ground" for the upcoming metastatic invasion by promoting a tolerogenic niche environment (Sleeman and Cremers, 2007).

1.3 The Lymphangiogenic VEGF-C/VEGFR-3 Signalling Pathway

While the advancements in understanding the role of lymphangiogenesis have been long delayed by a lack of adequate methodology for identification of its regulatory molecules as well as for lymphatic endothelium-specific markers (Pepper, 2001; Sleeman et al., 2001), their discovery at the end of the 1990s practically opened a new area in cancer research. In the past few years, it has became clear that intratumoral, peritumoral and even sentinel node formation of new lymphatics is ultimately controlled by a complex network of growth factors. Most of the techniques that are currently available to demonstrate the presence of lymphangiogenesis rely on the detection of lymphatic endothelial (LYVE-1, podoplanin, Prox-1, VEGFR-3) markers, as well as on an increase in lymphatic vessel density (Ran, 2009).

Once the initial problems associated with the positive identification of lymphangiogenesis were solved, two new questions arose: 1) what is the inherent mechanism responsible for the induction of lymphangiogenesis; and 2) what exactly makes the tumor cells leave the tumoral mass and enter lymphatics. In this sense, recent evidence suggests that one of the possible answers to both questions resides in the crosstalk between VEGF-C and chemokines. However, an absolute consensus has not been reached yet.

When it comes to the first question formulated above, the most investigated and best characterized signal-transduction pathway involved in lymphatic endothelial cell proliferation, migration, and survival is represented by VEGF-C and its cognate receptor VEGFR-3 (Alitalo, 2005; Thiele and Sleeman, 2006; Su et al., 2007; Da et al., 2008).

VEGFR-3, also known as FLT-4, is a tyrosine kinase receptor expressed during early embryonic development both by venous and lymphatic endothelium. Nevertheless, in the postnatal life, VEGFR-3 is predominantly expressed on the surface of lymphatic endothelial cells, as well as by monocytes, macrophages, and dendritic cells (Hamrah et al., 2003, Schoppmann et al., 2002). However, during pathological lymphangiogenesis, VEGFR-3 is expressed by the capillary endothelium of tumor tissues (Laakkonen et al., 2007).

Structurally, VEGFR-3 consists of an extracellular ligand-binding region composed of six immunoglobulin-like domains, a single transmembrane domain, and an intracellular conserved tyrosine kinase domain followed by a C-terminal tail (Pajusola et al., 1994; Ran et al., 2009; Nilsson et al., 2010). The tyrosine kinase receptor VEGFR-3 is present in two alternatively spliced isoforms, long and short, which differ in their C-terminal ends, and also have different signalling capabilities. The short splice variant is predominantly expressed in breast carcinoma and frequently correlates with lymphatic metastasis (Gunningham et al., 2000; Hughes et al., 2010). VEGFR-3 exists as an inactive monomeric protein in an unbound state. Upon binding of the proteolytically processed form of VEGF-C, VEGFR-3 can form homodimers or heterodimers (with VEGFR-2) leading to the activation of a special combinatorial signalling pathway (Olsson et al., 2006).

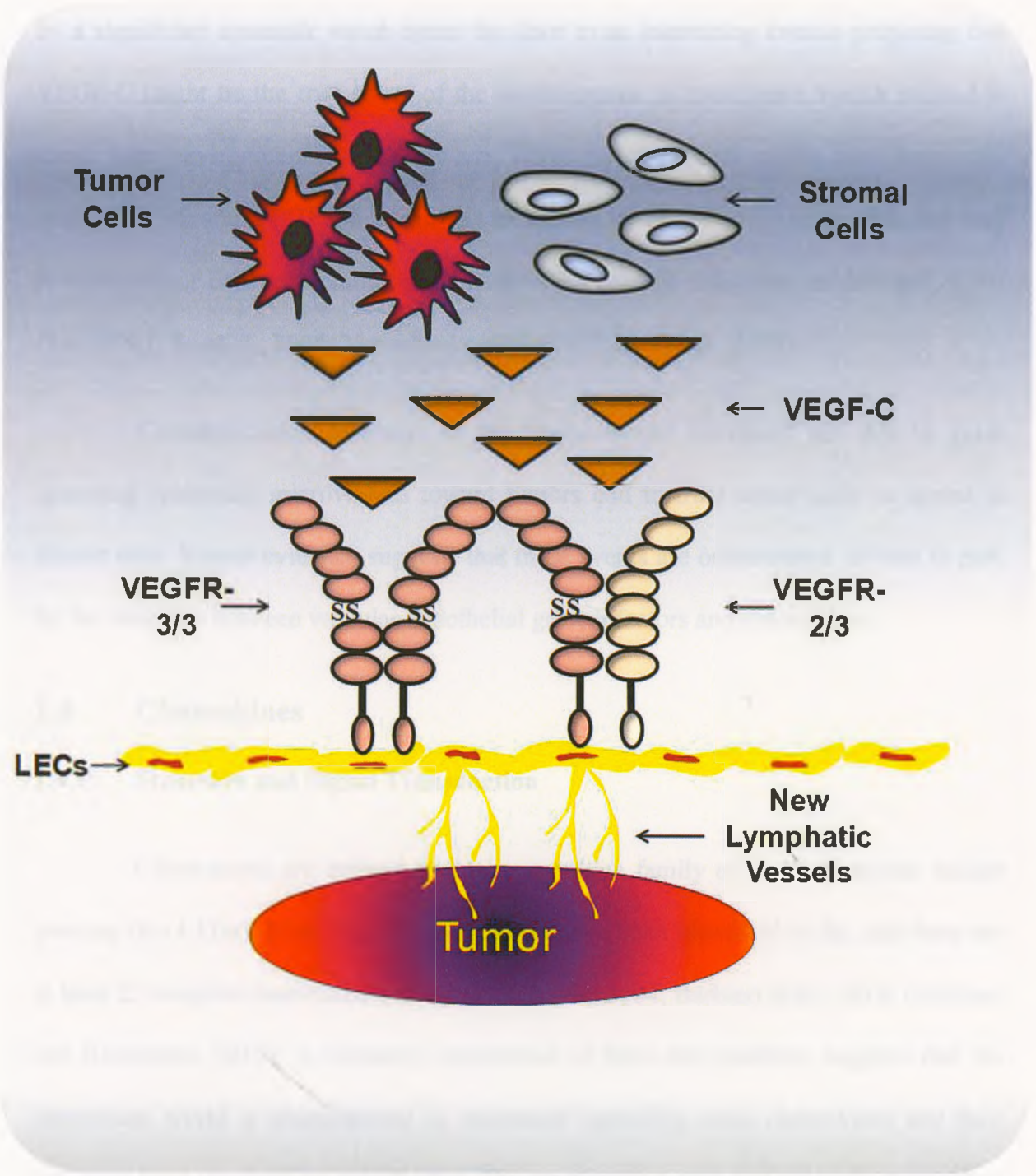
VEGF-C is a member of the VEGF family of growth factors, which are highly conserved secreted glycoproteins that are responsible for the regulation of vasculogenesis, hematopoiesis, angiogenesis, lymphangiogenesis, and vascular permeability and are implicated in many physiological and pathological processes

(Pepper, 2001). Observation of animal models has revealed that VEGF-C is an important regulator of lymphangiogenesis since VEGF-C-knockout mice fail to form primary lymphatic sprouts, lack all lymphatic vessels, and die before birth (He et al., 2004).

VEGF-C is secreted as a full-length inactive form consisting of NH_2- and COOH- terminal pro-peptides. After proteolytic cleavage, mature dimers bind and activate the VEGFR-3 receptor that – through tightly regulated pathways – controls the activation and sprouting of the lymphatic endothelial cells. VEGF-C is expressed in malignant, tumor infiltrating and stroma cells and creates an adequate tumor environment for generation of new lymphatic vessels (Figure 2). Also, VEGF-C qualitatively modulates the lymphatic vasculature to promote tumor metastasis. VEGF-C stimulates the formation of specialized intercellular gaps which in turn facilitates tumor entry into lymphatics (Tammela et al., 2007). VEGF-C also plays an active role in widening and enlarging the collecting lymphatic vessels that translates into: i) an increased lymph flow from the immediate tumor environment; ii) transportation of tumor cells; and iii) accommodation of larger tumor aggregates (He et al., 2005; Alitalo et al., 2005). The increased lymph drainage could activate the immunotolerant functions of lymph nodes through the upregulation of suppressor molecule expression as well as through an increased exposure of the lymph node to lymph-transported tumor antigens (Lund & Swartz, 2010).

Tumor derived VEGF-C can also attract macrophages and upregulate the expression of the CCL21 chemokine to further alter the tumor microenvironment and

Figure 2. Schematic of VEGF-C/VEGFR-3 Axis in the Regulation of Lymphangiogenesis. Tumor and stroma cells are major sources of VEGF-C. As a lymphangiogenic factor VEGF-C acts through activation of the tyrosine kinase receptor VEGFR-3 expressed by lymphatic endothelial cells. Upon activation, VEGFR-3 forms homodimers or heterodimers with VEGFR-2 and simulates formation of new lymphatic vessels.



Immunoglobulin Homology Domain

SS Cysteine Bridge

Tyrosine Kinase Domain

promote lymphatic invasion. Moreover, VEGF-C and CCL21 seem to be characterized by a significant crosstalk which opens the door to an interesting avenue proposing that VEGF-C might be the root cause of the immunogenic to tolerogenic switch noticed in tumor microenvironment (Lund & Swartz, 2010). Several recent studies have shown that both VEGF-C and VEGFR-3 are in fact expressed by tumor cells, suggesting that they promote tumor cell proliferation and invasiveness through autocrine mechanisms (Ueda et al., 2001; Su et al., 2006; Timoshenko et al., 2007; Issa et al., 2009).

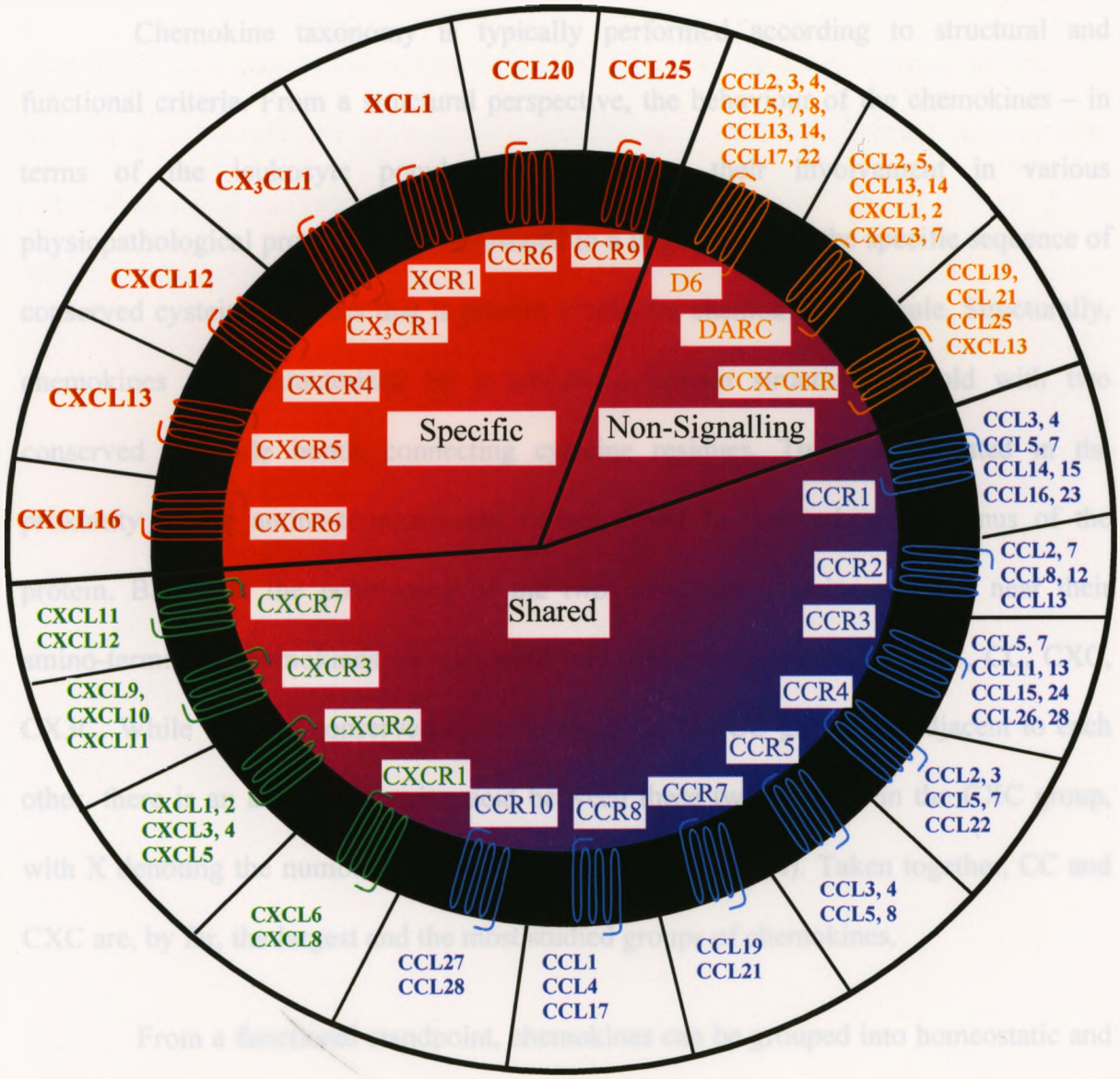
Communication pathways at the "tumor-vessel interface" are able to guide sprouting lymphatic microvessels toward tumors and instruct tumor cells to spread to distant sites. Recent evidence suggests that these events are orchestrated, at least in part, by the interplay between vascular endothelial growth factors and chemokines.

1.4 Chemokines

1.4.1 Structure and Signal Transduction

Chemokines are defined as a large cytokine family of small molecular weight proteins (8-14 kDa). More than 50 chemokines have been identified so far, and there are at least 22 receptors associated with them (Balkwill, 2004; Barbieri et al., 2010; Lazennec and Richmond, 2010). A summary comparison of these two numbers suggests that the chemokine world is characterized by redundant signalling since chemokines and their receptors have overlapping specificities. This practically means that multiple chemokines bind to more than one receptor and many of the chemokine receptors interact with more than one chemokine (Figure 3; after Lazennec and Richmond, 2010). Despite the lack of

Figure 3. Ligand-Binding Patterns of the Chemokine Families. Chemokines are characterized by redundant signalling. Most chemokines bind to more than one receptor and a single receptor can interact with multiple chemokines. This situation is typical for most of the CC (blue) and CXC (green) chemokine families. By contrast, some chemokine receptors (red) bind only one ligand. Decoy receptors (yellow) can bind multiple chemokines but act as "deceptors" since they do not signal.


CCL20
CCL25
CCL2, 3, 4,
CCL5, 7, 8,
CCL13, 14,
CCL17, 22
XCL1
CX3CL1
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CCL13, 14
CXCL1, 2
CXCL3, 7
CCR6
CCR9
XCR1
D6
CXCL12
CCL19,
CCL 21
CCL25
CXCL13
CX3CR1
DARC
CXCR4
CCX-CKR
CXCL13
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CXCR5
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CCL8, 12
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CCR3
CXCL11
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CXCR3
CCR4
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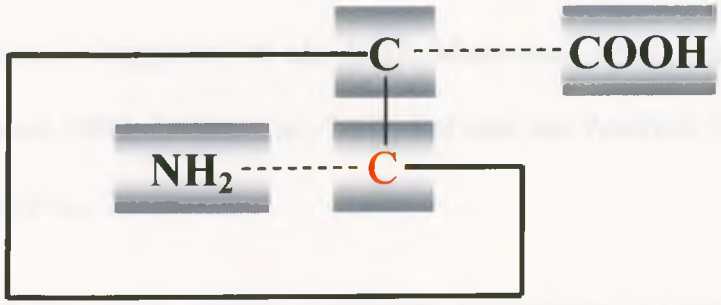
CXCL10
CXCL11
CXCR2
CCR5
CXCR1
CCR7
CCL2, 3
CCL5, 7
CCL22
CCR10
CCR8
CXCL1, 2
CXCL3, 4
CXCL5
CCL3, 4
CCL5, 8
CXCL6
CXCL8
CCL19
CCL21
CCL27
CCL28
CCL1
CCL4
CCL17

chemokine affinity for a unique receptor, each chemokine pair is characterized by distinctive functions (Devalaraja and Richmond, 1999; Murphy et al., 2000; Zlotnik et al., 2006; Hembruff and Cheng, 2009).

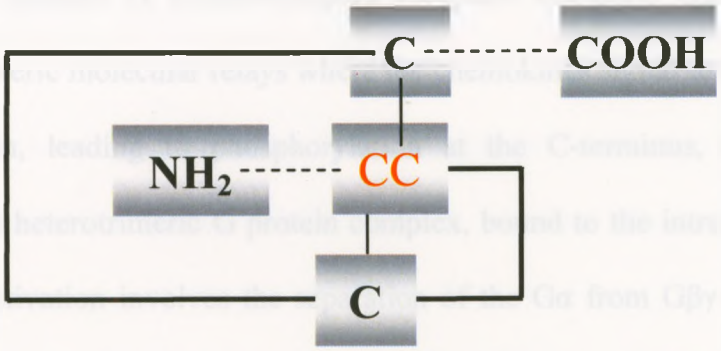
Chemokine taxonomy is typically performed according to structural and functional criteria. From a structural perspective, the behaviour of the chemokines – in terms of the leukocyte population targeted or their involvement in various physiopathological processes – is influenced to a large extent by the specific sequence of conserved cysteine residues that is present within the chemokine molecule. Structurally, chemokines are characterized by a conserved protein structure scaffold with two conserved disulfide bonds connecting cysteine residues. These are located in the proximity to the amino-terminus and further closer to the carboxyl-terminus of the protein. Based on the positioning of the two conserved cysteine residues near their amino-terminus, chemokines are classified into subfamilies/groups called C, CC, CXC, CX3C. While the two conserved cysteine motifs in the CC group are adjacent to each other, there is an additional amino acid between these two cysteins in the CXC group, with X denoting the number of amino acid residues (Figure 4). Taken together, CC and CXC are, by far, the largest and the most studied groups of chemokines.

From a functional standpoint, chemokines can be grouped into homeostatic and inflammatory types, depending on their origin and the tasks performed. Homeostatic chemokines are expressed in lymphoid organs with a role in homeostatic trafficking of leukocytes, while inflammatory chemokines are produced in response to inflammation

Figure 4. Schematic Representation of the Chemokine Classes. The structure of the chemokines encloses two conserved disulfide bonds connecting cysteine residues that are positioned in the vicinity of the amino-terminus and carboxyl-terminus. The position of the cysteins in the amino-terminus part of the chemokines defines each chemokine class. In the CC group, the first two cysteins are proximal to each other, while in the CXC and CX3C groups there are one and three amino acids between them, respectively.



C Group



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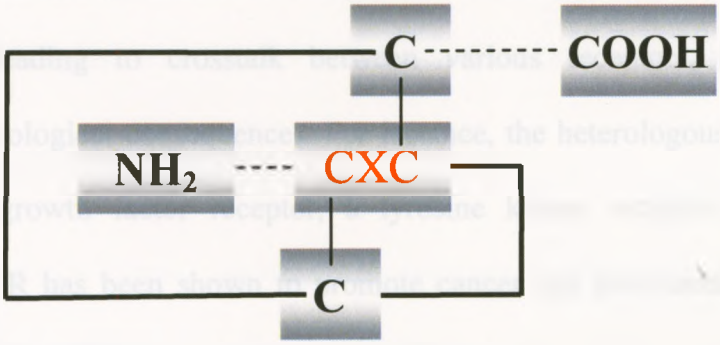
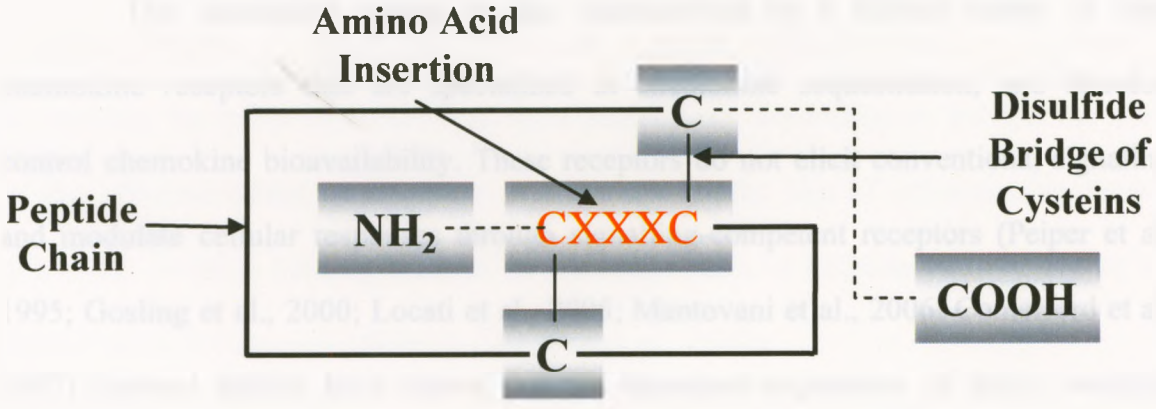


CXC Group



CX3C Group

and immune stimuli (Baggiolini et al., 1997; Mantovani 1999; Murphy et al., 2000; Zlotnik and Yoshie, 2000; Locati et al., 2002; Rot and von Andrian, 2004; Raman et al., 2011; Mantovani et al., 2010).

Chemokines elicit cellular responses by binding to their cognate seven transmembrane domain G protein-coupled receptors (GPCR). Chemokine receptors function as allosteric molecular relays where the chemokines signal to the extracellular N terminus domain, leading to phosphorylation at the C-terminus, and allowing the activation of the heterotrimeric G protein complex, bound to the intracellular domain of the receptor. Activation involves the separation of the Gα from Gβγ subunits and then subsequent activation of their downstream effectors (Neptune and Bourne, 1997; Raman et al., 2011). In addition, chemokine receptors can undergo constitutive homo or hetero dimerizations leading to crosstalk between various receptors, concomitant with significant physiological consequences. For instance, the heterologous transactivation of the epidermal growth factor receptor, a tyrosine kinase receptor, by an activated chemokine GPCR has been shown to promote cancer cell proliferation (Porcile et al., 2004; Porcile et al., 2005).

The chemokine system is also characterized by a distinct subset of silent chemokine receptors that are specialized in chemokine sequestration, and therefore control chemokine bioavailability. These receptors do not elicit conventional signalling and modulate cellular responses through signalling-competent receptors (Peiper et al., 1995; Gosling et al., 2000; Locati et al., 2005; Mantovani et al., 2006; Comerford et al., 2007). Several studies have shown that the decreased expression of decoy receptors correlates with lymph node metastasis and decreased survival rates in breast cancer. Their

expression controls the amount of intratumoral chemokines, and therefore modulates immune responses, vessel density, and tumorigenesis (Wu et al., 2008; Feng et al., 2009; Raman et al., 2011).

Chemokines trigger a variety of effector pathways after binding to their specific G protein coupled receptors. Signalling through phosphatidylinositol 3-kinase (PI3K) and its downstream mediator protein kinase B (AKT) promotes cancer cell survival, migration, and invasion (Barbero et al., 2003). Besides survival, chemokines activate the mitogen activated-protein kinase (MAPK) cascade, and Rho family of GTPases that are responsible for tumor growth (Fang and Hwang, 2009; Barbieri et al., 2010). Chemokine receptors also activate signalling pathways independent of G proteins, including p38MAPK (Goda et al., 2006) and JAK/Stat (Vila-Coro et al., 1999), to regulate cellular processes such as migration and gene transcription (Hembruff and Cheng, 2009).

1.4.2 The Role of Chemokine Signalling

Under physiological conditions, chemokines and their receptors are generally regarded as key mediators of cellular recruitment with strong consequences on both native and adaptive immune responses. Chemokines communicate with their target immune effectors through their G-protein coupled receptors and are capable of modulating the influx of certain leukocyte populations, depending on the specific needs of the afflicted tissue. Chemokines are secreted at the site of inflammation thereby becoming veritable homing beacons for the chemotaxis of immune cells guided by the chemokine concentration gradients (Dubinett et al., 2010).

While the chemotactic role of the chemokines in inflammation caused by injury or infection is probably the most acknowledged one, these molecules are in fact involved in many other physiological processes concerning lymphoid tissue ontogenesis, organogenesis, vasculogenesis and tissue repair (Garin and Proudfoot, 2011). The wide plethora of roles played by chemokines is generally regarded as a direct result of their significant regulatory effects, exerted on migration, proliferation and survival signals in multiple cell types (Hembruff and Cheng, 2009).

The physiological expression of chemokines is controlled by an extremely fine tuned system, whose delicate balance cannot be broken without significant implications on the well being of the entire human organism. On one hand, a subverted expression of the chemokines often intertwines with the pathobiology of chronic inflammation. On the other hand, the uncontrolled amplification of chemokine expression represents one of the primary causes of autoimmune diseases whose pathology is sometimes manifested in conjunction with tumor development. As such, taking advantage of the highly conserved chemokine expression between humans and mice, the physiological functionalities of the chemokines were thoroughly explored in both *in vivo* and *in vitro* studies (DeVries et al., 2006; Zlotnik et al., 2006).

1.4.3 The Role of Chemokines in Cancer

1.4.3.1 Expression Pattern in Cancer

Among many other functions, normal breast tissue is capable of expressing a broad panel of chemokines, typically belonging to the CXC and CC classes. However, their physiological levels, measured in human milk (Maheshwari et al., 2003) or primary

cultures of normal breast epithelial cells from healthy patients (Basolo et al., 1993) are extremely low.

Numerous studies have indicated that chemokine patterns change drastically after the onset of tumoral activity in mammary tissues. According to the most commonly accepted theory, the malignant switch in breast epithelial cells is responsible for a considerable up regulation in chemokine levels (Ali and Lazennec, 2007). By contrast, serial analysis of gene expression (SAGE) suggested that some of the chemokine-regulating genes that were highly expressed in normal breast epithelium were in fact lost during normal-to-carcinoma transition, while others were upregulated (Porter et al., 2001). Regardless of the case, these evident phenotypic changes could be exploited in the future for targeted therapy.

Extensive immunohistochemistry and RNA *in situ* analyses have revealed that an elevated expression of certain members of the chemokine family and their cognate receptors correlates reasonably well with poor prognosis, as well as with lymph node metastases in cancer patients (Hembruff and Cheng, 2009). Indeed, the epithelial-specific expression of chemokines and their receptors has been established at protein and RNA levels in breast cancer, and in many other types of malignancies (Wente et al., 2008). Interestingly, the upregulation of the chemokines in breast cancer is generally associated with the downregulation of the decoy receptors (Wu et al., 2008), which may imply that tumor cells are characterized by intense chemokine signalling activities.

When it comes to the identification of the specific chemokine molecules that are upregulated in breast carcinomas, without exhibiting an absolute consensus, the surveyed

literature seems to imply that CXCL8, CXCL12, CCL2, CCL4 and CCL5 are some of the most studied members of the chemokine class (Ali and Lazennec, 2007). Sometimes the studies performed on a particular member of the chemokine family were extensive to justify a certain "role model" status, often attributed to the CXCL12/CXCR4 chemokine pair due to its involvement in both sentinel lymph node and remote metastasis associated with mammary tumors (Ben-Baruch, 2008). It is extremely important to point out that the expression of a certain chemokine receptor does not automatically translate into its functionality with respect to its ligand. CXCR4 is a classic example in this sense, since it has a relatively uniform expression in several breast cancer cell lines as measured through Western Blot, flow cytometry or ligand binding, but its signalling capabilities have only been detected in metastatic cell lines (Holland et al., 2006). The elevated levels of these chemokines was associated with elevated levels of tumor-associated macrophages (Ueno et al., 2000) and it was later found that not only tumor cells, but also tumor stroma could be a source for chemokine production (Finak et al., 2008).

However, when it comes to the particularities of breast tumors, newer research studies suggest a broadened palette of chemokine involvement. The enlarged spectrum now includes less investigated members of the CC chemokine class, among which the CCL21/CCR7 pair is presently believed to play a role that is at least of equal significance with CXCL12/CXCR4. When compared to normal breast tissue, CCR7 was found to be upregulated in primary tumors, in particular to human invasive lobular and ductal carcinomas (Cabioglu et al., 2005b; Andre et al., 2006). CCL21, the ligand binding to CCR7, is physiologically present in lymph nodes, but its expression was found to be considerably increased in lymph node-positive versus lymph-node negative breast cancer

patients (Wilson et al., 2006). Taken together, these findings provide a foundation for the lymph node selectivity of mammary tumor cells and reinforce once more that the normal to tumoral switch in breast tissue is associated with a dramatic change in chemokine pattern expression.

1.4.3.2 Regulation of Chemokine Expression

Since there is no doubt that tumor development is linked to changes in chemokine expression, intensive efforts are currently directed towards identifying the inherent mechanisms responsible for these alterations. When it comes to chemokine expression in epithelial and mesenchymal cells, specific environmental and soluble factors are often cited as major players involved in their upregulation. Among them, hypoxia-related and/or numerous growth factors (HGF, EGF, TNF-α, IL-6, LMP1) were shown to contribute decisively to the regulation of chemokines and their cognate receptors in both epithelial and endothelial cells (Matteucci et al., 2007; Maroni, et al., 2007; Buettner et al., 2007; Kulbe et al., 2005). Notable results along this direction were obtained, for example, through the correlations established in context of breast tumors between IL-1β/TNFα and CCL2 (Freund et al., 2004), EGF and CXCL8 (Azenshtein et al., 2005). The most acceptable explanation proposed for this correlative expression resides in the commonality of the signalling pathways involved both in the upregulation of chemokines and that of the aforementioned proteins (Shim et al., 2006).

Other important connections have been made between the expression of tyrosine kinase receptors with well-defined roles in mammary malignancy and chemokines. The functional links already established between CXCR4 and HER2 (Li et

al., 2004; Cabioglu et al., 2005a), and IGF-1R (Akekawatchai et al., 2005), might mean that at least some of the tumor promoting factors are also active players in the regulation of chemokine expression.

Finally, in addition to the mechanisms outlined above, the most recent evidences seem to suggest that genetic mutations are also accountable for chemokine expression abnormalities (Hembruff and Cheng, 2009). Chemokines constitute direct targets of several oncogenes involved in the pathogenesis of a broad range of human tumors. The genetic lesions caused by these oncogenes are responsible for the upregulation of the chemokines that in turn induce the activation of an entire inflammatory cascade (Allavena et al., 2011). To illustrate this concept, for instance, CCR7 has been proved to be upregulated by the p53 mutation in T cell-acute lymphocytic leukemia as a result of the activation of the Notch1 signalling pathway (Buonamici et al., 2009). However, the surveyed literature presents numerous other examples connecting chemokines and their receptors with various other oncogenes like mutant p53, Ras, and Myc as well as with the deregulation of the transcriptional factors involved in chemokine transactivation (Mantovani et al., 2010).

1.4.3.3 Chemokine Signalling in Cancer Progression

Chemokines are one of the key players involved in tumor progression since many of the multiple pathways involved in tumorigenesis, including primary tumor growth, leukocyte recruitment, angiogenesis, survival, and metastasis rely on chemokines to fulfil their functions. However, prior studies seem to suggest that they might be responsible for antithetical roles, since in some situations the presence of chemokines is

vital for tumor progression, while in other cases chemokines become veritable drivers of anti-tumor defence. The actual role played by certain chemokines in malignancy generally depends on their type and on microenvironmental characteristics. Evidently, the balance between pro- and anti-tumor chemokines has a major impact on tumor development (Balkwill and Mantovani, 2001; Huang et al., 2002; Raman et al., 2007; Garin and Proudfoot, 2010).

The presence of certain chemokines in the tumor microenvironment enables tumor cells to acquire the essential characteristics required for malignant growth: self-renewal, activation of anti-apoptotic pathways, unlimited replicative potential, sustained angiogenesis, and acquisition of an invasive phenotype (Hanahan et al., 2000).

With respect to the angiogenic effect of chemokines, it has been established that several members of this family can mediate angiogenesis directly through the activation of matrix metalloproteases, as well as the stimulation of endothelial cells migration and proliferation (Giraudo et al., 2004; Ma et al., 2007). In addition to the aforementioned mechanism, some chemokines can indirectly mediate angiogenesis through the recruitment of leukocytes and subsequent production of vascular endothelial growth factors (Mantovani et al., 2006; Lewis et al., 2006; Sozzani et al., 2007). Furthermore, chemokines were found to be active mediators in the recruiting of vascular smooth muscle cells toward endothelial cells. They are also capable of regulating the angiogenic switch that in turn will provide the tumor cells with essential nutrients and oxygen (Ben-Baruch, 2006; Strieter et al., 2006; Mehrad et al., 2007; Keeley et al., 2008; Waugh and Wilson, 2008; Mishra et al., 2010).

Analogous to their physiological role in the regulation of cell trafficking, one of the first identified cancer-related effects of chemokines was related to their participation in tumor cell migration and organ selective metastasis (Ben-Baruch, 2008). A plethora of studies has indicated that the selective, non-random distribution of tumor cells to remote tissues in a chemokine-dependent manner relies on the perfect match between chemokines and their cognate receptors according to "seed and soil" theory developed by Paget (1889) and reviewed by Ribatti et al. (2006). Due to their chemokine expressing capabilities, various body organs become veritable metastatic targets since they attract the chemokine receptors expressed by tumor cells. Also, it was found that tumor cells tend to increase their adhesiveness and invasiveness under the action of chemokines released by remote organs, partly due to the activation of focal adhesion kinase and the actin polymerization signalling pathway (Lee et al., 2004; Prasad et al., 2004; Fernandis et al., 2004; Ben-Baruch, 2008).

1.4.4 CCL21/CCR7 Axis

1.4.4.1 Expression Pattern of the CCL21/CCR7 Pair

Like other chemokines, CCL21 and its cognate receptor CCR7 are physiologically involved in the control of leukocyte trafficking to secondary lymphoid organs (peripheral lymph nodes). However, in addition to the well acknowledged roles of CCL21/CCR7 pair in chemotactic guidance and in the adhesion of circulating leukocytes to the vascular endothelium of high endothelial venules, more recent studies also emphasize their involvement in cytoarchitecture and the maturation of leukocytes (Sanchez-Sanchez et al., 2006).

The two major leukocyte subsets whose surfaces express CCR7 are dendritic and T cells, but there is an important distinction between them: while naive T cells express CCR7 constitutively in order to allow their continuous recirculation through secondary lymph nodes (Forster et al., 1999), the surface of dendritic cells begins to express CCR7 only after tissue injury or cytokine-induced activation (Saeki et al., 1999). This behaviour suggests that CCR7 has a strong implication in both central and peripheral tolerance (Worbs and Forster, 2007). On the other hand, CCL21 is constitutively expressed by the lymphatic endothelium of multiple organs, high endothelial venules of lymph nodes and Peyer's patches, as well as stromal cells in T cell rich areas of lymph nodes, spleen and Peyer's patches (Fang and Hwang, 2009). The wide physiological distribution, combined with the complex and multifaceted roles in lymph node trafficking is probably what makes the CCL21 chemokine and its cognate receptor an extremely suitable candidate for the fast dissemination of the breast cancer cells developed in immediate proximity of the lymphatics. Following the discovery by Muller et al. (2001) that a number of the chemokine receptors – including CCR7 – are upregulated in a distinct and non-random pattern in breast cancer, and that CCL21 is abundantly expressed by lymph nodes, the involvement of CCL21/CCR7 chemokine pair in mammary malignancies became more than a simple speculative theory. Subsequent research studies have demonstrated that the overexpression of CCR7 correlates well with enhanced lymph node metastasis in animal models (Pan et al., 2009; Cunningham et al., 2010). CCR7 upregulation in human cancer correlates with tumor size, increased incidence of lymph node metastasis, and poor survival rates (Andre et al., 2009; Liu et al., 2010). Interestingly, the CCR7-expressing breast cancer cells are capable of secreting

CCL21 in an autocrine manner, especially in 3D environments (Shields et al., 2007), demonstrating the importance of the microenvironment on chemokine signalling.

1.4.4.2 Mechanisms that Promote CCR7 Upregulation

Despite the unambiguous correlation between CCR7 upregulation and the poor outcome of breast carcinomas, the mechanisms underlying CCR7 expression by tumor cells continue to remain relatively obscure (Forster et al., 2008). One of the possible mechanisms suggested for an increase in CCR7 expression by tumor cells is epigenetic changes, such as deacetylation and DNA methylation (Mori et al., 2005). Another potential factor responsible for CCR7 upregulation in breast cancer cells is cyclooxygenase-2 (COX-2) mediated prostaglandin E2 (PGE2) production. COX-2 was shown to upregulate CCR7 expression via E-prostanoid receptors (EP2, EP4) in breast cancer cells (Pan et al., 2008).

Other studies have revealed that CCR7 upregulation at both the mRNA and protein levels could also be caused by the overexpression of endothelin receptors (Wilson et al., 2006). Since the expression of the endothelins and their receptors in breast cancer cells is induced by hypoxic conditions and inflammatory cytokines (Grimshaw, 2005), it can be inferred that local factors present within the tumor microenvironment might play a determinant role on CCR7 upregulation.

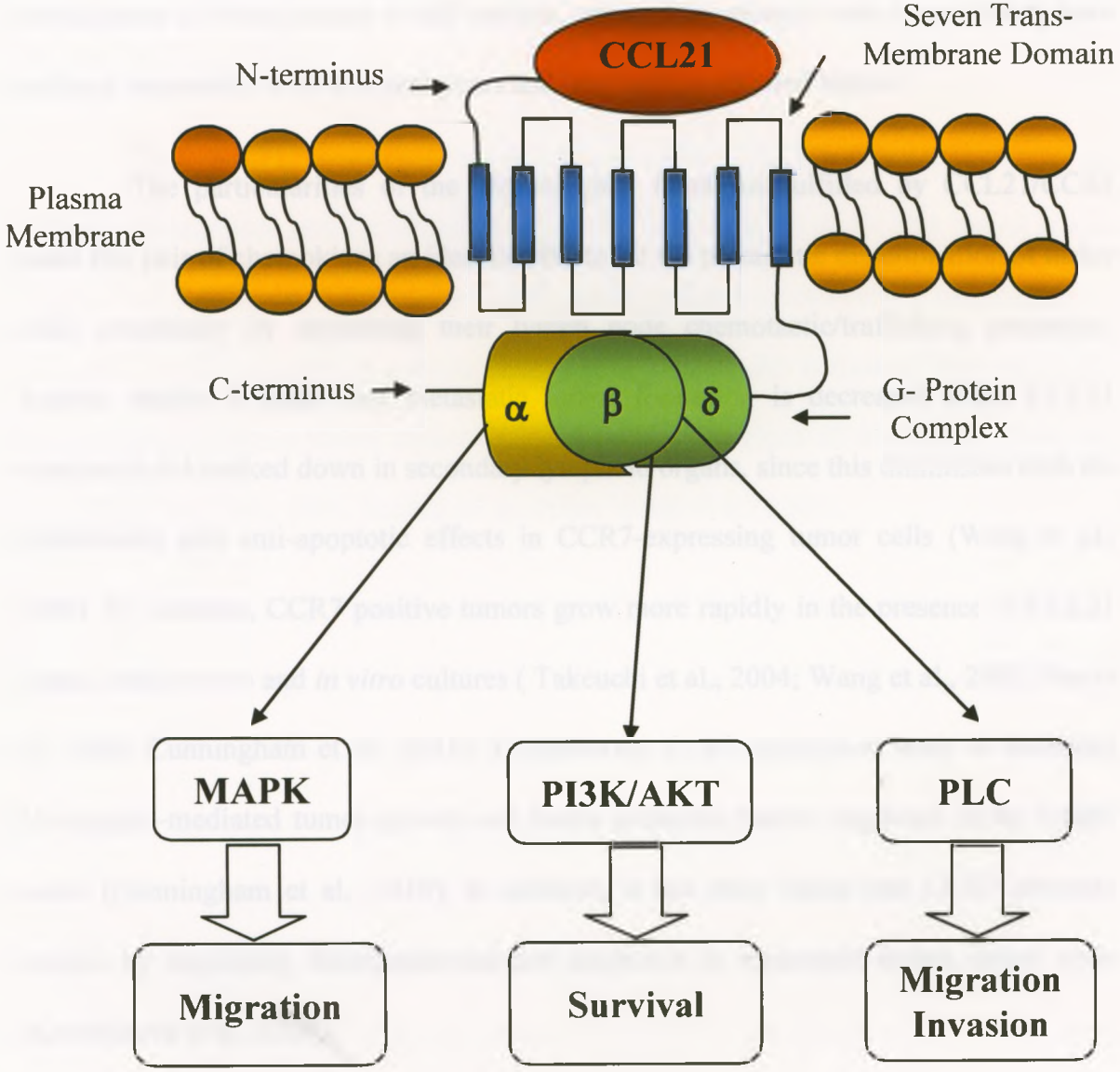
1.4.4.3 CCR7 and G Proteins Signalling

Similar to all other chemokine receptors, CCR7 belongs to the family of seven transmembrane domain heterotrimeric G protein-coupled receptors (GPCR). A cascade of

downstream intracellular events is triggered by CCR7 activation by its CCL21 ligand (Rubin et al., 2009). The immediate consequence of the binding between CCR7 and its CCL21 ligand is constituted by the phosphorylation and activation of the downstream G protein complex, adjacent to this receptor (Arai and Charo, 1996; Kuang et al., 1996). Following activation, G proteins associate with their effectors and further propagate the intracellular signals.

However, it is important to note that G protein activation is accompanied by a dissociative process that separates the Gα and Gβγ subunits. The two distinct subunits yielded after separation play distinct roles on the intracellular signalling scene. On one hand, the Gβγ subunit interacts with p101, a protein associated with the p110 catalytic subunit of PI3K and thereby induces signalling through PI3K (Stephens et al., 1994) and its downstream mediator AKT. Given the well established role of the PI3K/AKT pathway on protection from cellular apoptosis (Datta et al., 1999), it is easy to assert that activation of PI3K following CCL21/CCR7 binding is one of the multiple mechanisms invoked by breast cancer cells in order to survive the attack of the immune system (Fang and Hwang, 2009). An analogous premise has already been validated for CCR7 signalling in the context of dendritic and effector CD8+ T cells (Sanchez-Sanchez et al., 2004; Kim et al., 2005). Although investigated considerably less so far, the Gα subunit released after G protein activation following CCL21/CCR7 binding activates the mitogen-activated protein kinase (MAPK) signalling cascade, with established impact on tumor cell proliferation, invasiveness, and migration (Redondo-Munoz et al., 2008) (Figure 5).

Figure 5. Schematic of the CCR7 Signalling Pathways. Upon CCR7 chemokine receptor activation by CCL21 stimulation, the G-protein complex dissociates and triggers different signal cascades. Gβγ subunits activate phosphatidylinositol 3 kinase (PI3K) and phospholipase C (PLC) whereas the Gα subunit activates mitogen-activated protein kinase (MAPK)/extracellular regulated kinase (ERK) pathway. Protein kinase B (AKT) is activated downstream of PI3K and is mainly responsible for cell survival.


CCL21
N-terminus
Seven Trans-
Membrane Domain
Plasma
Membrane
C-terminus
α
β
δ
G- Protein
Complex
MAPK
PI3K/AKT
PLC
Migration
Survival
Migration
Invasion

1.4.4.4 The Role of CCL21/CCR7 Axis in Breast Cancer

The CCL21/CCR7 pair has several different grades of involvement in the growth and dissemination of human tumors. While the complete picture of CCL21/CCR7 participation in breast cancer is still unclear, some of its primary roles have already been outlined reasonably well in recent years and they will be detailed below.

The particularities of the physiological functions fulfilled by CCL21/CCR7 make this pair of chemokines an ideal candidate for the metastatic dissemination of tumor cells, essentially by exploiting their lymph node chemotactic/trafficking properties. Animal studies indicate that metastatic tumor formation is decreased when CCL21 expression is knocked down in secondary lymphoid organs, since this diminishes both the chemotactic and anti-apoptotic effects in CCR7-expressing tumor cells (Wang et al., 2008). By contrast, CCR7 positive tumors grow more rapidly in the presence of CCL21 ligand, both *in vivo* and *in vitro* cultures (Takeuchi et al., 2004; Wang et al., 2005; Sun et al., 2009; Cunningham et al., 2010). Furthermore, CCR7 expression leads to increased β1-integrin–mediated tumor growth and hence promotes tumor migration to the lymph nodes (Cunningham et al., 2010). In addition, it has been found that CCR7 prevents anoikis by regulating detachment-induced apoptosis in metastatic breast cancer cells (Kochetkova et al., 2009).

Many members of the CC chemokine family were found to play active roles in tumor dissemination to specific tissues throughout the body, such as the skin, gut and liver (Lazennec and Richmond, 2010). When it comes to the specifics of CCL21 involvement in chemotactic signalling, researchers have shown that the CCL21/CCR7

pair plays a prominent role in the metastatic dissemination of breast tumor cells to lymph nodes (Muller et al., 2001; Cabioglu et al., 2005b; Cabioglu et al., 2007; Cunningham et al., 2010). Moreover, a recent study revealed that in addition to lymph node metastasis, this pair also mediates brain infiltration by T-cell acute lymphoblastic leukemia (Buonamici et al., 2009).

Furthermore, analogous to the physiological contribution of this pair to leukocyte maturation and cytoarchitecture, CCR7 activation by its CCL21 ligand is also responsible for rapid intracellular actin polymerization/cytoskeleton reorganization, followed by the formation of pseudopodia, which dramatically enhances the motility and invasiveness of human breast cancer cells as well as other types of tumor cells (Muller et al., 2001; Ding et al., 2003; Sancho et al., 2006; Wang et al., 2005).

Among various roles played by the CCL21/CCR7 pair in breast cancer, perhaps the one related to migration and guidance of the cells detached from the primary tumor towards draining lymphatics has a crucial role on the subsequent metastatic evolution of the disease. Questions remain as to whether there is any link between the CCL21/CCR7 axis with the lymphangiogenic factors VEGF-C and VEGF-D. Evidently, the autocrine secretion of VEGF-C and D by tumor cells facilitates tumor cell dissemination, since these growth factors favour intra/peritumoral lymphatic development. As such, since primary tumors are capable of creating their future propagation pathways, these events may explain why the overexpression of VEGF-C and D in the experimental tumor models leads to enhanced metastasis to lymph nodes (Skobe et al., 2001; Mandriota et al., 2001; Stacker et al., 2001; He et al., 2005).

However, while all these observations constitute rather indirect proofs of the crosstalk between VEGF-C and CCL21, new evidence has demonstrated that the interplay between these two molecules influences breast cancer progression by multiple distinct, but complementary mechanisms (Shields et al., 2007; Issa et al., 2009):

i) The autocrine secretion of VEGF-C by tumor cells increases tumor invasiveness by increasing the proteolytic activity and motility of tumor cells.

ii) VEGF-C also acts in a paracrine manner to increase the lymphatic endothelial secretion of CCL21.

iii) The autocrine secretion of CCL21 by tumor cells guides the transcellular migration of CCR7-expressing tumor cells under the slow interstitial flow.

iv) Once the tumor cells have departed from the matrix, the paracrine secretion of CCL21 takes over and guides the CCR7-expressing tumor cells towards draining lymphatics.

Taken together, these observations – essentially built around the paracrine and autocrine mechanisms associated with VEGF-C and CCL21 – point out that the synergy between these two axes is a major factor in the metastatic propagation of breast tumors.

One of the newest established roles of the CCL21/CCR7 pair is related to its lymphangiogenic potential. In the tested mouse models, CCR7 expression correlated well with lymphatic vessel density and lymph node metastasis, therefore having a major impact on proliferation and invasiveness of colon cancer (Yu et al., 2008). Another study has concluded that the interaction between CCL21 chemokine and its cognate receptor

constitute a critical event in progression of pancreatic cancer due to the induction of the lymphangiogenic process (Zhao et al., 2011).

Perhaps the most intriguing role of CCL21/CCR7 in tumor dissemination is related to its modulatory effects on the tumor microenvironment. According to a recent study, Shields et al. (2010) showed that CCL21-secreting tumor cells are responsible for a tolerogenic switch in the host's immune response. Consistent with prior findings which emphasize that the lymph node stroma plays a major role in promoting tolerance to self antigens, it was found that CCL21-expressing tumors are capable of evading immune surveillance by developing stromal zones reminiscent of lymph node paracortex stroma. The intrinsic mechanism of the tolerogenic switch in the microenvironment induced by CCL21-expressing tumors relies on regulatory shifts in T cell populations. As an overall result, the CCL21 overexpressing tumors grew significantly larger when implanted in immunosuppressed mice, reinforcing the effectiveness of CCL21/CCR7 signalling on the promotion of immune tolerance to tumors.

1.5 Rationale

The chemokine ligand/receptor CCL21/CCR7 pair plays a significant role in tumorigenesis and in the migration of tumor cells into the sentinel lymph nodes in mammary malignancies. In fact, CCR7 receptor has emerged as an important marker in the prediction of axillary lymph node metastasis in breast cancers, since CCR7 expression correlates with larger primary tumors, and deeper lymphatic invasion.

In human breast cancer cell lines, the epithelial expression of CCR7 chemokine receptor has been validated both at the mRNA and protein levels (Muller et al., 2001).

Several studies have shown that a number of different CCR7 positive tumor cell lines can create themselves a gradient of CCL21 chemokine, which increases under the slow interstitial fluid flow towards draining lymphatics (Shields et al., 2007; Shields et al., 2010). Additionally, CCL21 secretion by endothelial cells has been reported to be upregulated by the lymphangiogenic factor, VEGF-C (Issa et al., 2009).

The crosstalk between vascular growth factors and chemokines is now believed to play an important role in tumorigenesis (Barbieri et al., 2010) and in this regard, the existence of a novel link between CCL21 chemokine and VEGF-C has been recently uncovered. Moreover, it seems that CCL21 and VEGF-C act synergistically in the process of invasion (Issa et al., 2009; Cohen-Kaplan et al., 2008; Yu et al., 2007).

Another link was established between COX-2/VEGF-C and CCR7 chemokine receptor. Studies from our laboratory reported that COX-2 expression by breast cancer cells plays a key role in VEGF-C secretion via the EP1/EP4 receptor by endogenous PGE2 (Timoshenko et al., 2006). Subsequently, Pan et al. (2008) established that COX-2 increased CCR7 expression via the EP2/EP4 receptor, suggesting that CCR7 is a downstream target for COX-2 to enhance migration of breast cancer cells and to promote lymphatic invasion. However, they did not verify whether CCR7 plays any role in VEGF-C production by breast cancer cells.

New reports have also suggested the involvement of CCL21/CCR7 pair in lymphangiogenesis (Yu et al., 2008; Zhao et al., 2011). Since the latest data published on this topic concluded that the activation of CCR7 signalling constitutes a critical event in the progression of pancreatic cancer, due to the induction of the lymphangiogenic

process, it is very likely that a similar mechanism is also present in mammary carcinomas. Moreover, due to its ability to induce migration and proliferation, the CCL21/CCR7 chemokine axis is an ideal candidate for the lymphangiogenic spread.

1.6 Hypothesis and Objectives

Based on the foregoing findings, the present study proposes that *activation of CCR7 signalling is responsible for upregulation of VEGF-C secretion associated with breast cancer-induced lymphangiogenesis* (Figure 6).

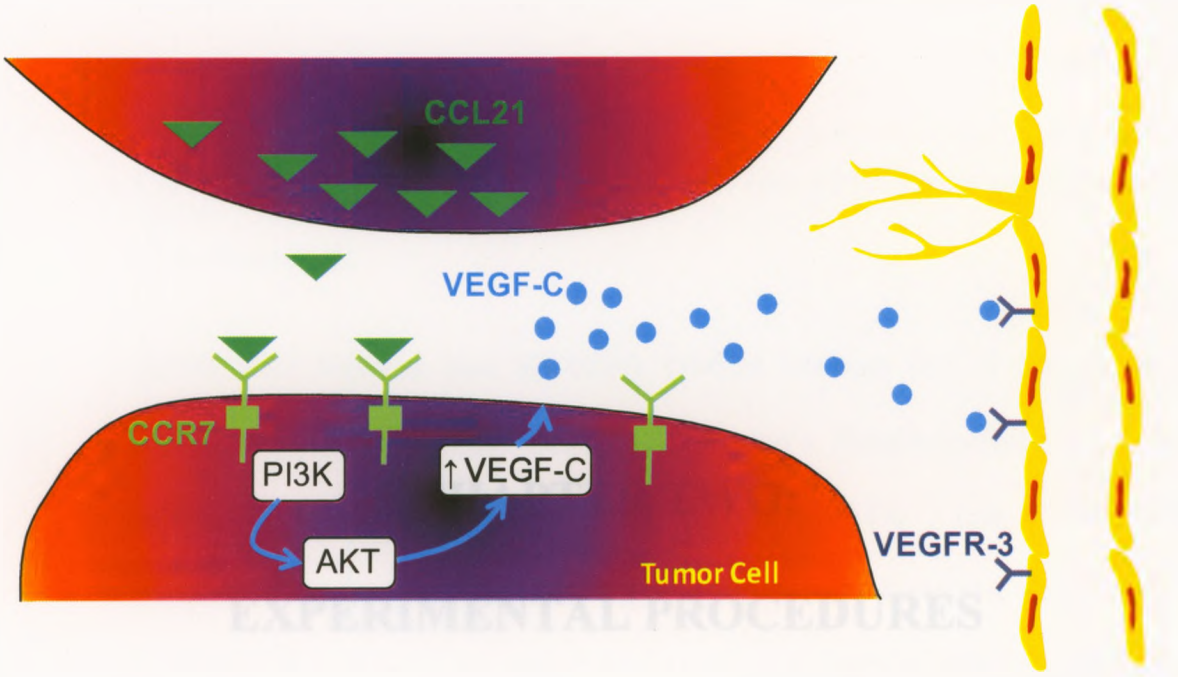
The proposed hypothesis will be tested through the following objectives:

1. To determine whether CCR7 expression plays a regulatory role in VEGF-C secretion by breast cancer cells.

2. To identify the underlying molecular mechanism of CCL21/CCR7-induced VEGF-C secretion by breast cancer cells.

3. To test the lymphangiogenic potential of CCL21/CCR7 chemokine pair *in vitro*.

Figure 6. Hypothesis. Activation of CCR7 signalling is responsible for up regulation of VEGF-C secretion associated with breast cancer-induced lymphangiogenesis.


CCL21
VEGF-C
CCR7
PI3K
↑VEGF-C
AKT
Tumor Cell
VEGFR-3
Lymphatic Vessel

CHAPTER TWO:

EXPERIMENTAL PROCEDURES

2.1 Materials

- BD Falcon cell culture flasks (75 cm^2), 6-well plates, 96-well plates, Transwell inserts (24-well plate, 6.5 mm diameter, 8 μm pore size), BD Cell Recovery Solution (cat. #354253), and Growth Factor Reduced (GFR) Matrigel (cat. #356231) were from BD Biosciences, CA.

- Corning Ultra-Low Attachment 6-well plates (cat. #3473) were purchased from Cole-Parmer, IL.

- RPMI 1640 Medium (cat. #224000), DMEM Medium (cat. # 12634), Fetal Bovine Serum (FBS) (cat. #12483), Dulbecco's Phosphate-Buffered Saline (DPBS) (cat. #14190), 0.25% Trypsin-EDTA (cat. #325200), Penicillin/Streptomycin (cat. #15140), Platinum® PCR SuperMix High Fidelity (cat. #12532-016) were purchased from Invitrogen, GIBCO, ON.

- M-PER® Mammalian Protein Extraction Reagent (cat. #78501), HALT™ Protease Inhibitor Cocktail (cat. #PI78410), BCA™ Protein Assays kit (cat. # 23225), DAPI (4', 6-diamidino-2-phenylindole, dihydrochloride) (cat. # 46190) were from Thermo Scientific, IL.

- Phosphatase Inhibitor Cocktail 2 (cat. #P5726), Phosphatase Inhibitor Cocktail 3 (cat. #P0044), and Albumin from Bovine Serum (BSA) (cat. #030M1610) were from Sigma-Aldrich®, MO.

- Recombinant human CCL21/6 Ckine (cat. #366-6C), mouse monoclonal anti-human CCR7 antibody (cat. #MAB197), goat anti-human 6 Ckine antibody (cat.

#AF366), mouse IgG1 isotope control-PE (cat. #IC002P), anti-human CCR7 phycoerythin conjugated mouse IgG (cat. #FAB197P), Quantikine® Human CCL21/6Ckine Immunoassay (cat. #D6C00), and Quantikine® Human VEGF-C Immunoassay (cat. #DVEC00) were acquired from R&D Systems, MN.

- Phospho-AKT (Ser 473) rabbit monoclonal antibody (cat. #4060), AKT (pan) mouse monoclonal antibody (cat. #2920), LY294002 (PI3 Kinase Inhibitor) (cat. #9901) were from Cell Signalling Technology®, MA.

- 6 Ckine (FL-134) rabbit polyclonal antibody (cat. #SC-25445), VEGF-C (C-20) goat polyclonal antibody (cat. #SC-1881) were from Santa Cruz Biotechnology, CA.

- CKR7/CCR7 (N-term) rabbit monoclonal antibody (cat. #2059-1) was purchased from Epitomics®, CA.

- Odyssey blocking buffer (cat. #Q0391), goat anti-rabbit (cat. #3926-68021), donkey anti-goat (cat. #926-68024), and donkey anti-mouse (cat. #926-68020) IRDye polyclonal secondary antibodies were from LI-COR, NE.

- Immobilon-FL PVDF membrane (cat. #IPFL10100), goat anti-mouse IgG, FITC conjugate antibody (cat. #AP181F) and donkey anti-goat IgG, FITC conjugate antibody (cat. #AP180F) were from Millipore, MA.

- RNeasy MiniKit (cat. #74104) and RNase-Free DNase set (cat. #79254) were purchased from QIAGEN, MD.

- Amaxa Cell Line Nucleofector Kit V (cat. #VCA-1003), Microvascular Endothelial Cell Growth Medium EGM®-2-MV Bulletkit® (CC-3202) were obtained from Lonza, MO.

- TaqMan® Universal PCR Master Mix (cat. #4304437), TaqMan® Pre-Developed Assay Reagent, Hu GAPDH (cat. #1103172), TaqMan® Gene Expression Assay, CCR7 probe (ID: 96261), TaqMan® Gene Expression Assay, VEGF-C probe (ID: 992071), CCR7 Silencer® Select Pre-design siRNA (ID: S3217), Silencer® Select Negative Control siRNA (cat. #4392420), High Capacity cDNA Reverse Transcription Kit (cat. # 0810065) were purchased from AB Applied Biosystems, CA.

- GelRed™ Nucleic Acid Gel Stain (cat. #41002) was from Biotium, CA.

- Cell Proliferation ELISA, BrdU (colorimetric) (cat. # 11647229001) was purchased from Roche, IN.

2.2 Research Methodology

2.2.1 Cell Lines and Culture

Adult Human Dermal Lymphatic Microvascular Endothelial Cells (HMVEC-dLyAd) (cat. # CC-2810T25) were obtained from Clonetics®/ Lonza (Walkersville, MO) and maintained in an endothelial growth medium containing growth supplements provided by the supplier (EGM®-2-MV Bulletkit®, cat. # CC-3202, Lonza). The initial expansion and subsequent passages (maximum of 5) were done according to the manufacturer's instructions.

Human mammary MDA-MB-231cells (passage # 3) from American Type Culture Collection (ATCC) (Rockville, MD) were grown as a monolayer in RPMI 1640 medium (Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator at 37°C with 5% CO_2.

2.2.2 Flow Cytometry Analysis

2.2.2.1 CCR7 Expression

To characterize the cell surface phenotype of CCR7 chemokine receptor, MDA-MB-231 and HMVEC-dLy cells were grown up to 80-85% confluence in T75 flasks, and then gently treated with a Trypsin-EDTA solution to facilitate the removal from the substrate. Further, cells were incubated in complete medium for four hours on an ultra low attachment cluster plate to enable the regeneration of the receptors. For staining purposes, cells were then washed and resuspended in DPBS, supplemented with 0.5% BSA and 0.01% NaN_3 (sodium azide). The monoclonal anti-human CCR7 antibodies were diluted in this buffer and used at a final concentration of 10 μl/ml for 45 minutes, at 4°C. After incubation, cells were washed with DPBS three times by centrifugation at 500 g for 5 minutes and labelled with R-Phycoerythrin conjugated IgG antibody for 30 minutes, at 4°C, in the dark. Following the final washing steps, 10^4 labelled cells were analyzed on a FACS Calibur Cytometer (BD Biosciences) at the London Regional Flow Cytometry Facility (Robarts Research Institute, London, On). FlowJo software version 7.6.5 (Treestar, Ashland, OR) was used for data analysis.

2.2.3 Western Blot Analysis

2.2.3.1 CCR7 Protein Expression

MDA-MB-231 and HMVEC-dLy cells were plated at a concentration of $2x10^5$/well in six well plates and grown to near confluence. The day before protein extraction, cells were placed in serum free media and incubated overnight. For the analysis of the total cell lysates, cells were washed with ice-cold DPBS and treated with M-Per lysies buffer supplemented with HALT protease inhibitor cocktail. After five minutes of shaking on ice, cells were scraped from the wells and transferred to 1.5 ml Eppendorf tubes. Lysates were sonicated (eight pulses, at level four) and then centrifugated at 13,000 RCF for 20 minutes at 4°C, to remove cell debris. The supernatants were collected and protein concentration was quantified in triplicate, using the BCA protein assay kit following the manufacturer's protocol. Fifteen micrograms of total protein from the cell lysate was run per well on a 1.5 mm 10% SDS-PAGE gel (polyacrylamide gel electrophoresis) at 90V (voltage) for one hours. The proteins were then transferred to an Immobilon-FL PVDF membrane at 85V for two hours on ice (semi-dry transfer). After the transfer, the membrane was blocked for one hour in a blocking buffer which consisted of 60% TBS (20mM tris-base, 0.14M NaCl, pH 7.4) and 40% Odyssey blocking buffer. The membrane was then incubated with a mixture of primary antibodies: rabbit monoclonal CCR7 (1:10000) and mouse monoclonal GAPDH (1:5000), diluted in a solution of 60% TBST(20mM tris-base, 0.14M NaCl, 0.01% Tween, pH 7.4) and 40% Odyssey blocking buffer overnight at 4°C. After being washed in TBST (pH 7.4)(3 X 15 minutes), the membrane was probed with a mixture of goat anti-rabbit (1:5000), donkey anti-mouse (1:10000) IRDye polyclonal secondary

antibodies diluted in a solution of 60% TBST and 40% Odyssey blocking buffer, for two hours in the dark. Finally, the membrane was washed three times (15 minutes each) in TBST (pH 7.4) before scanning on an Odyssey infrared imaging system (LI-COR, Lincoln, NE).

2.2.3.2 CCL21 Protein Expression

Western blot of CCL21 protein expression by MDA-MB-231 and HMVEC-dLy cells was performed as in 2.2.3.1 with modification as followed. Fifteen micrograms of total protein was electrophoresed per well on a 1.5 mm 12% SDS-PAGE gel at 80V for two hours. The protein was then transferred to an Immobilon-FL PVDF membrane at 5V for three hours (dry transfer). After the transfer, the membrane was blocked in 3% BSA in TBST (20mM tris-base, 0.14M NaCl, 0.01% Tween, pH 7.4) for one hour at room temperature. The membrane was incubated in a mixture of primary antibodies: rabbit polyclonal antibodies against human 6Ckine (1:200) and mouse monoclonal GAPDH antibody (1:5000) diluted in a solution consisting of 3% BSA in TBST overnight at 4°C. The membrane was than washed three times in TBST (15 minutes each) followed by two hours incubation in goat anti-rabbit (1:5000), donkey anti-mouse (1:10000) IRDye polyclonal secondary antibodies diluted in 3% BSA in TBST (pH 7.4). Finally, the membrane was washed three times (15 minutes) in TBST. The detection of CCL21 was done in the same manner as described in 2.2.3.1.

2.2.3.3 VEGF-C Protein Expression

MDA-MB-231 cells transiently transfected with CCR7 siRNA or control vector were harvested 48 hours post-nucleofection and lysed in M-Per lysies buffer following the protocol described in 2.2.3.1. Proteins (15 μg /well) from the cell lysates were separated by 10% SDS-PAGE gel, transferred to an Immobilon-FL PVDF membrane at 5V for three hours. After the transfer, the membrane was blotted with a mixture of goat polyclonal antibodies against human VEGF-C (1:500) and mouse monoclonal GAPDH antibody (1:5000) diluted in 2% BSA in TBS (20mM tris-base, 0.14M NaCl, pH 7.4) overnight at 4°C. The membrane was then washed three times in TBST (15 minutes each) followed by two hours of incubation in donkey anti-goat (1:5000) and donkey anti-mouse (1:10000) IRDye polyclonal secondary antibodies diluted in 2% BSA in TBS (pH 7.4). Finally, the membrane was washed three times (15 minutes) with TBST. The detection of VEGF-C was done in the same manner as described in 2.2.3.1.

2.2.3.4 Phospho-AKT Detection

To test whether CCL21 promotes the activation of AKT signalling pathway downstream of CCR7, MDA-MB-231 cells were plated at $2x10^5$/well in six well plates. The next day, cells were serum starved by culturing them in serum free RPMI overnight. Prior to the protein extraction, cells were incubated in the absence (negative control) and presence of recombinant human CCL21/6Ckine (350 ng/ml) over different time intervals (5, 10, 15, 30, and 45 minutes). Also, to analyze the effect of blocking the CCR7 receptor, cells were treated with monoclonal anti-human CCR7 neutralizing antibodies (1, 5, 10, 15 μg/ml) for two hours before stimulation with human CCL21/6Ckine (350

ng/ml) for 30 minutes. Western blot analysis of the total cell lysates was then performed as in 2.2.3.1 with the following modification. For analysis of the total cell lysates, cells were washed with ice-cold DPBS and treated with M-Per lysies buffer supplemented with Phosphatase Inhibitor Cocktail 2 (10 µl/ml) and Phosphatase Inhibitor Cocktail 3 (10 µl/ml). Fifteen micrograms of the total protein from the cell lysates was run per well on a 1.5mm 10% SDS-PAGE gel at 90V for 1 hour. The proteins were then transferred to an Immobilon-FL PVDF membrane at 85V for two hours on ice. After the transfer, the membrane was blocked for one hour in a blocking buffer consisting of 60% TBS (20mM tris-base, 0.14M NaCl, pH 7.4) and 40% Odyssey blocking buffer. Immunoblotting was performed with the following primary antibodies: Akt (pan) mouse monoclonal antibody (1:500), Phospho-Akt (Ser473) XP rabbit monoclonal antibody (1:500) diluted in a solution consisting of 60% TBST(20mM tris-base, 0.14M NaCl, 0.01% Tween, pH 7.4) and 40% Odyssey blocking buffer overnight at 4°C. Blots were washed three times with TBST and probed afterwards with the following IRDye polyclonal secondary antibodies: donkey anti-mouse (1:10000) and goat anti-rabbit (1:5000) diluted in 60% TBST and 40% Odyssey blocking buffer before detection.

2.2.3.5 Densitometry

For quantitative analysis of the protein levels, NIH Image J (National Institutes of Health) software was used to determine the average density of each band. For each condition, the band density of analyzed protein was normalized to the band density of the house keeping gene (GAPDH) or to the corresponding total protein (total-AKT).

2.2.4 Real-Time Polymerase Chain Reaction Analysis

2.2.4.1 CCR7 mRNA Expression

For RNA extraction and cDNA synthesis, cells were grown up to 80-85% confluence, trypsinized and collected as a cell pellet, then gently washed with DPBS prior to lyses. Total RNA was extracted with RNeasy Minikit following the manufacturer's instructions. In order to eliminate genomic contamination, DNase digestion was performed (RNase-Free DNase kit). The total RNA was quantified with a spectrophotometer (NanoDrop 2000, Thermo Scientific) and cDNA was synthesized with a High Capacity cDNA Reverse Transcription kit using up to 2 μg of total DNase-treated RNA per 20 μl volume reaction. Reverse transcription was performed in a thermal cycler (*C1000*^{TM}, Bio Rad) under the following parameters: 25°C for 10 minutes, 37°C for 120 minutes, followed by 85°C for 5 minutes. Primers for the human CCR7 chemokine receptor and housekeeping gene (GAPDH) were synthesised at the University of Western Ontario's Oligo Factory (London, ON). Their product sizes were as follows: CCR7 PCR product, forward 5'-GACCGATACCTACCTGCTCAACC-3', and reverse 5'-GCTCACTGCTGCTCCTCTGG-3',341 bp, GAPDH PCR product, forward 5'-ACCACAGTCCATGCCATCAC-3', and reverse 3'- TCCACCACCCTGTTGCTGTA-5',452 bp. Samples were analyzed using the Platinum PCR SuperMix High Fidelity. A PCR reaction volume of 50 μl was prepared for amplification, including 2.2 μl primers, and 2.8 μl of DNA template solution. PCR cycling conditions were specific to each primer pair, consisting of initial denaturation at 94°C for 60 seconds and then 28 cycles of denaturation at 94°C (30 seconds), annealing at 59°C (30 seconds), and extension at 72°C (45 seconds) for CCR7, and 20 cycles of denaturation at 94°C (30 seconds),

annealing at 55°C (30 seconds), and extension at 72°C (60 seconds) for GAPDH. Real-time PCR products were separated by electrophoresis (90V for 45 minutes) on 2% agarose gel and visualized by GelRed Nucleic Acid Gel Stain using a gel imaging system (Gel Doc™ XR System, Bio Rad).

2.2.4.2 CCL21 mRNA Expression

Real-time PCR for CCL21 mRNA expression was performed as in 2.2.4.1 with the following modification. Primers for the human CCL21 chemokine ligand, synthesised by the University of Western Ontario Oligo Factory (London, ON), and their product size were : forward 5'-CGCAGCTACCGGAAGCAG-3', and reverse 5'-CTGCCTGAGAGCGCTTGC-3',176 bp. Real-time PCR products were separated by electrophoresis (90V for 1 hour) on a 2.5% agarose gel electrophoresis (90V for 1 hour) and visualized by GelRed Nucleic Acid Gel Stain using a gel imaging system (Gel Doc™ XR System, Bio Rad).

2.2.4.3 VEGF-C mRNA Expression

Real-time PCR for VEGF-C was performed following the previously described protocol (2.2.4.1) with the following modification. The primers for VEGF-C, synthesised by the University of Western Ontario Oligo Factory (London, ON), and their product size were: forward 5' CGGGAGGTGTGTATAGATGTG-3, and reverse 3'-ATTGGCTGGGGAAGAGTTTG-5', 583 bp. The cycling parameters for VEGF-C consists of initial denaturation at 94°C for 60 seconds and then 28 cycles of denaturation 94°C for 30 seconds, annealing 56°C for 30 seconds, and extension 72°C for 45 seconds. Real-time PCR products were separated on a 2% agarose gel electrophoresis (90V for 30

minutes) and visualized by GelRed Nucleic Acid Gel Stain using a gel imaging system (Gel Doc™ XR System, Bio Rad).

2.2.5 CCR7 siRNA Nucleotransfection

MDA-MB-231 cells were grown up to 80% confluence in T75 flasks and then gently harvested from the substrate. The harvest cells, at a concentration of 10^6 cells/ml, were distributed into certified cuvettes and transfected with 6 μM of either silencer pre-designed siRNA targeting CCR7 or silencer negative control siRNA (scramble siRNA) using the Amaxa Cell Line Nucleofactor Kit and X-013 program according to the manufacturer's protocol. After nucleofection, cells were placed in an antibiotic free-medium and incubated at 37°C, 5% CO_2 for 48 hours. The nucleofection efficiency was assayed with quantitative real-time PCR, conventional real-time PCR, and Western blot.

2.2.6 Quantitative Real-Time Polymerase Chain Reaction Analysis

2.2.6.1 CCR7 and VEGF-C Expression

MDA-MB-231 cells transiently transfected with CCR7 siRNA or control vector were harvested 48 hours post-nucleofection and RNA was extracted with an RNeasy Minikit following the manufacturer's instructions. DNA was synthesized with a High Capacity cDNA Reverse Transcription kit following the previously described protocol (2.2.4.1). Quantitative real-time PCR was performed in single micro capillary tubes on a LightCycler (Roche Diagnostic, Que.) with TaqMan® Universal PCR Master Mix for both the control (TaqMan® Pre-Developed Assay Reagent, Hu GAPDH) and the target gene expression primer probes (TaqMan® Gene Expression Assay, CCR7 probe and TaqMan® Gene Expression Assay, VEGF-C probe). Twenty microliters of PCR reaction

volume was prepared for the amplification, including 1 μl TaqMan primer probe, and 2 μl template DNA solution. The quantitative real-time PCR profile was 95°C/15 seconds denature, 58°C/1 minute anneal-extension for 40 cycles.

2.2.6.2 CCR7 and VEGF-C Relative Quantification

Quantitative real-time PCR reactions were done in triplicate. Delta-delta Ct method was employed. For delta Ct calculation, the cycle threshold (Ct) values of the sample under investigation and reference (control) sample were normalized to the endogenous housekeeping gene (GAPDH). Delta-delta Ct was then calculated by subtracting ΔCt of the reference sample from ΔCt of the gene under investigation and fold difference ($2^{-\Delta\Delta Ct}$) was obtained (Applied Biosystems).

2.2.7 Enzyme-Linked Immunosorbent Assay (ELISA) Analysis

2.2.7.1 CCL21 Protein Secretion

In order to quantify CCL21 protein secretion from MDA-MB-231 and HMVEC-dLy cells, both cell lines were maintained in 2D and 2D-matrix culture conditions. The cells grown in T75 flasks were serum starved overnight, trypsinized and suspended in serum free media at a concentration of $6x10^4$ cells /ml. For 2D samples, cells were seeded onto six-well plates and basal conditioned media was collected after 24 hours culture. For 2D-matrix samples, GFR Matrigel was diluted in cold basal medium (1:1 dilution) and added to the six-well plates (250 μl of GFR Matrigel/well) and allowed to solidify for 30 minutes. Cells were then seeded on the solidified Matrigel and incubated for 24 hours. After culture, the apical compartment (basal medium) was collected and the Cell Recovery Solution (2 ml/well) was added to the cells and

subcellular matrix together to digest the Matrigel matrix. After the matrix was completely dissolved, the solution containing cells and matrix was centrifugated for five minutes at 1500 RPM. The supernatant, basal compartment was collected for further analysis and the pellet, cellular compartment, was lysed with M-PER buffer (200 µl/well) for five minutes. Lysates were sonicated (six pulses), then centrifugated at 13,000 RCF for 20 minutes at 4°C to remove cell debris, and the supernatants were collected. After collection, the apical, basal, and cell compartments were analyzed for CCL21 by ELISA (Quantikine Human 6Ckine Immunoassay) according to the manufacturer's protocol. Measurements were done in triplicate for two independent experiments. The optical density at 570 nm and 450 nm was determined for each well using an Infinite M200 (TECAN) plate reader. Then the reading at 570 nm was subtracted from the reading at 450 nm for each well.

2.2.7.2 VEGF-C Protein Secretion

To determine whether the activation of CCR7 signalling regulates VEGF-C secretion, MDA-MB-231 cells transiently transfected with CCR7 siRNA or control vector (scramble siRNA) were cultured in serum free media onto six-well plates and treated with human CCL21/6Ckine (350 ng/ml) for 24 hours before supernatants were collected for VEGF-C quantification. VEGF-C concentration in conditioned media was measured by ELISA following the manufacturer's instructions. Each measurement was done in triplicate. The optical density at 570 nm and 450 nm was determined for each well using an Infinite M200 (TECAN) plate reader.

To determine whether the AKT signalling pathway regulates VEGF-C protein secretion, MDA-MB-231 cells were grown in T75 flask to near confluence in complete medium. Then, cells were serum starved by culturing in serum free RPMI overnight, trypsinized and resuspended in serum free media to a final concentration of 10^6 cells/ml. Cells were pre-treated with various concentrations of PI3 kinase inhibitor LY294002 (0, 0.5, 1, 1.5 μl/ml) for one hour before being plated onto six-well plates. Tumour cells were then stimulated with human CCL21/6Ckine (350 ng/ml) and incubated at 37°C, 5% CO_2 for 24 hours. The next day, supernatants were collected and an ELISA was carried out according to the manufacturer's protocol. Each measurement was done in triplicate. The optical density at 570 nm and 450 nm was determined for each well using an Infinite M200 (TECAN) plate reader.

2.2.8 Cell Proliferation ELISA, BrdU Assay Analysis

2.2.8.1 CCL21-Stimulated HMVEC-dLy Proliferation

To examine whether CCL21 stimulates HMVEC-dLy proliferation, cells were grown up to 80% confluence in T75 flasks. HMVEC-dLy cells were then serum starved overnight by culturing in an endothelial basal medium (EBM) without any growth supplements, treated with a Trypsin-EDTA solution to facilitate removal from the substrate, then resuspended in EBM to a final concentration of $2x10^4$ cells/ml. Serum-starved cells were seeded onto 96-well tissue-culture microplates, stimulated with various concentrations of human CCL21/6Ckine (0, 100, 200, and 350 ng/ml), and incubated at 37°C, 5% CO_2 for 24 hours. After incubation, the quantification of cell proliferation was performed by the measurement of BrdU incorporation in newly synthesized cellular DNA, Cell Proliferation ELISA assay. HMVEC-dLy cells were labelled by the addition

of BrdU labelling solution for two hours. During this labelling period, BrdU was incorporated into the DNA of cycling cells. After removing the labelling medium, the cells were fixed, and the DNA was denatured in one step by adding FixDenat. After removing FixDenat, the anti-BrdU-POD antibody was added, and then bound to the BrdU incorporated into the newly synthesized cellular DNA. The immune complexes were detected by the subsequent substrate reaction. The reaction product was measured with a plate reader, Infinite M200 (TECAN) at wavelength of 370 nm.

2.2.8.2 Inhibition of CCL21-Stimulated HMVEC-dLy Proliferation

To determine whether CCR7 antibodies inhibit CCL21 induced HMVEC-dLy proliferation, cells were harvested as previously described and pre-treated with various concentrations of mouse monoclonal anti-human CCR7 antibodies (0, 5, 10, 20 μg/ml) in EBM for two hours before being plated onto 96-well tissue-culture microplates. Human CCL21/6Ckine (200 ng/ml) was added to each well and the system was incubated at 37°C, 5% CO_2 for 24 hours. The following day, a cell proliferation ELISA assay was performed according to the manufacturer's protocol.

2.2.9 Boyden Chamber Assay Analysis

2.2.9.1 CCL21-Stimulated HMVEC-dLy Migration

To establish the role of CCL21/CCR7 interaction on migration of lymphatic endothelial cells (LECs), a Boyden chamber assay was performed using Trans-well inserts. This assay consists of two chamber plates separated by a porous membrane. Polycarbonate membrane inserts with 8 μm pore opening placed within 24-well plates

were used. HMVEC-dLy cells were grown as described in 2.2.8.1. A two hundred microliter suspension of serum-starved cells at a concentration of $2x10^5$/ml was added in the upper chamber of the cell culture inserts. Increasing concentrations of human CCL21/6Ckine (0, 100, 200, and 350 ng/ml) were added to serum-free media in the lower chamber. The assembled cell culture insert chambers were then incubated at 37°C, 5% CO_2 for different time periods (12, 24, and 48 hours). The cells were then fixed with methanol and stained with eosin/thiasine. Direct microscopic counting at 40X magnification (LEICA DFC 295) of the LECs that have migrated to the lower side of the membrane was performed. Three independent experiments with each condition tested in triplicate were conducted. For each sample, the cells in ten random high power fields were counted and a mean value for each sample was calculated.

2.2.9.2 Inhibition of CCL21-Stimulated HMVEC-dLy Migration

To test whether CCR7 antibodies inhibit CCL21-induced HMVEC-dLy migration, cells were harvested as previously described and pre-treated with various concentration of the mouse monoclonal anti-human CCR7 antibodies (0, 5, 10, 20 μg/ml) for two hours, before being seeded in the upper chambers. Human CCL21/6Ckine (350 μg/ml) in serum-free media was added to the lower chambers. The assembled cell culture insert chambers were then incubated at 37°C, 5% CO_2 for 24 hours. Direct microscopic counting of the LECs that have migrated to the lower side of the membrane (after eosin-thiasine staining) was performed. Three independent experiments with each condition tested in triplicate were conducted. For each sample, the cells in ten randomly chosen high power fields were counted and a mean value for each sample was calculated.

2.2.10 Lymphatic Endothelial-Like Tube Formation Analysis

2.2.10.1 CCL21-Induced HMVEC-dLy Tube Formation

Tube formation assay is based on the ability of lymphatic endothelial cells to form capillary like tube structures when cultured on a gel of basement extract. This assay represents a simple, but powerful model to study the effect of either lymphangiogenic activators or inhibitors on LECs' properties (Berndt et al., 2008; Banziger-Tobler et al., 2008; Cueni et al., 2009). To determine whether the CCL21/CCR7 interaction stimulates lymphatic endothelial-like tube formation, HMVEC-dLy grown up in T75 flasks to near confluence (80%) were trypsinized and resuspended in endothelial basal media + 0.5% BSA (without any growth supplements) to a final concentration of $2x10^5$ cells/ml. GFR Matrigel was thawed overnight at 4°C, diluted with cold EBM (1:1 dilution) and placed in six-well plates to solidify. HMVEC-dLy cells (2 ml of cell suspension) were seeded on the solidified Matrigel and stimulated with various concentrations of recombinant human 6Ckine/CCL21 (0, 100, 200, 350, ng/ml). The system was incubated at 37°C, 5% CO_2 for 24 hours and tube formation was examined on an inverted microscope at 100 x magnification at different time intervals. Pictures were taken with a phase contrast Leica (DFC340FX) camera at different magnifications (5X, 10X). Five images were randomly taken in different areas of the wells by selecting fields of view that were distinct and distant enough to not overlap with each other.

2.2.10.2 Inhibition of CCL21-Induced HMVEC-dLy Tube Formation

To test whether CCR7 antibodies inhibit CCL21-induced HMVEC-dLy tubular network formation, $2x10^5$cells/ml were harvest as previously described, and treated with various concentrations of mouse monoclonal anti-human CCR7 antibody (0, 5, 10, 20 μg/ml) for two hours. Recombinant human 6Ckine/CCL21 (350 ng/ml) was then added and cells were seeded onto the solidified Matrigel and incubated at 37°C, 5% CO_2 for 24 hours. Pictures were then taken with the Leica EC3 camera. Five images were randomly taken in different areas of the wells by selecting fields of view that were distinct and distant enough to not overlap with each other.

2.2.10.3 Lymphatic Endothelial Tube Quantification

The total length of the interconnected cells forming tubular structures was measured with ImageJ (National Institutes of Health) software.

2.2.11 Statistical Analysis

Statistical calculations were performed using GraphPad Prism software version 5 (GraphPad Software, La Jolla, CA). All data were analyzed with one-way ANOVA followed by Tukey's multiple comparisons test, with the exception of densitometry data which were analyzed with one-way ANOVA followed by Dunnett's test. Data from quantitative real-time PCR were analyzed with a Student's T-test. Statistically relevant differences between mean values were determined based on $p < 0.05$ criterion.

CHAPTER THREE:

RESULTS

3.1 Expression of CCR7 Chemokine Receptor by MDA-MB-231 and HMVEC-dLy Cells

Previous studies have reported that breast cancer cells express chemokine receptors in a defined rather than in a random manner. Muller et al. (2001) analyzed the mRNA expression of CCR7 receptor in several breast cancer cell lines and found that CCR7 was highly expressed in metastatic cell lines, compared to low metastatic or normal mammary epithelial cells. In this research, the constitutive expression of CCR7 chemokine receptor by MDA-MB-231 and HMVEC-dLy cell lines was analyzed at the mRNA and protein levels.

Real-time PCR analysis showed that CCR7 chemokine receptor is expressed at the mRNA level by both cell lines. CCR7 was detected at 341 bp and GAPDH was used as an internal control (Figure 7).

To verify the expression of CCR7 protein, western blot analysis was performed. The representative images shown in Figure 8A and the quantitative analysis in Figure 8B demonstrate that CCR7 is expressed at the protein level by both cell lines analyzed. GAPDH was used to control for equal loading. Blots shown are representative of three independent experiments. CCR7 protein expression on the surface of both cell lines was then assayed by flow cytometry. Data analysis revealed that 89.1% of MDA-MB-231 and 59.3% of HMVEC-dLy express CCR7 receptor. IgG isotype-PE was used as control (Figure 9).

Based on these results, it can be concluded that CCR7 chemokine receptor is expressed at the mRNA and protein levels by MDA-MB-231 and HMVEC-dLy cells.

Figure 7. CCR7 Chemokine Receptor Is Expressed at the mRNA Level by MDA-MB-231 and HMVEC-dLy Cells. Cells were cultured under standard conditions before RNA extraction. Real-time PCR was performed using primers specific for human CCR7. GAPDH was used as an internal control. Real-time PCR analysis demonstrates that CCR7 receptor is expressed at the mRNA level by both cell lines. Representative results from a series of three independent experiments are shown.

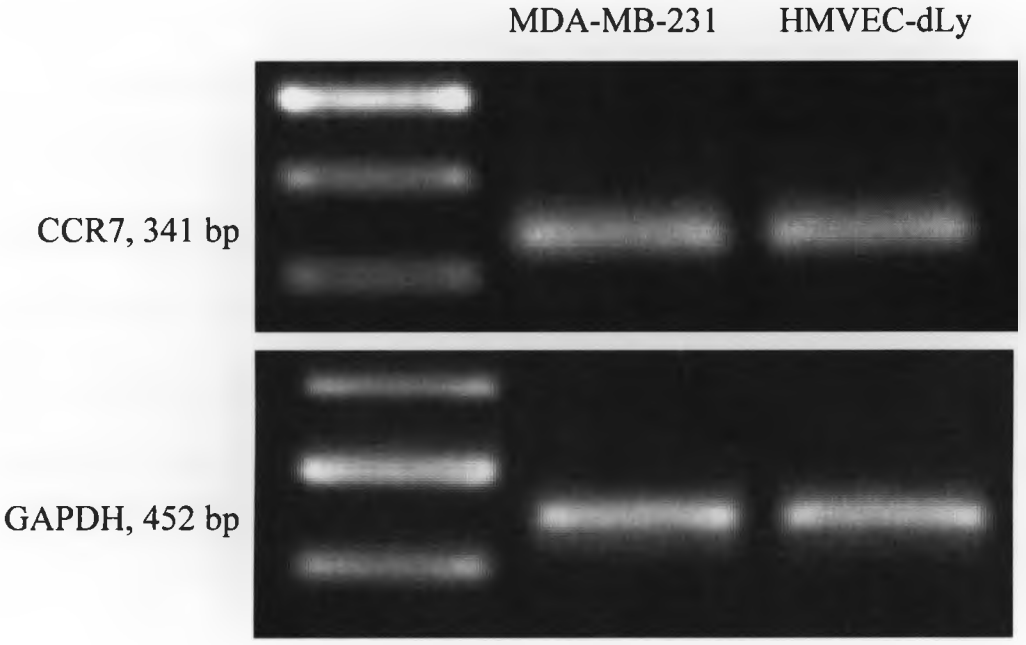
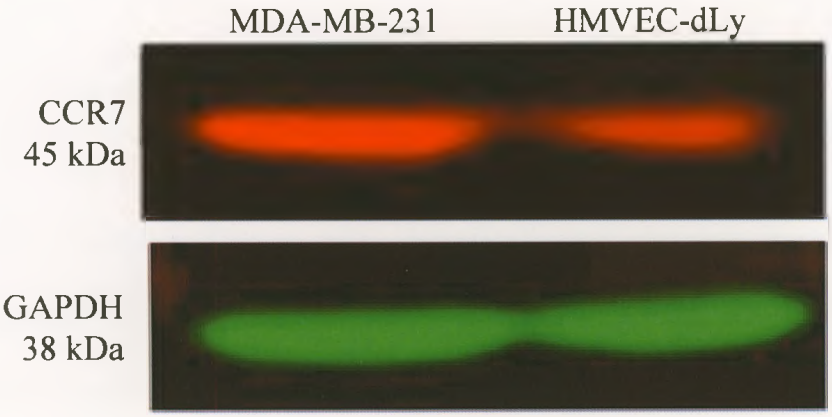
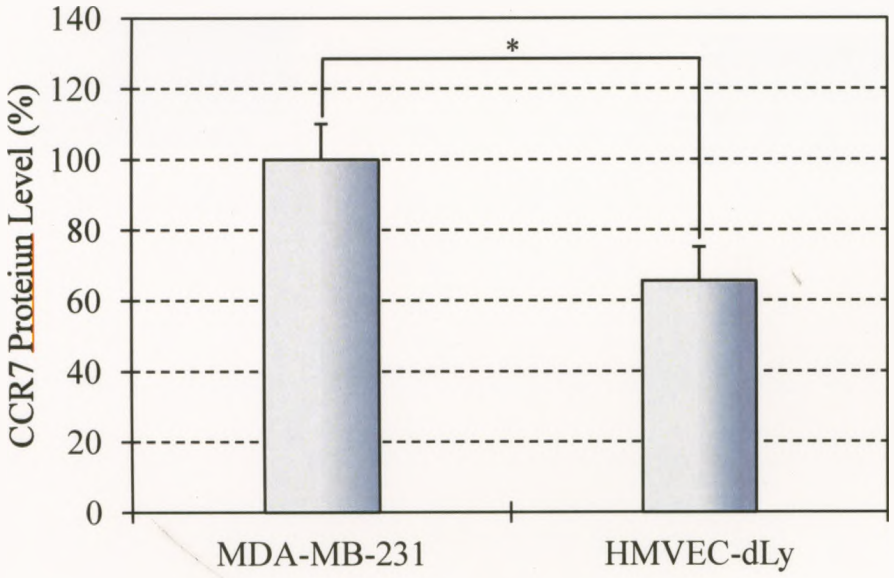

MDA-MB-231
HMVEC-dLy
CCR7, 341 bp
GAPDH, 452 bp

Figure 8. CCR7 Chemokine Receptor Is Expressed at the Protein Level by MDA-MB-231 and HMVEC-dLy Cells. A) Lysates from cultured MDA-MB-231 and HMVEC-dLy cells were assayed by Western blot and CCR7 protein expression was detected at 45 kDa. GAPDH (house-keeping gene) was used as an internal control. **B)** CCR7 quantification through densitometry of Western blots revealed that the protein level of CCR7 is 1.5 fold higher in tumor cells compared to lymphatic endothelial cells. Data are represented as a mean normalized expression ± SD for three independent experiments. (*) indicates significant difference ($p < 0.05$).

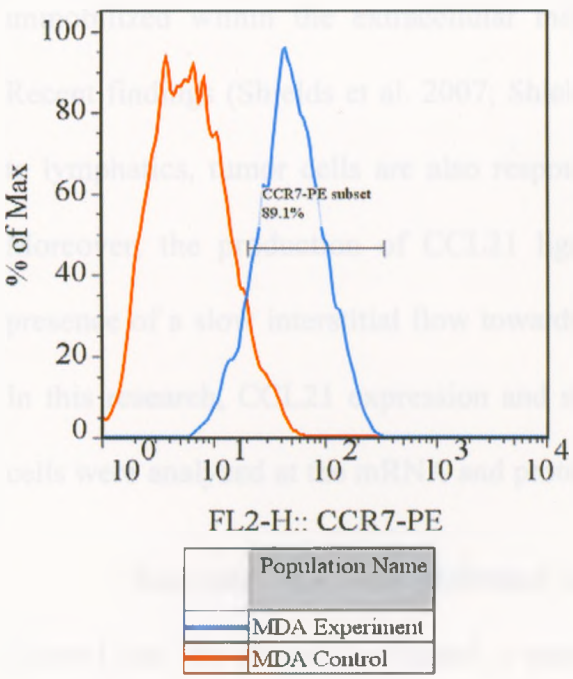

MDA-MB-231
HMVEC-dLy
CCR7
45 kDa
GAPDH
38 kDa

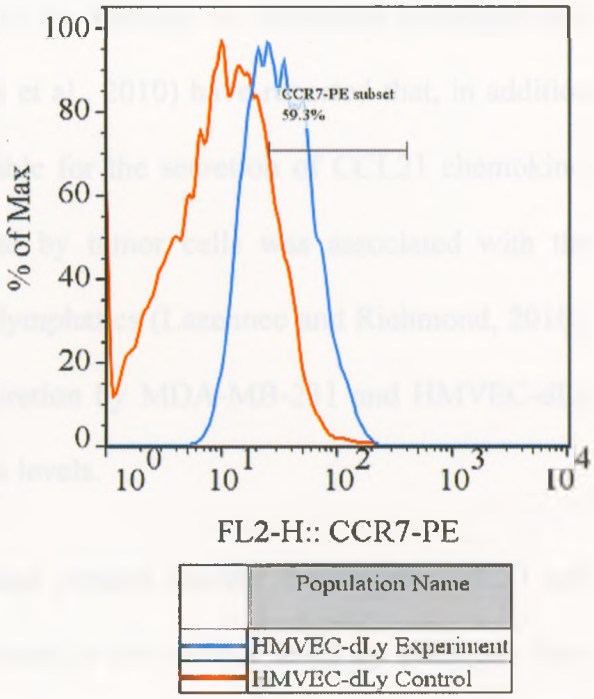
A)


CCR7 Proteiun Level (%)
140
120
100
80
60
40
20
0
*
MDA-MB-231
HMVEC-dLy

B)

Figure 9. CCR7 Chemokine Receptor Is Expressed on the Surface of MDA-MB-231 and HMVEC-dLy Cells. To characterize the cell surface phenotype of CCR7 receptor, cells were analyzed through flow cytometry. The cyan histograms represent the florescence activity of MDA-MB-231 and HMVEC-dLy cells after incubation with phycoerythrin monoclonal antibody directed against CCR7. Red histograms denominate the signal of the isotype control. Data analysis shows that 89.1% of MDA-MB-231 and 59.3% of HMVEC-dLy express CCR7 receptor.


100
80
60
40
20
0
% of Max
CCR7-PE subset
89.1%
10^0 10^1 10^2 10^3 10^4
FL2-H:: CCR7-PE
Population Name
MDA Experiment
MDA Control


100
80
60
40
20
0
% of Max
CCR7-PE subset
59.3%
10^0 10^1 10^2 10^3 10^4
FL2-H:: CCR7-PE
Population Name
HMVEC-dLy Experiment
HMVEC-dLy Control

3.2 Expression and Secretion of CCL21 Chemokine Ligand by MDA-MB-231 and HMVEC-dLy Cells

The CCL21 chemokine ligand is secreted as a 12 kDa protein that is readily immobilized within the extracellular matrix by binding to sulfatated proteoglycans. Recent findings (Shields et al. 2007; Shields et al., 2010) have reported that, in addition to lymphatics, tumor cells are also responsible for the secretion of CCL21 chemokine. Moreover, the production of CCL21 ligand by tumor cells was associated with the presence of a slow interstitial flow towards lymphatics (Lazennec and Richmond, 2010). In this research, CCL21 expression and secretion by MDA-MB-231 and HMVEC-dLy cells were analyzed at the mRNA and protein levels.

Real-time PCR was performed using primers specific for human CCL21 and showed that the chemokine ligand is expressed at the mRNA level by both cell lines analyzed. CCL21 was detected at 176 bp and GAPDH was used as an internal control (Figure 10).

The protein expression of CCL21 chemokine in MDA-MB-231 and HMVEC-dLy cells was analyzed by Western blot. The representative images of blots shown in Figure 11A and the quantification of densitometry data in Figure 11B indicate that CCL21 is expressed by tumor cells while only traces of this ligand were observed in lymphatic cells. Three independent experiments were conducted and GAPDH was used as an internal control.

Figure 10. CCL21 Chemokine Is Expressed at the mRNA Level by MDA-MB-231 and HMVEC-dLy Cells. Real-time PCR was performed using primers specific for human CCL21. Agarose gel electrophoresis of real-time PCR products from MDA-MB-231 and HMVEC-dLy cells showed the expected amplicon size (176 bp). GAPDH was used as an internal control. Representative results from a series of three independent experiments are shown.

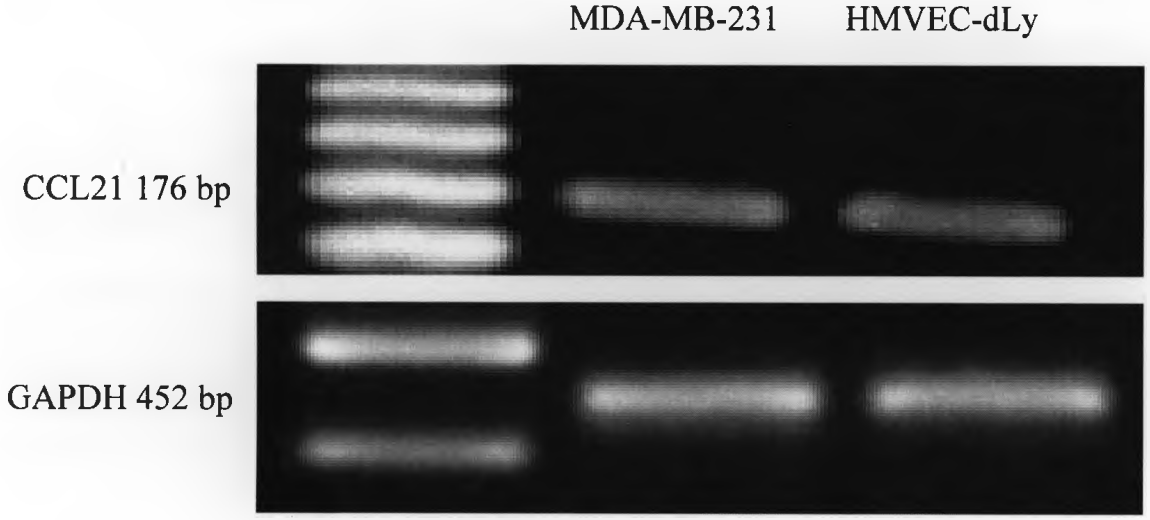
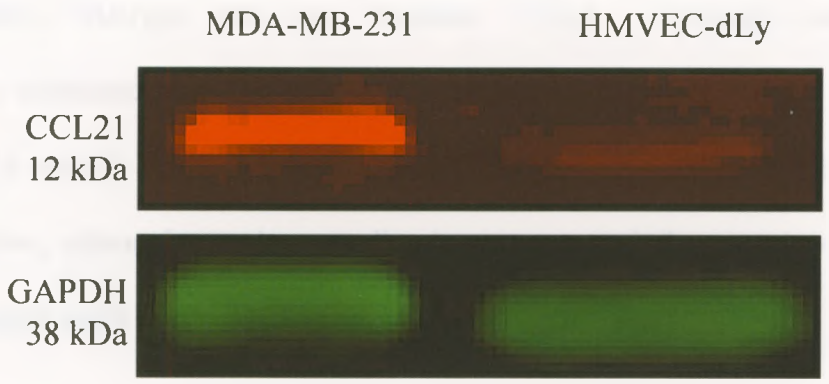
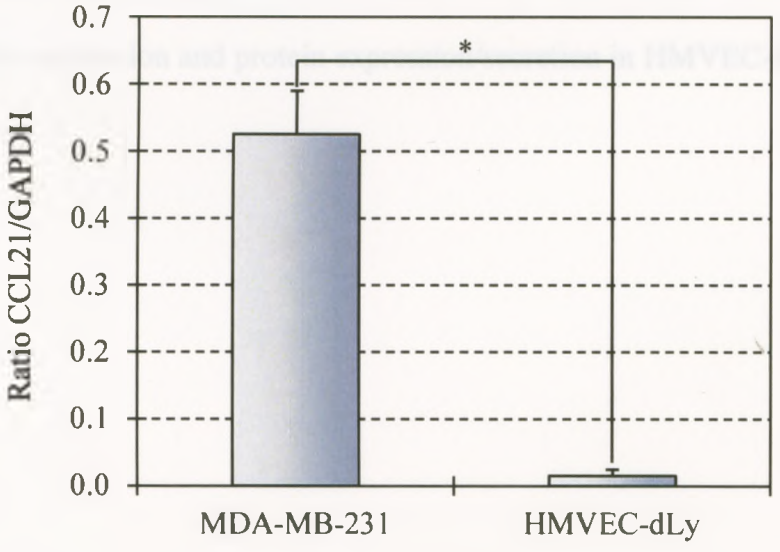

MDA-MB-231
HMVEC-dLy
CCL21 176 bp
GAPDH 452 bp

Figure 11. CCL21 Chemokine Is Expressed at the Protein Level by MDA-MB-231 Cells. A) Western blot analysis of MDA-MB-231 total cell lysates detected CCL21 at 12 kDa while only traces were observed in HMVEC-dLy. **B)** Densitometry analysis of CCL21 expression in tumor and lymphatic cells. CCL21 band intensity from densitometry of Western blots was normalized relative to GAPDH. Data are represented as a mean normalized expression ± SD for three independent experiments. (*) indicates significant difference ($p < 0.05$).


MDA-MB-231
HMVEC-dLy
CCL21
12 kDa
GAPDH
38 kDa

A)

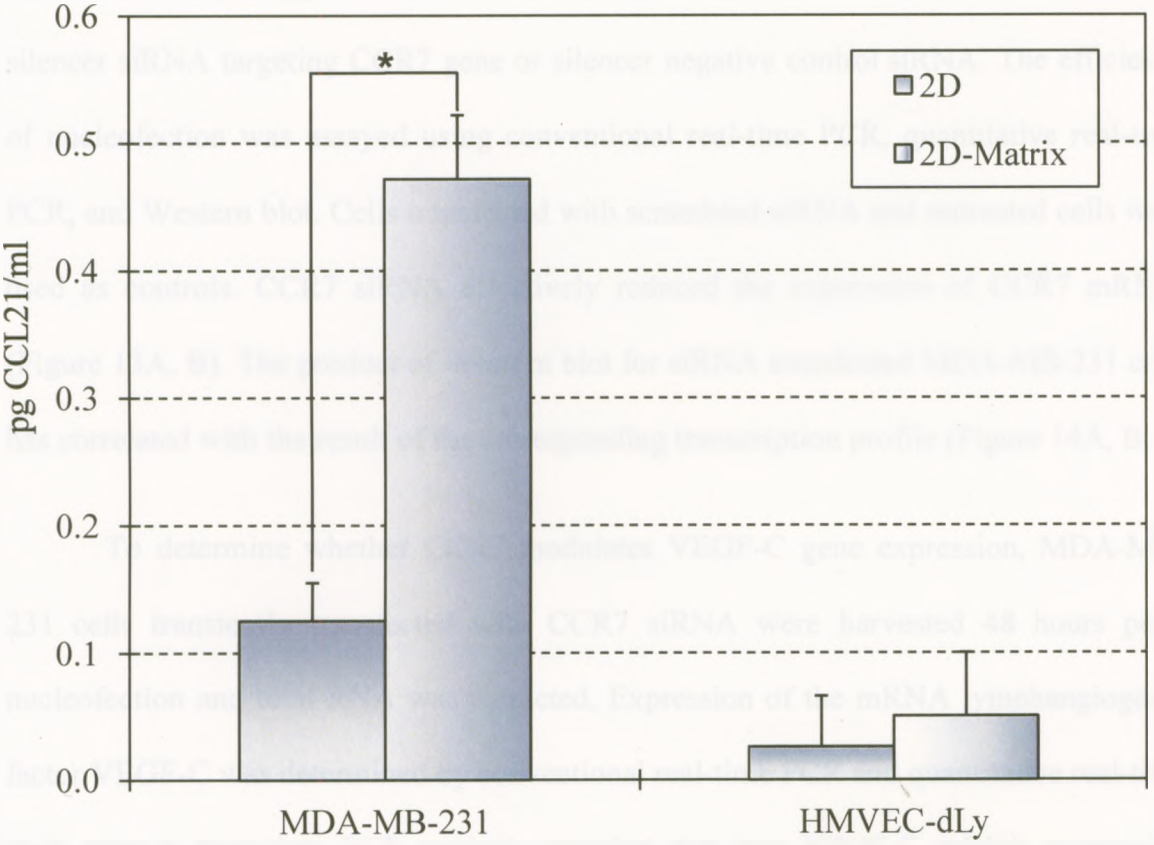

Ratio CCL21/GAPDH
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
MDA-MB-231
HMVEC-dLy

B)

CCL21 protein secretion was quantified from tumor and lymphatic cells using a Human CCL21 Quantikine ELISA kit. For generation of conditioned media, cells in basal media were plated onto 6-well plates coated with or without a growth factor-reduced Matrigel matrix. Matrigel was used because CCL21 is strongly matrix-binding particularly to sulphated proteoglycans. The bound CCL21 protein fraction was 3.7 fold higher than the soluble fraction, as expected. On the contrary, HMVEC-dLy cells were CCL21 negative, although previous studies have suggested that *in vitro* and *in vivo* settings lymphatic cells secrete CCL21 chemokine (Figure 12).

To conclude, CCL21 chemokine ligand is expressed at the mRNA and protein levels by MDA-MB-231 cells. On the other hand, no correlation was found between CCL21 mRNA expression and protein expression/secretion in HMVEC-dLy cells.

Figure 12. CCL21 Chemokine Is Secreted by MDA-MB-231 Cells. CCL21 protein concentration in conditioned media was measured by enzyme-linked immunosorbent assay (ELISA). Serum-starved cells were cultured onto 6-well plates coated with or without Matrigel. The apical, basal, and cell compartments were then analyzed after using Cell Recovery Solution for matrix digestion and M-PER buffer for cell lysis. CCL21 secretion by tumor cells was 3.7 fold higher in 2D-matrix condition than in 2D culture conditions, a result that was expected due to the matrix binding properties of this chemokine. On the contrary, CCL21 protein secretion by HMVEC-dLy cells was observed as being at very low levels. Data are represented as a mean $\pm$ SD ($n = 3$). (*) indicates significant difference ($p < 0.05$).


0.6
0.5
0.4
0.3
0.2
0.1
0.0
pg CCL21/ml
*
2D
2D-Matrix
MDA-MB-231
HMVEC-dLy

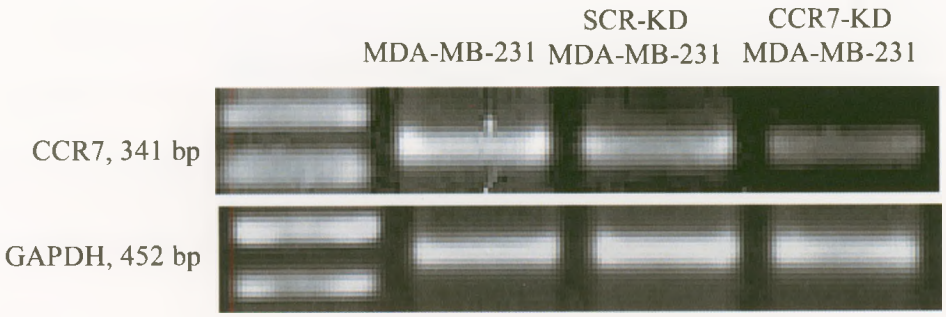
3.3 CCR7 Expression Regulates VEGF-C Secretion

The working hypothesis tested was that in CCR7 positive tumor cells the secretion of lymphangiogenic factor VEGF-C is stimulated by CCL21/CCR7 interaction. To demonstrate this, MDA-MB-231 cells were transiently transfected with 6μM of either silencer siRNA targeting CCR7 gene or silencer negative control siRNA. The efficiency of nucleofection was assayed using conventional real-time PCR, quantitative real-time PCR, and Western blot. Cells transfected with scrambled siRNA and untreated cells were used as controls. CCR7 siRNA effectively reduced the expression of CCR7 mRNA. (Figure 13A, B). The product of Western blot for siRNA transfected MDA-MB-231 cells has correlated with the result of the corresponding transcription profile (Figure 14A, B).

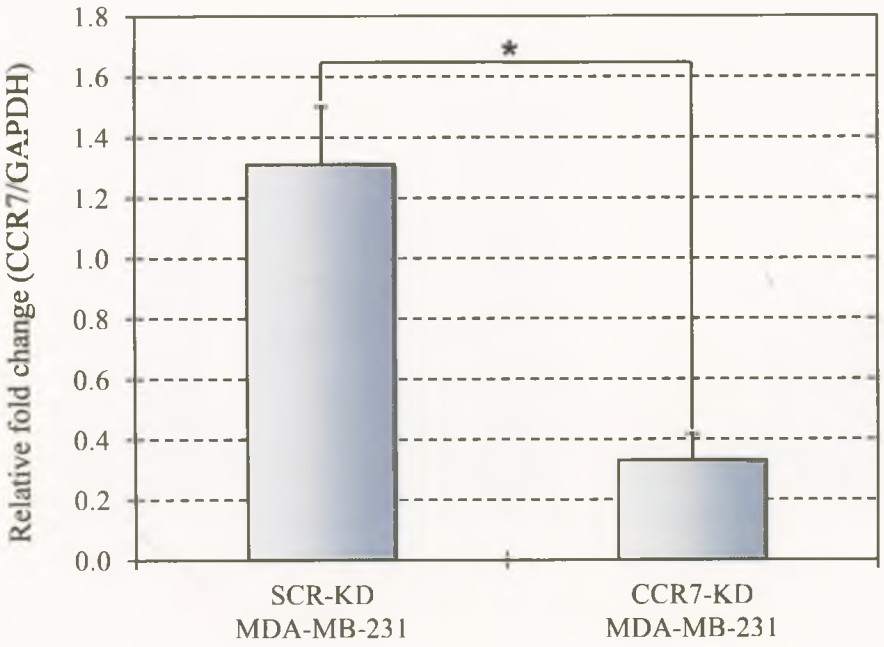
To determine whether CCR7 modulates VEGF-C gene expression, MDA-MB-231 cells transiently transfected with CCR7 siRNA were harvested 48 hours post-nucleofection and total RNA was extracted. Expression of the mRNA lymphangiogenic factor VEGF-C was determined by conventional real-time PCR and quantitative real-time PCR assays. Real-time PCR analysis revealed that low VEGF-C mRNA expression translates in correspondingly reduced band intensity when compared with control transfected cells. Also, compared to control-transfected MDA-MB-231 cells, CCR7 siRNA-transfected MDA-MB-321 cells showed a twofold decrease in VEGF-C mRNA expression (Figure 15A, B)

Furthermore, to verify if transient transfection of siRNA against human CCR7 results in low levels of VEGF-C protein expression, MDA-MB-231 cells were harvested

Figure 13. siRNA Against Human CCR7 Leads to Low Levels of CCR7 mRNA Expression. MDA-MB-231 cells were transiently transfected with 6μM of either silencer siRNA targeting CCR7 gene or silencer negative control siRNA. Total RNA was extracted 48 hours after nucleofection and subject to : **A)** semi-quantitative real-time PCR and **B)** quantitative real-time PCR. GAPDH was used as an internal control. Results for quantitative real-time PCR are presented in relative fold change ($2^{-\Delta\Delta Ct}$) normalized to GAPDH. A four fold change in CCR7 mRNA expression was observed in CCR7 knockdown cells with respect to scrambled knockdown cells. Data are represented as a mean ± SD for three independent experiments. (*) indicates significant differences ($p < 0.05$)) between treatment and control.

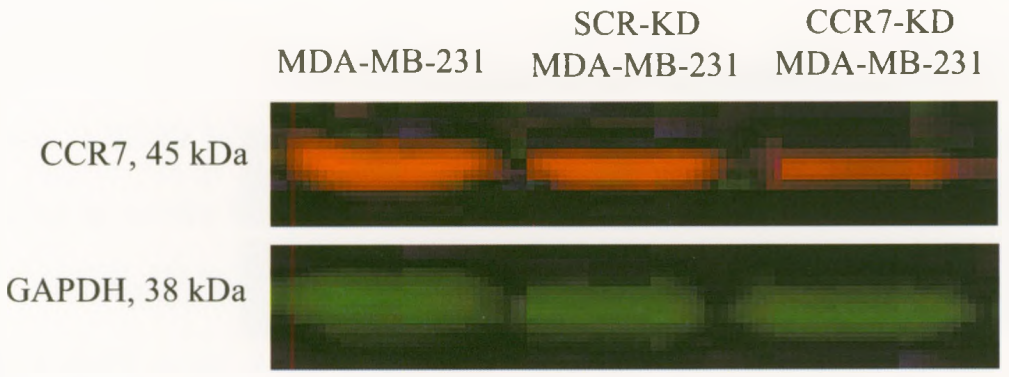

MDA-MB-231
SCR-KD
MDA-MB-231
CCR7-KD
MDA-MB-231
CCR7, 341 bp
GAPDH, 452 bp

A)

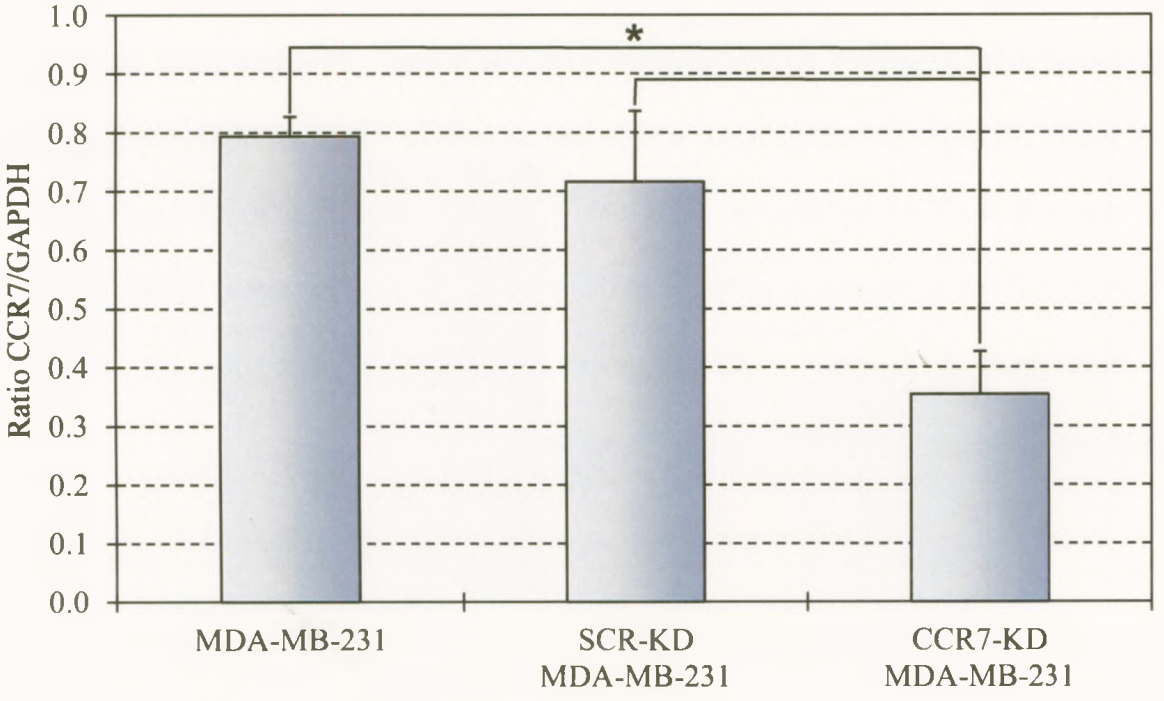

Relative fold change (CCR7/GAPDH)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
SCR-KD
MDA-MB-231
CCR7-KD
MDA-MB-231

B)

Figure 14. siRNA Against Human CCR7 Results in Low Levels of CCR7 Protein Expression. MDA-MB-231 cells transiently transfected with 6 μM of either silencer CCR7 siRNA or negative control siRNA were harvested 48 hours post-nucleofection. Cell lysates were then prepared and analyzed by: **A)** Western blot and **B)** densitometry. siRNA reduced the expression of CCR7 protein in knockdown cells by two fold when compared to scrambled knockdown cells. GAPDH was used as a loading control. Densitometry data are represented as a mean ± SD for three independent experiments. (*) indicates significant differences ($p < 0.05$) between treatment and control.

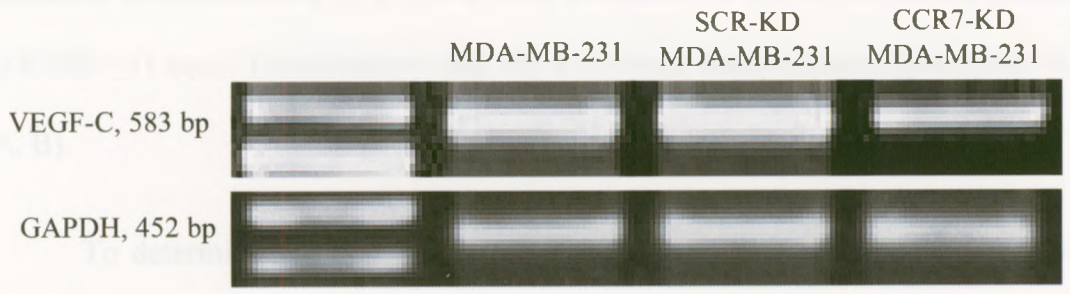

MDA-MB-231
SCR-KD MDA-MB-231
CCR7-KD MDA-MB-231
CCR7, 45 kDa
GAPDH, 38 kDa

A)

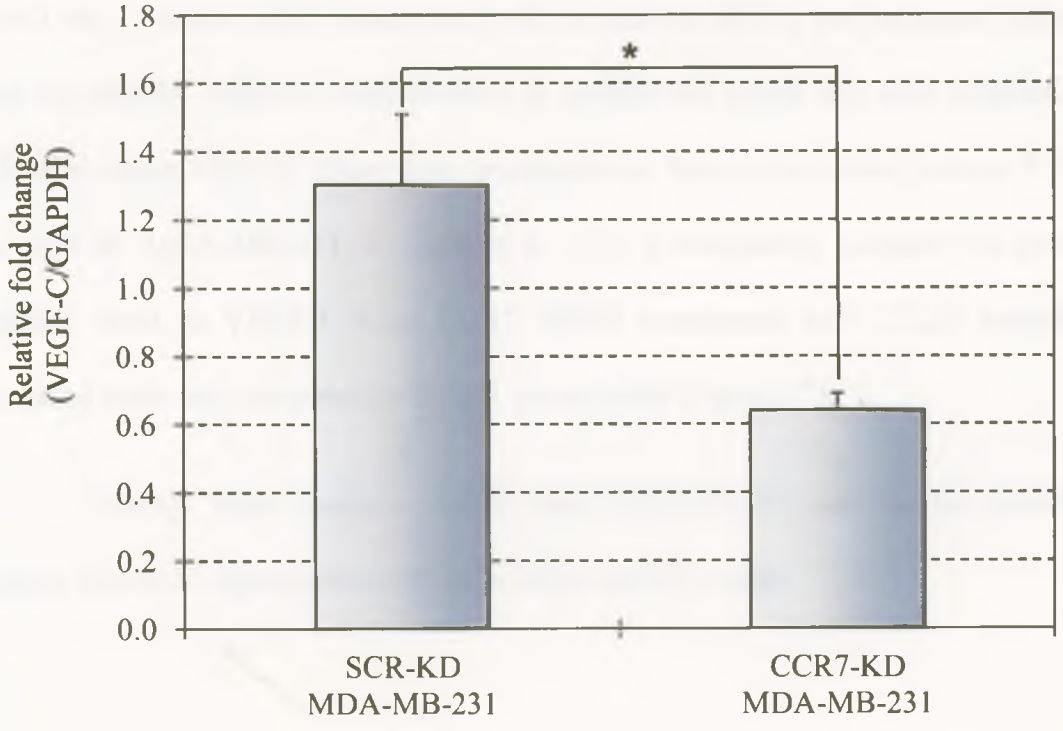

Ratio CCR7/GAPDH
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
MDA-MB-231
SCR-KD MDA-MB-231
CCR7-KD MDA-MB-231

B)

Figure 15. siRNA Against Human CCR7 Results in Low Levels of VEGF-C mRNA Expression. MDA-MB-231 cells transiently transfected with CCR7 siRNA or negative control siRNA at 6 μM concentration were harvested 48 hours post nucleofection and total RNA was extracted. Gene expression of the lymphangiogenic factor VEGF-C was determined by A) semi-quantitative real-time PCR and **B)** quantitative real-time PCR. Real-time PCR analysis revealed that low VEGF-C mRNA expression in CCR7 transfected cells translates in correspondingly reduced band intensity when compared with control, transfected cells. Also, a two fold change in VEGF-C mRNA expression was observed in CCR7 knockdown cells with respect to control. Results for quantitative real-time PCR are presented in relative fold change ($2^{-\Delta\Delta Ct}$) normalized to GAPDH. Data are represented as a mean ± SD for three independent experiments. (*) indicates significant differences ($p < 0.05$) relative to control.


MDA-MB-231
SCR-KD MDA-MB-231
CCR7-KD MDA-MB-231
VEGF-C, 583 bp
GAPDH, 452 bp

A)


Relative fold change (VEGF-C/GAPDH)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
SCR-KD MDA-MB-231
CCR7-KD MDA-MB-231

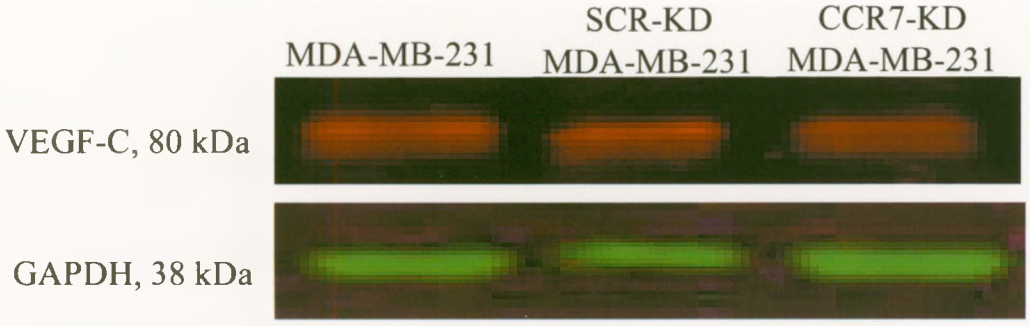
B)

48 hours post nucleofection and cell lysates were prepared and analyzed by Western blot and densitometry. Intracellular VEGF-C protein expression was detected at 80 kDa. GAPDH was used as an internal control. The protein level of VEGF-C in CCR7 knockdown cells decreased by 2.6 fold when compared to control scrambled knockdown MDA-MB-231 cells. Densitometry data are from three independent experiments (Figure 16A, B).

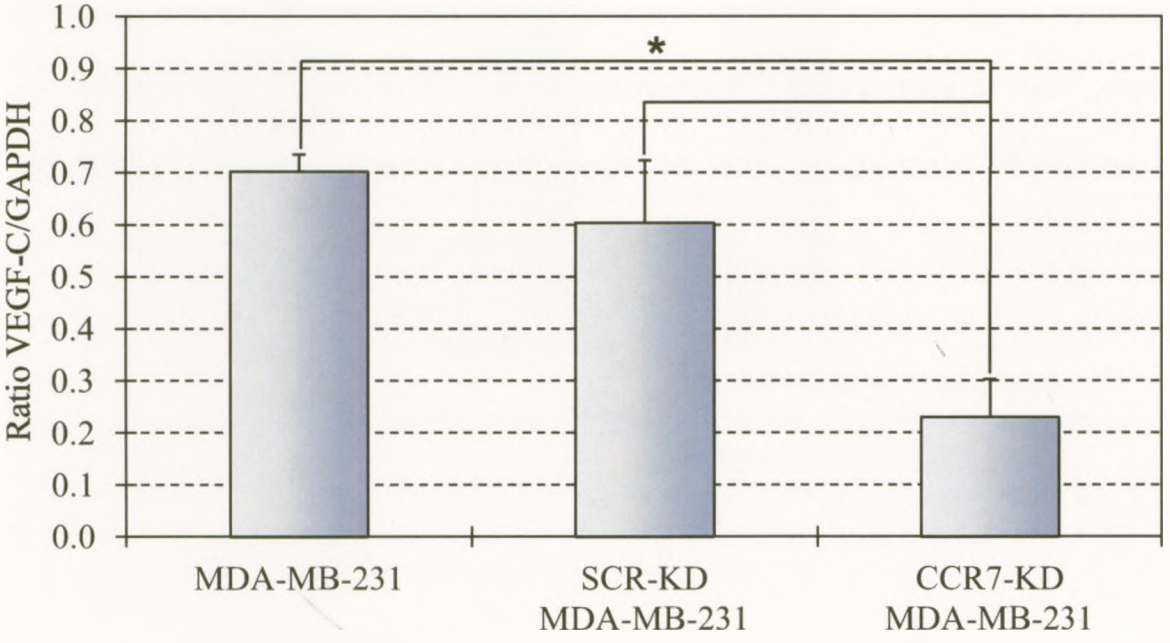
To determine whether the CCL21/CCR7 interaction regulates the secretion of lymphangiogenic factor VEGF-C, MDA-MB-231 cells transiently transfected with CCR7 siRNA were cultured in serum-free media and treated with human CCL21/6Ckine (350 ng/ml) for 24 hours. Cells transfected with scrambled siRNA and untreated cells were used as controls. VEGF-C concentration in conditioned media was then measured with ELISA by using VEGF-C Quantikine Immunoassay. Relative to resting culture, VEGF-C secretion by MDA-MB-231 in response to CCL21 stimulation increase two fold. The secretion level of VEGF-C from CCR7 siRNA transfected and CCL21 treated cells decreased three fold compared to CCL21 treated cells (Figure 17).

Overall, these findings suggest that CCL21/CCR7 pair has the potential to regulate VEGF-C expression/secretion in MDA-MB-231 cells.

Figure 16 siRNA Against Human CCR7 Results in Low Levels of VEGF-C Protein Expression. MDA-MB-231 cells transiently transfected with either silencer CCR7 siRNA or negative control siRNA were harvested 48 hours post-nucleofection. Protein sample were collected, separated by 10% SDS-PAGE and analyzed by: **A)** Western blot and **B)** densitometry. VEGF-C protein levels in knockdown cells decreased by 2.6 fold when compared to control, scrambled knockdown cells. GAPDH was used as a loading control. Densitometry data are represented as a mean ± SD for 3 independent experiments. (*) indicates significant differences ($p < 0.05$) between treatment and control.

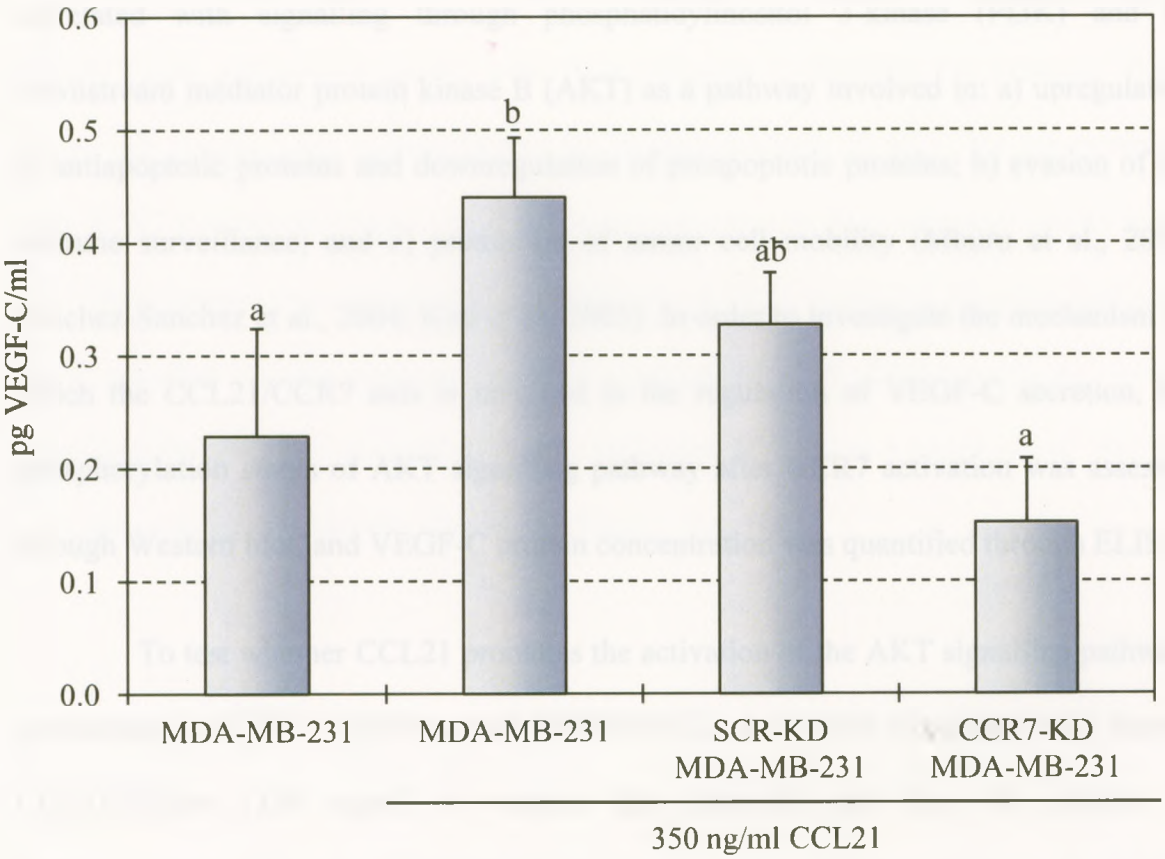

MDA-MB-231
SCR-KD MDA-MB-231
CCR7-KD MDA-MB-231
VEGF-C, 80 kDa
GAPDH, 38 kDa

A)


Ratio VEGF-C/GAPDH
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
MDA-MB-231
SCR-KD MDA-MB-231
CCR7-KD MDA-MB-231

B)

Figure 17. CCL21/CCR7 Chemokine Pair Regulates VEGF-C Protein Secretion. MDA-MB-231 cells transiently transfected with CCR7 siRNA or negative control siRNA were cultured in serum-free media and treated with human CCL21/Ckine (350 ng/ml) for 24 hours. The level of VEGF-C protein secretion was then measured through ELISA by using human VEGF-C Quantikine Immunoassay. The optical density of each well was determined using a plate reader by substracting the reading at 570 nm from the reading at 450 nm. VEGF-C content of CCR7 siRNA transfected cells decreased by three fold when compared to the control group. Data are represented as a mean $\pm$ SD ($n = 3$). Different superscripts indicate statistically significant differences, while shared superscripts are assimilated with no statistical differences ($p < 0.05$).


0.6
0.5
0.4
0.3
0.2
0.1
0.0
pg VEGF-C/ml
a
b
ab
a
MDA-MB-231
MDA-MB-231
SCR-KD
MDA-MB-231
CCR7-KD
MDA-MB-231
350 ng/ml CCL21

3.4 CCR7 Activation Regulates VEGF-C Secretion via the AKT Signalling Pathway

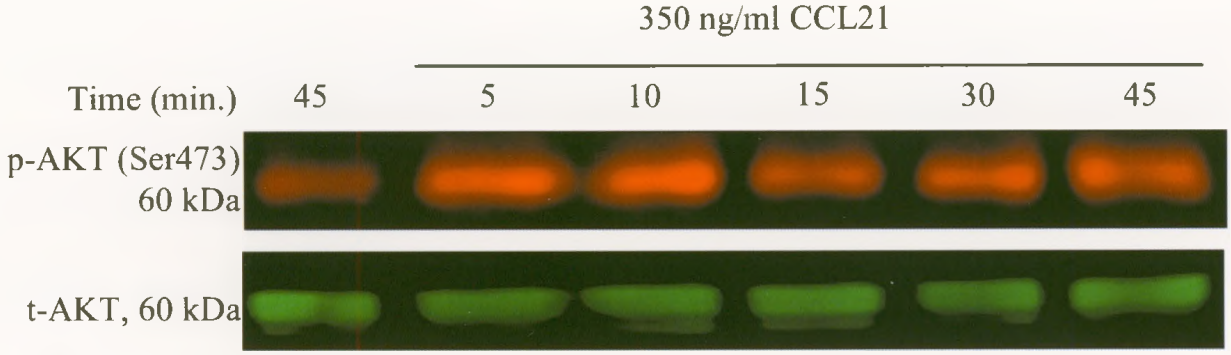
The working hypothesis tested was that the CCL21/CCR7 axis regulates VEGF-C secretion through the AKT signalling pathway. CCR7 activation has been positively correlated with signalling through phosphatidylinositol 3-kinase (PI3K) and its downstream mediator protein kinase B (AKT) as a pathway involved in: a) upregulation of antiapoptotic proteins and downregulation of proapoptotic proteins; b) evasion of the immune surveillance; and c) promotion of tumor cell mobility (Mburu et al., 2006; Sanchez-Sanchez et al., 2004; Kim et al., 2005). In order to investigate the mechanism by which the CCL21/CCR7 axis is involved in the regulation of VEGF-C secretion, the phosphorylation status of AKT signalling pathway after CCR7 activation was assessed through Western blot, and VEGF-C protein concentration was quantified through ELISA.

To test whether CCL21 promotes the activation of the AKT signalling pathway, downstream of CCR7, serum-starved MDA-MB-231 cells were stimulated with human CCL21/6Ckine (350 ng/ml) at various time intervals and then the amount of phosphorylated AKT was analyzed within the cell lysates. Phosphorylation of AKT at Ser 473 increased after five minutes of stimulation with CCL21. An increase in the phosphorylation status of AKT was evident for the entire duration of stimulation, suggesting that the CCL21 chemokine promotes AKT activation. Phosphorylation of AKT was also observed in untreated cells, a faint band in the lane containing cell lysates under serum-free conditions, suggesting the existence of an autocrine signalling loop.

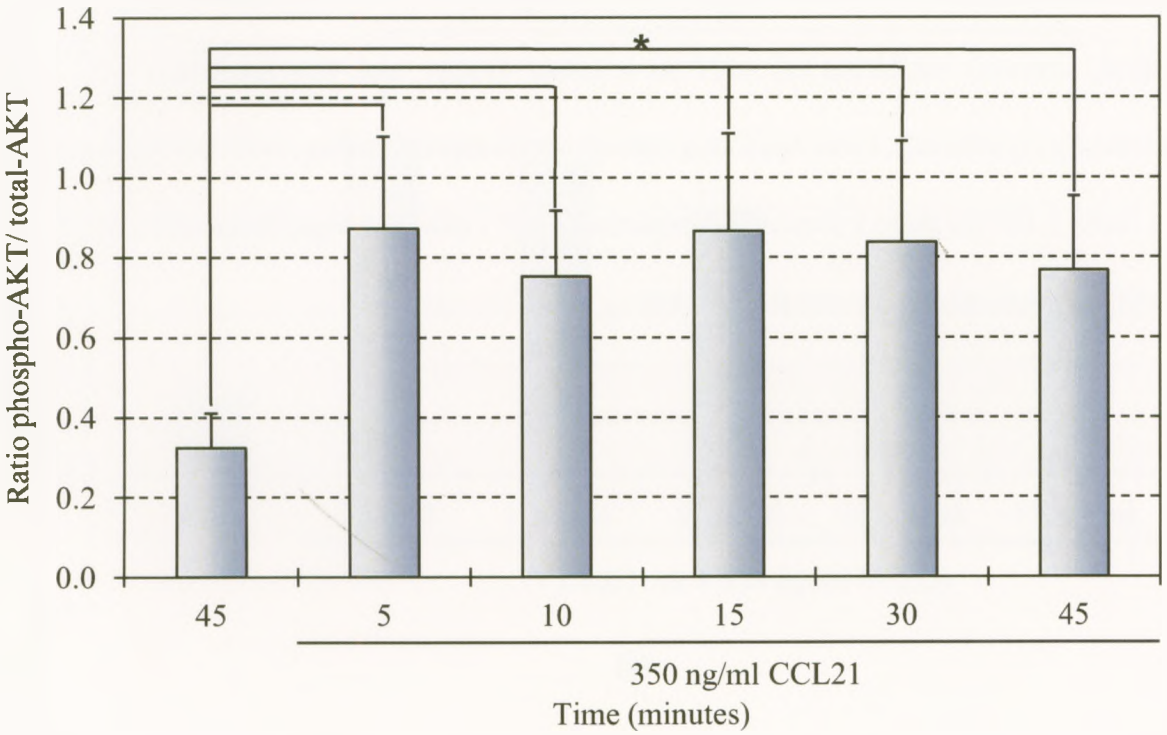
Densitometry data revealed that AKT reached maximum phosphorylation at five minutes of stimulation compared to the control level (Figure 18A, B).

To verify whether CCR7 antibody blocks CCL21 induced AKT activation, serum-starved MDA-MB-231 cells were pre-treated with various concentrations of CCR7 neutralizing antibody (1, 5, 10, 15 μg/ml) before stimulation with CCL21/6Ckine (350 ng/ml). Equal amounts of protein were then subjected to Western blot analysis. When whole cell lysates were immunoblotted for phosphorylation of AKT, the pre-treatment with CCR7 specific MAb was associated with a decrease in AKT phosphorylation at Ser 473. Densitometry data confirmed that inhibition of CCL21-induced phosphorylation of AKT occurred in the presence of high concentration of CCR7 antibody. Lower concentration of CCR7 antibodies showed inhibition of AKT to a lesser extent, and this inhibition was not statistically significant (Figure 19A, B).

Figure 18. CCL21 Induces Phosphorylation of AKT at Ser 473 in MDA-MB-231 cells. Cells were serum starved for 24 hours before stimulation with human CCL21/6Ckine (350 ng/ml) for various amounts of time (5, 10, 15, 30, and 45 minutes). Cell lysates were then prepared and analyzed by: A) Western Blot and B) densitometry. Western blot analysis of MDA-MB-231 total cell lysates detected AKT at 60 kDa. Phosphorylation of AKT was observed over the entire duration of stimulation. Densitometry data revealed that phosphorylation of AKT was significantly induced at five minutes of stimulation, and this effect was sustain over the entire duration of stimulation. Total AKT confirmed the equivalent loading of lanes. Data are represented as a mean ± SD for three independent experiments. (*) indicates significant differences ($p < 0.05$) between treatments and control.

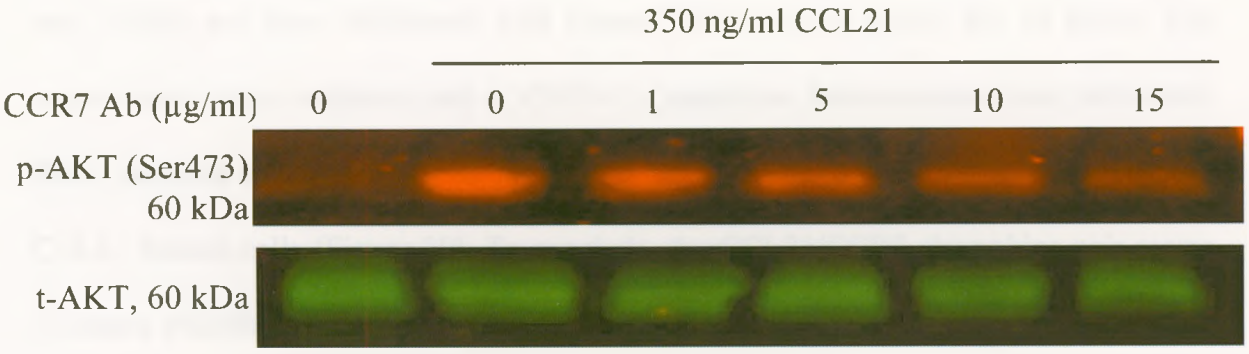

350 ng/ml CCL21
Time (min.)
45
5
10
15
30
45
p-AKT (Ser473)
60 kDa
t-AKT, 60 kDa

A)

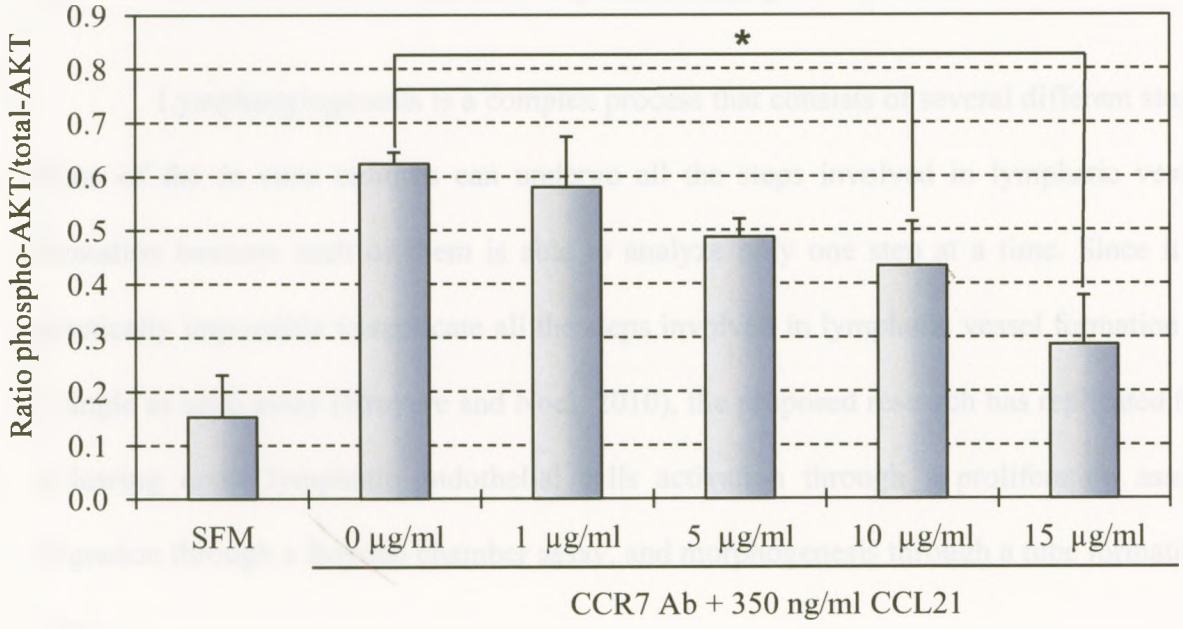

Ratio phospho-AKT/ total-AKT
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
45
5
10
15
30
45
350 ng/ml CCL21
Time (minutes)

B)

Figure 19. CCR7 Antibody Inhibits CCL21-Induced Phosphorylation of AKT in MDA-MB-231 Cells. Serum-starved MDA-MB-231 cells were pre-treated with varying concentration of monoclonal anti-human CCR7 neutralizing antibodies (1, 5, 10, 15 μg/ml) for two hours before stimulation with human CCL21/6Ckine (350 ng/ml). Cell lysates were prepared and analyzed by: **A)** Western Blot and **B)** densitometry. CCL21-induced AKT phosphorylation of MDA-MB-231 cells was inhibited in the presence of high concentrations of CCR7 antibody (10, 15 μg/ml). Lower concentrations of CCR7 antibody showed inhibition of AKT to a lesser extent, and this inhibition was not statistically significant. Total AKT was used to verify equal loading. Data are represented as a mean ± SD for three independent experiments. (*) indicates significant differences ($p < 0.05$) between treatment and stimulation.


350 ng/ml CCL21
CCR7 Ab (µg/ml)
0
0
1
5
10
15
p-AKT (Ser473)
60 kDa
t-AKT, 60 kDa

A)


Ratio phospho-AKT/total-AKT
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
SFM
0 µg/ml
1 µg/ml
5 µg/ml
10 µg/ml
15 µg/ml
CCR7 Ab + 350 ng/ml CCL21

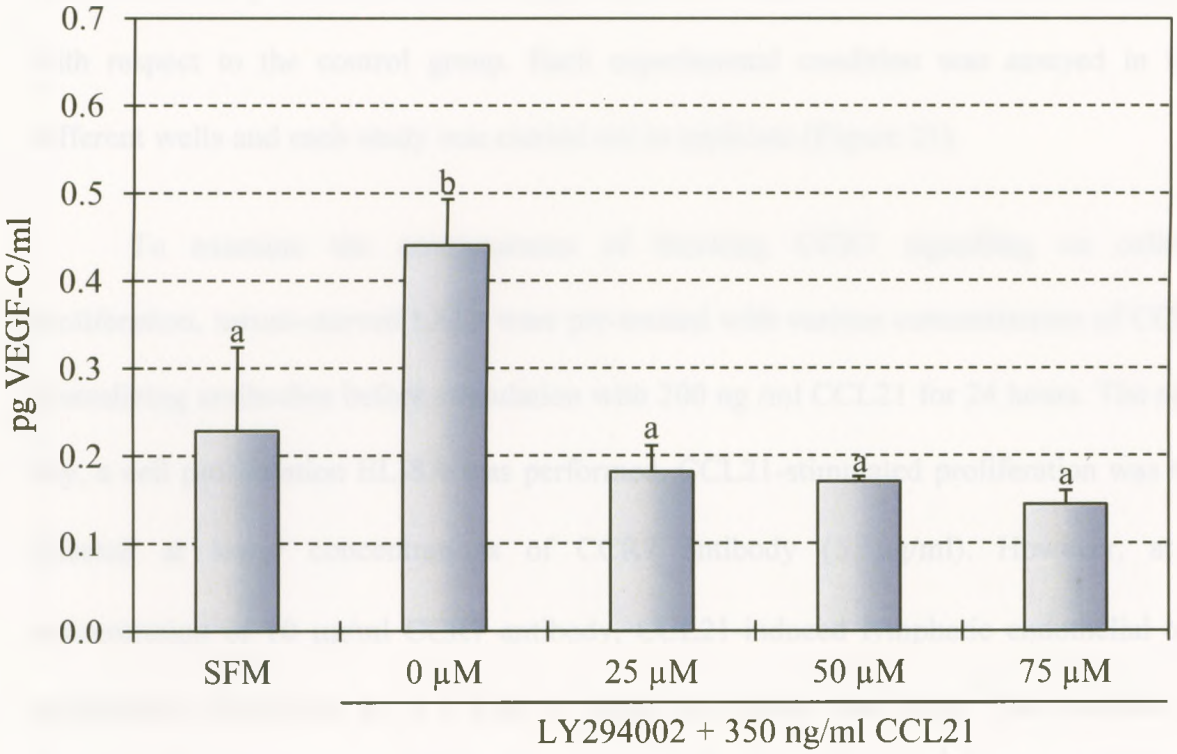
B)

Finally, the functional role of the AKT signalling pathway in the induction of VEGF-C secretion was investigated. For this purpose, serum-starved MDA-MB-231 cells were pre-treated with various concentrations of PI3 kinase inhibitor LY294002 (0, 25, 50, and 75μM) and then stimulated with human CCL21 (350 ng/ml) for 24 hours. The supernatants were collected and a VEGF-C Quantikine Immunoassay was performed. PI3K inhibitor reduced VEGF-C protein secretion by three fold when compared with CCL21 treated cells (Figure 20). To conclude, the CCL21/CCR7 chemokine pair seems to induce phosphorylation of AKT in MDA-MB-231 cells. These results also support that the activation of PI3K/AKT signalling pathway downstream of CCR7 is involved in the regulation of VEGF-C secretion.

3.5 CCL21/CCR7 Axis Has Lymphangiogenic Potential *in Vitro*

Lymphangiogenesis is a complex process that consists of several different steps. None of the *in vitro* cultures can undergo all the steps involved in lymphatic vessel formation because each of them is able to analyze only one step at a time. Since it is practically impossible to replicate all the steps involved in lymphatic vessel formation in a single *in vitro* assay (Bruyere and Noel, 2010), the proposed research has replicated the following ones: lymphatic endothelial cells activation through a proliferation assay, migration through a Boyden chamber assay, and morphogenesis through a tube formation assay.

Figure 20. CCL21/CCR7 Chemokine Pair Modulates VEGF-C Secretion via AKT Signalling Pathway. Serum starved MDA-MB-231 cells were treated with varying concentrations of PI3K inhibitor LY294002 (0, 25, 50, and 75μM). After two hours incubation, PI3K-treated cells were stimulated with human CCL21/6Ckine (350 ng/ml) for 24 hours. The supernatants were then collected for VEGF-C Quantikine ELISA assay. PI3K inhibitor reduced VEGF-C protein secretion by three fold compared to untreated cells (SFM). Data are represented as a mean ± SD (n = 3). Different superscripts indicate statistically significant differences ($p < 0.05$).


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
pg VEGF-C/ml
a
b
a
a
a
SFM
0 µM
25 µM
50 µM
75 µM
LY294002 + 350 ng/ml CCL21

BrdU colorimetric assay was performed to determine the effect of CCL21 chemokine on HMVEC-dLy proliferation. Serum-starved HMVEC-dLy cells were treated with various concentrations of human CCL21/6Ckine for 24 hours before quantification. The number of proliferating cells increased by 1.7 fold after stimulation. The outcome is presented as percentages of BrdU incorporation and data are normalized with respect to the control group. Each experimental condition was assayed in five different wells and each study was carried out in triplicate (Figure 21).

To examine the consequences of blocking CCR7 signalling on cellular proliferation, serum-starved LECs were pre-treated with various concentrations of CCR7 neutralizing antibodies before stimulation with 200 ng /ml CCL21 for 24 hours. The next day, a cell proliferation ELISA was performed. CCL21-stimulated proliferation was not affected at lower concentrations of CCR7 antibody (5 μg/ml). However, at a concentration of 10 μg/ml CCR7 antibody, CCL21-induced lymphatic endothelial cell proliferation decreased by 1.9 fold, to reach the serum free level. The outcome is presented as percentages of BrdU incorporation and data are normalized with respect to the control group. Each experimental condition was assayed in five different wells and each study was carried out in triplicate (Figure 22). From these findings, it can be inferred that CCL21 promotes HMVEC-dLy cell proliferation.

Figure 21. CCL21 Promotes HMVEC-dLy Proliferation. Serum-starved HMVEC-dLy cells were plated onto 96-well tissue-culture microplates and stimulated with varying concentrations of human CCL21/6Ckine (0, 100, 200, 350 ng/ml) for 24 hours. After incubation, the quantification of cell proliferation was performed by the measurement of BrdU incorporation in newly synthesized cellular DNA (Cell Proliferation ELISA assay). The number of proliferating cells increased by 1.7 fold. Each experimental condition was assayed in five different wells and the study was carried out in triplicate. Data are presented as mean ± SD. Different superscripts indicate statistically significant differences ($p < 0.05$) between treatments and control.

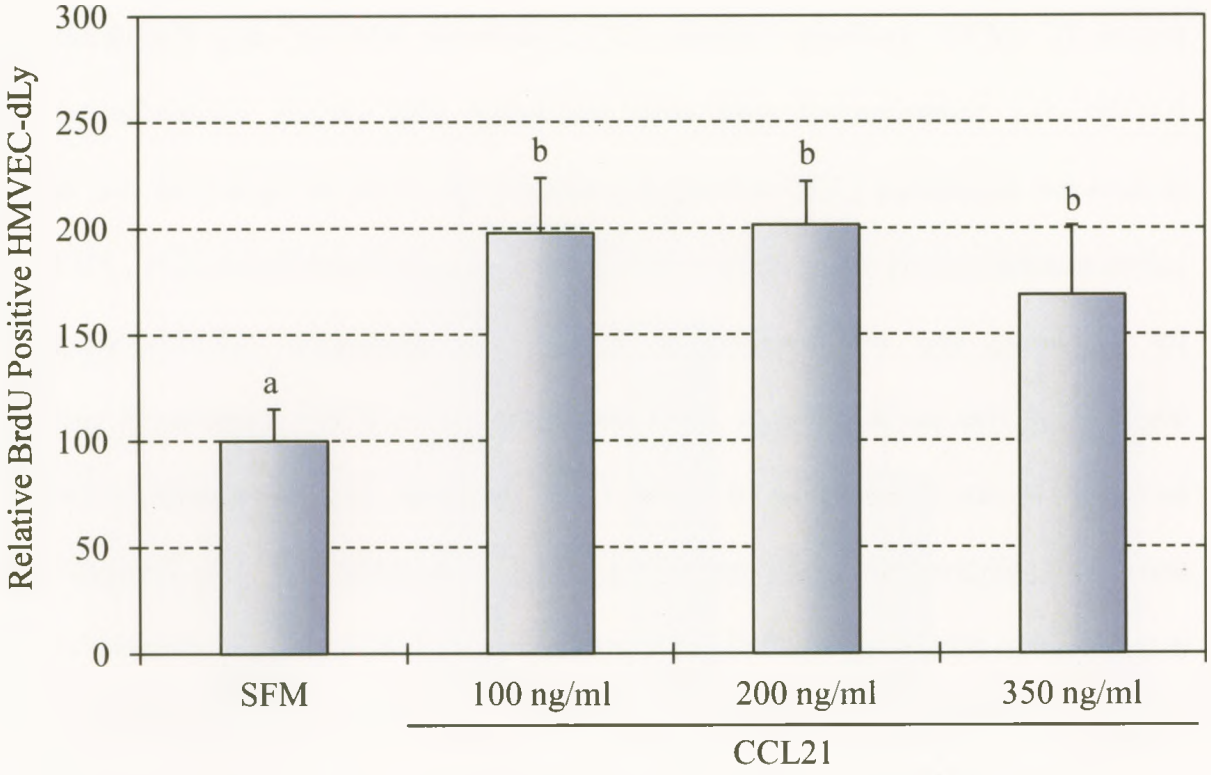
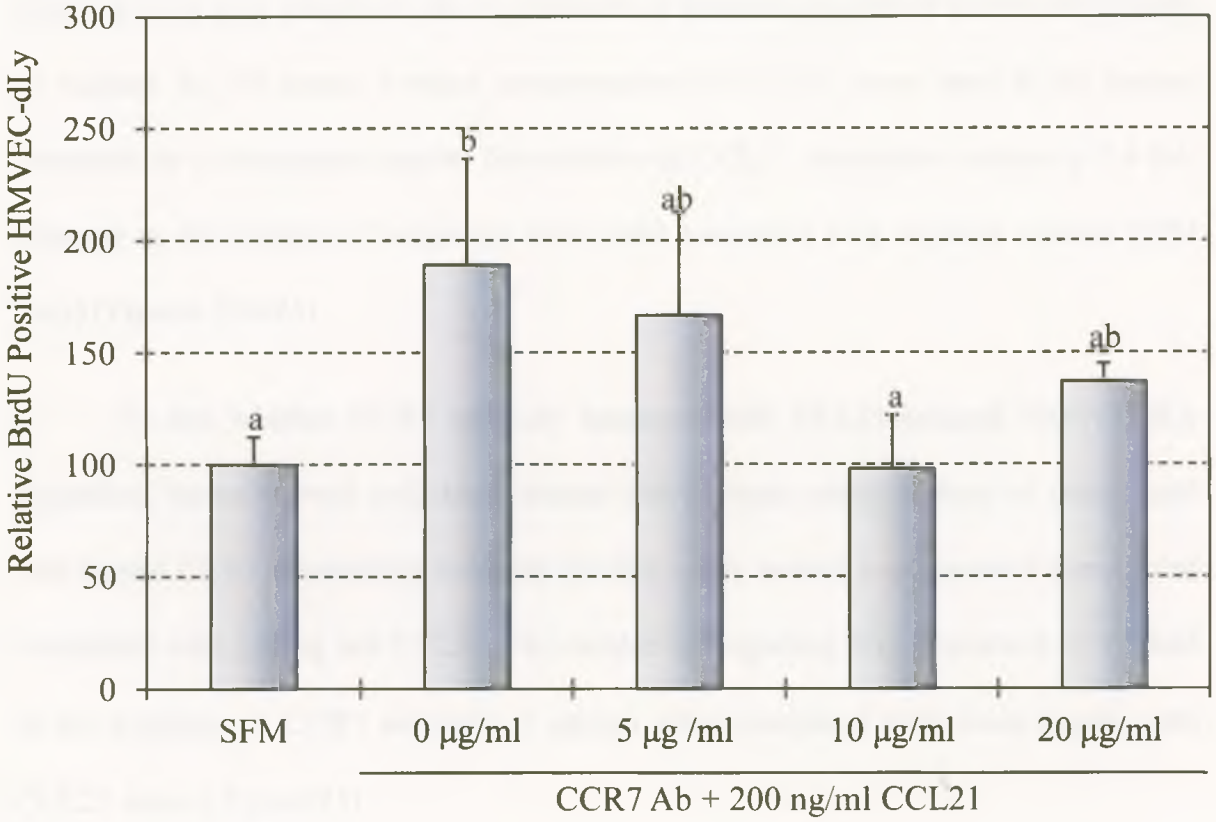

Relative BrdU Positive HMVEC-dLy
300
250
200
150
100
50
0
a
b
b
b
SFM
100 ng/ml
200 ng/ml
350 ng/ml
CCL21

Figure 22. CCR7 Antibody Inhibits CCL21-induced HMVEC-dLy Proliferation. HMVEC-dLy serum-starved cells were pre-treated with varying concentrations of monoclonal anti-human CCR7 neutralizing antibody (0, 5, 10, 20 μg/ml) for two hours before stimulation with 200 ng/ml human CCL21. Cells were incubated at 37°C, 5% CO_2 for 24 hours and cell proliferation ELISA was performed. CCL21-stimulated proliferation was not affected at lower concentrations of CCR7 antibody (5 μg/ml). However, in the presence of 10 μg/ml CCR7 antibody, CCL21-induced lymphatic endothelial cell proliferation decreased by 1.9 fold, to the serum free level. The outcome is presented as percentages of BrdU incorporation normalized to the control group. Each experimental condition was assayed in five different wells and each study was carried out in triplicate. Data are presented as mean ± SD. Different superscripts indicate statistically significant differences, while shared superscripts are assimilated with no statistical differences ($p < 0.05$).


Relative BrdU Positive HMVEC-dLy
300
250
200
150
100
50
0
a
b
ab
a
ab
SFM
0 µg/ml
5 µg /ml
10 µg/ml
20 µg/ml
CCR7 Ab + 200 ng/ml CCL21

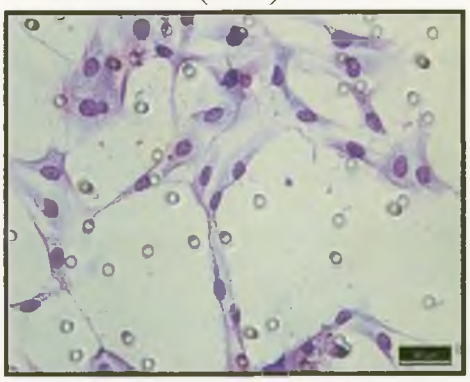
To examine the effect of CCL21 chemokine on lymphatic endothelial cell migration, transwell migration assays were performed. Initially, dose and time response to CCL21 was determined. Following the time course experiments, the chemotactic response of lymphatic endothelial cells to CCL21 was then analyzed. Serum-starved HMVEC-dLy were placed on the top chamber of transwell migration inserts and allowed to migrate for 24 hours. Various concentrations of CCL21 were used in the bottom chambers as a chemotactic agent. The addition of CCL21 chemokine induced a 2.6 fold increase in the number of migratory cells when compared with negative control (SFM only) (Figures 23&24).

To test whether CCR7 antibody interferes with CCL21-induced HMVEC-dLy migration, serum-starved cells were treated with various concentrations of monoclonal anti-human CCR7 neutralizing antibody for two hours, seeded into transwell inserts, and stimulated with 350 ng /ml CCL21. The number of migrating cells decreased by 2.5 fold in the presence of CCR7 antibody (5 μg/ml) when compared with those treated with CCL21 alone (Figure 25).

Taken together, these results suggest that lymphatic endothelial cells are capable of responding to the chemokine gradients and migrate. CCR7 antibody is a potent inhibitor of CCL21 induced HMVEC-dly migration.

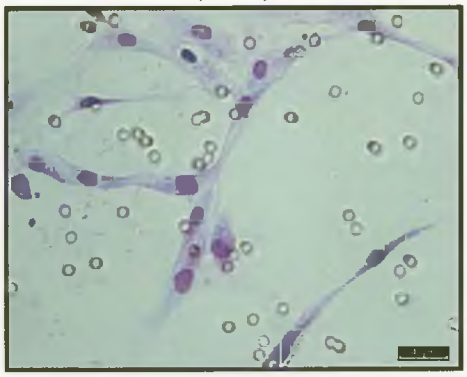
Figure 23. Representative Images of the Migration Slices Obtained After 24 Hours Incubation. HMVEC-dLy cells under different treatments were placed on the top chamber of transwell migration inserts and allowed to migrate for 24 hours. Direct microscopic counting of the lymphatic endothelial cells that have migrated to the lower side of the membrane after eosin-thiasine staining was performed. Three independent experiments with each condition tested in triplicate were conducted. For each sample, cells in 10 randomly chosen high power fields were counted and a mean value for each sample was calculated. Microphotographs were taken at 40X magnification.

HMVEC-dLy
(40X)

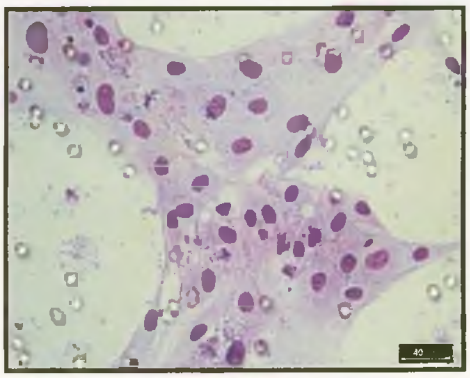


Control: SFM

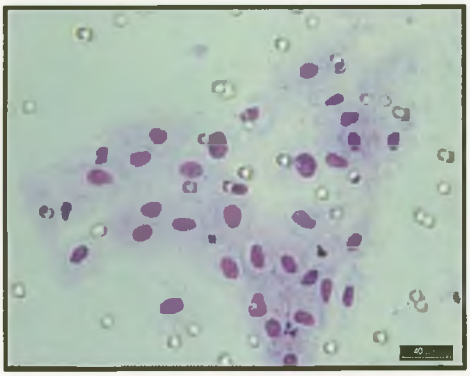
HMVEC-dLy
(40X)



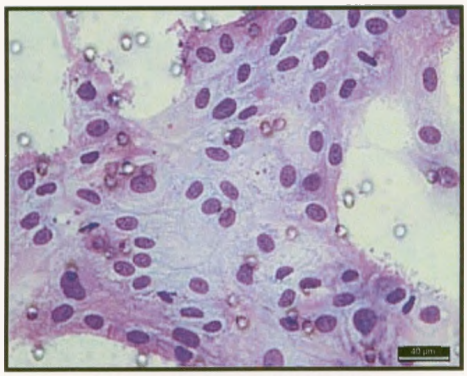
Inhibition: CCR7 Ab (5 µg/ml) +
CCL21 (350 ng/ml)



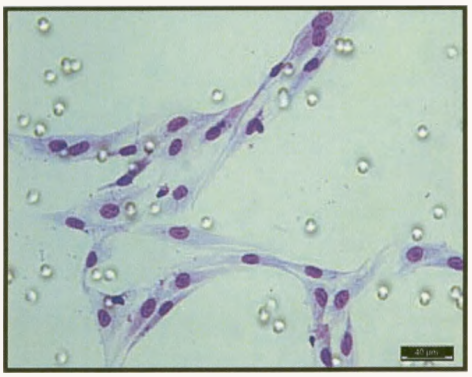
Stimulation: CCL21
(200 ng/ml)



Inhibition: CCR7 Ab (10 µg/ml)+
CCL21 (350 ng/ml)



Stimulation: CCL21
(350 ng/ml)



Inhibition: CCR7 Ab (20 µg/ml) +
CCL21 (350 ng/ml)

Figure 24. CCL21 Stimulates HMVEC-dLy Cell Migration. Chemotaxis of HMVEC-dLy cells was assessed in transwell migration assay. Serum-starved cells were seeded in the upper chamber and increasing concentrations of human CCL21/6Ckine (0,100, 200, and 350 ng/ml) were added to serum-free media in the lower chamber. The assembled cell culture insert chambers were then incubated at 37°C, 5% CO_2 for 24 hours. Following ligand stimulation, lymphatic endothelial cells had a 2.6 fold higher capacity to migrate than un-stimulated cells. Migration data are represented as a mean ± SD for three independent experiments with each condition being tested in triplicate. Statistically significant differences ($p < 0.05$) in the means are indicated by different superscripts.

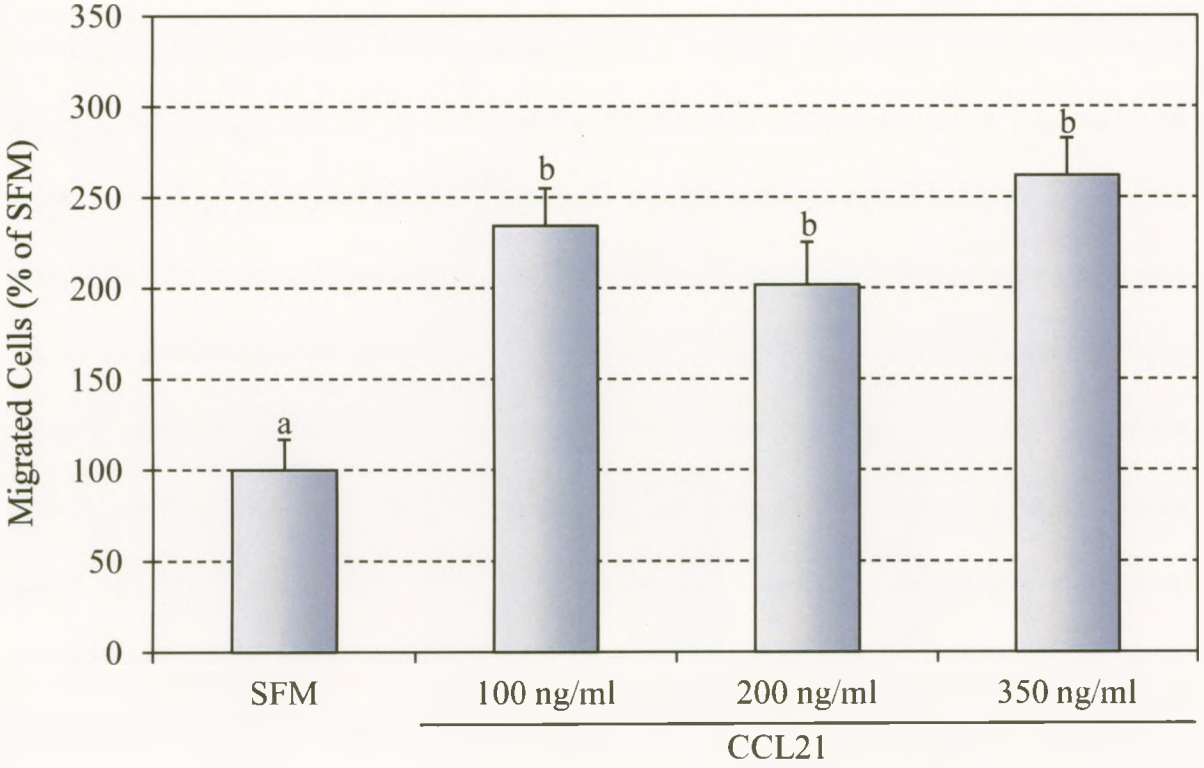
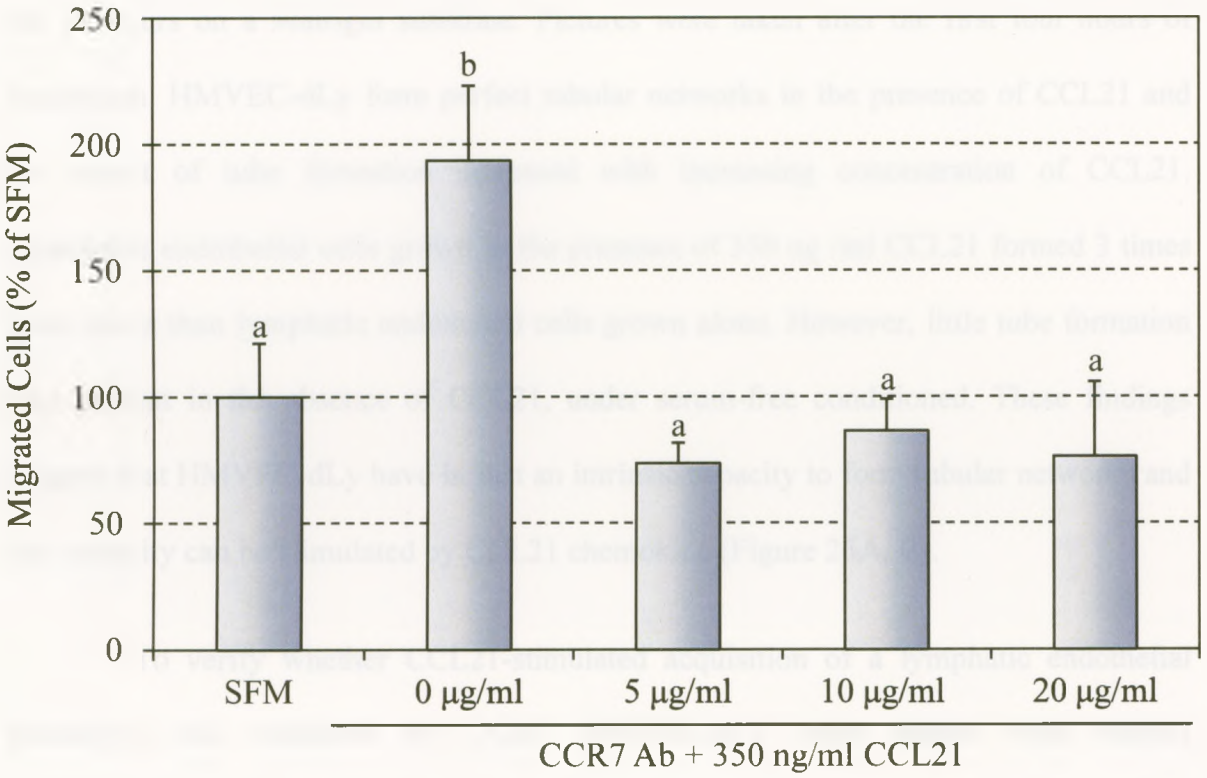

Migrated Cells (% of SFM)
350
300
250
200
150
100
50
0
a
b
b
b
SFM
100 ng/ml
200 ng/ml
350 ng/ml
CCL21

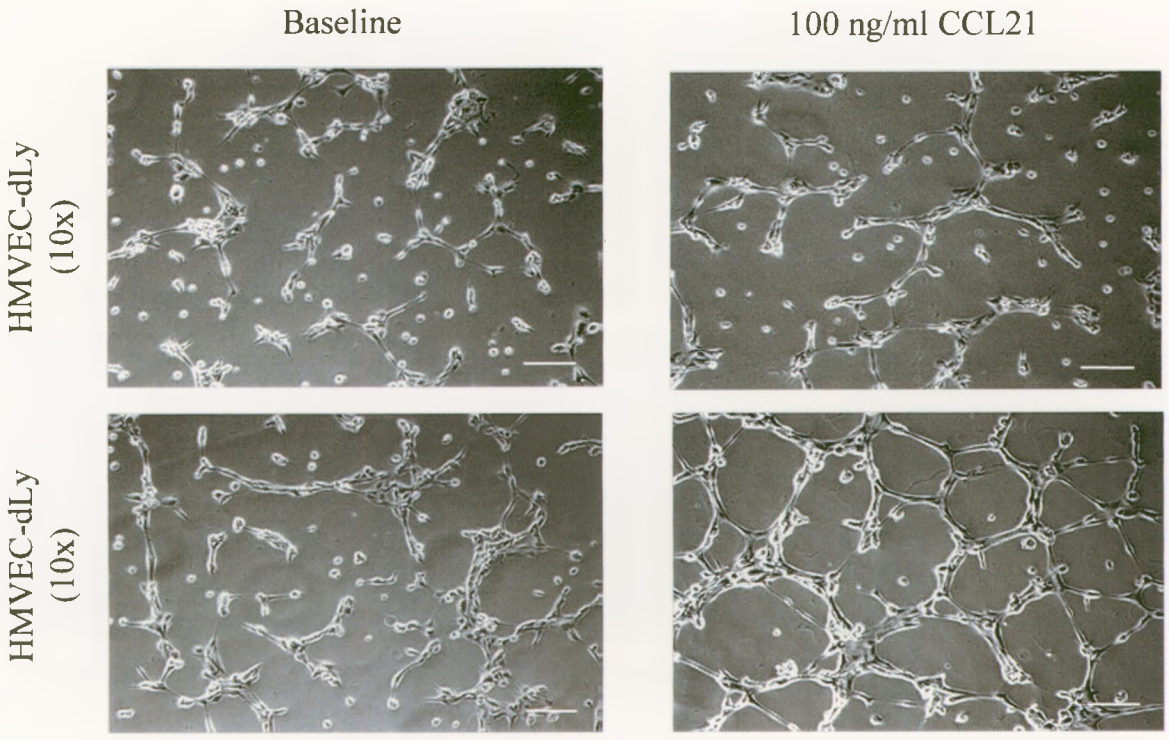
Figure 25. CCR7 Antibody Inhibits CCL21-Induced HMVEC-dLy Cell Migration. HMVEC-dLy were pre-treated with CCR7 antibody (0, 5, 10, 20 μg/ml) for 2 hours before being seeded in the upper chambers. Human CCL21/6Ckine (350 ng/ml) in serum-free media was added to the lower chambers. The assembled cell culture insert chambers were then incubated at 37°C, 5% CO_2 for 24 hours. All tested concentrations of CCR7 antibody decreased lymphatic endothelial cells migration almost to the same degree. However, peak inhibition was observed for 5 μg/ml CCR7 antibody, with a 2.5 fold migration reduction noticed. Migration data are represented as a mean ± SD for three independent experiments with each condition being tested in triplicate. Statistically significant differences ($p < 0.05$) in the means are indicated by different superscripts.


250
200
150
100
50
0
Migrated Cells (% of SFM)
a
b
a
a
a
SFM
0 µg/ml
5 µg/ml
10 µg/ml
20 µg/ml
CCR7 Ab + 350 ng/ml CCL21

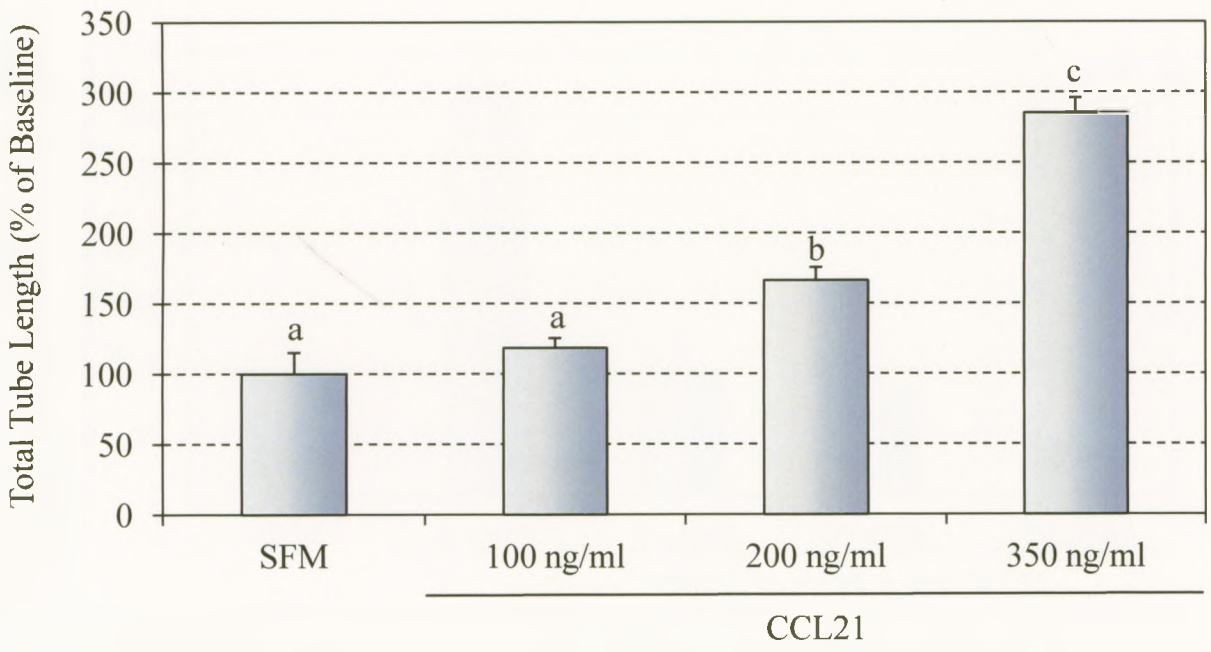
Lymphatic endothelial-like tube formation assay was performed to verify whether the CCL21/CCR7 pair has the ability to stimulate the formation of tube-like structures when cultured on a gel of a basement extract. HMVEC-dLy in serum free-media were treated with varying concentration of human CCL21/6Ckine and incubated for 24 hours on a Matrigel substrate. Pictures were taken after the first four hours of incubation. HMVEC-dLy form perfect tubular networks in the presence of CCL21 and the extent of tube formation increased with increasing concentration of CCL21. Lymphatic endothelial cells grown in the presence of 350 ng /ml CCL21 formed 3 times more tubes than lymphatic endothelial cells grown alone. However, little tube formation was present in the absence of CCL21, under serum-free conditioned. These findings suggest that HMVEC-dLy have in fact an intrinsic capacity to form tubular networks and this capacity can be stimulated by CCL21 chemokine (Figure 26A, B).

To verify whether CCL21-stimulated acquisition of a lymphatic endothelial phenotype was mediated by CCR7, HMVEC-dLy were treated with various concentrations of CCR7 neutralizing antibody for two hours before being stimulated with human CCL21/6Ckine (350 ng/ml) and incubated on a Matrigel substrate for 24 hours. Pictures were taken after four hours of incubation. Tubular network formation was blocked in the presence of CCR7 antibody, with a peak of inhibition at 20 μg/ml antibody. These results suggest that CCR7 antibody is a potent inhibitor of CCL21-induced lymphatic endothelial like tubular formation (Figure 27A, B).

Figure 26. CCL21 Stimulates Tubular Network Formation by HMVEC-dLy. HMVEC-dLy cells in serum free-media were treated with various concentration of human CCL21/6Ckine (0, 100, 200, 350, ng/ml) and incubated for 24 hours on a Matrigel substrate. **A)** Representative micrographs of tubular network formation were taken after the first four hours of incubation. **B)** Through quantification of the length of connected cells forming tubular structures it was inferred that tube formation increase with CCL21 concentration. Quantification of the length of tubular structures in at least three fields was determined using Image J. Total tube lengths are represented as a mean ± SD for three independent experiments. Statistically significant differences ($p < 0.05$) in the means are indicated by different superscripts.

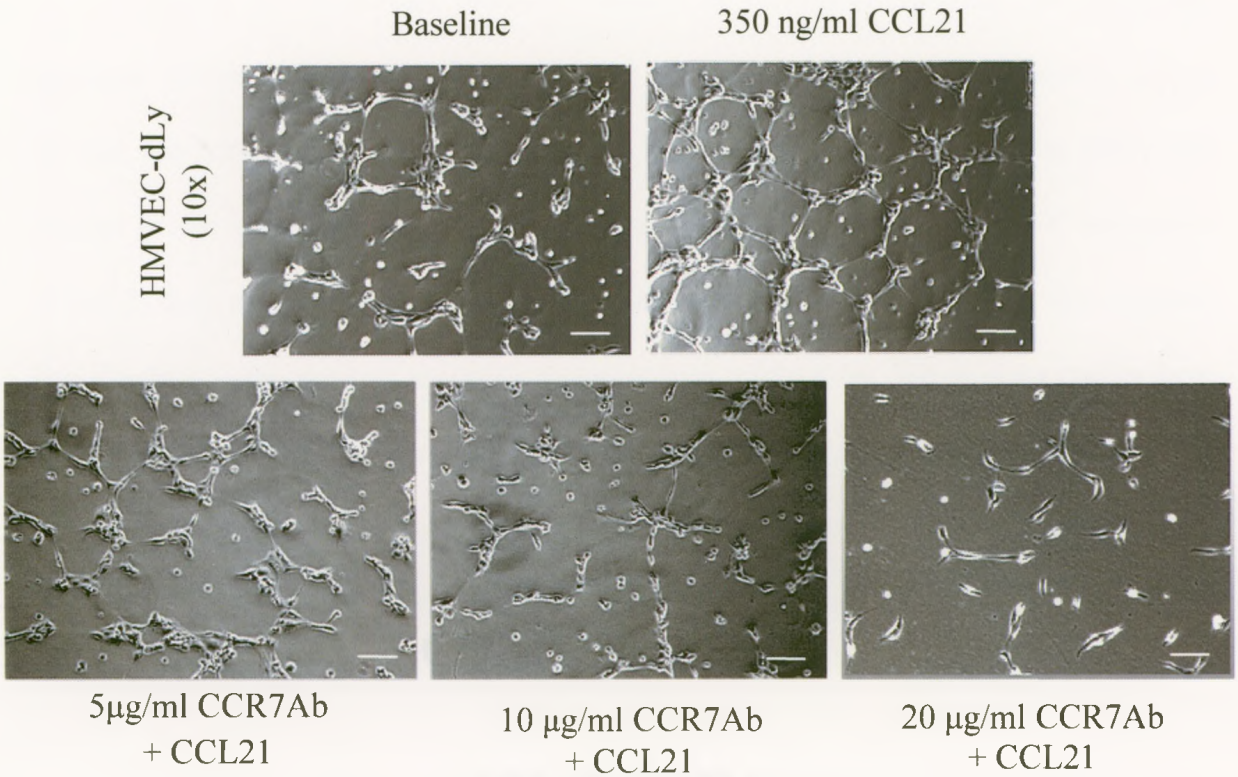

Baseline
100 ng/ml CCL21
HMVEC-dLy (10x)
HMVEC-dLy (10x)
200 ng/ml CCL21
350 ng/ml CCL21

A)

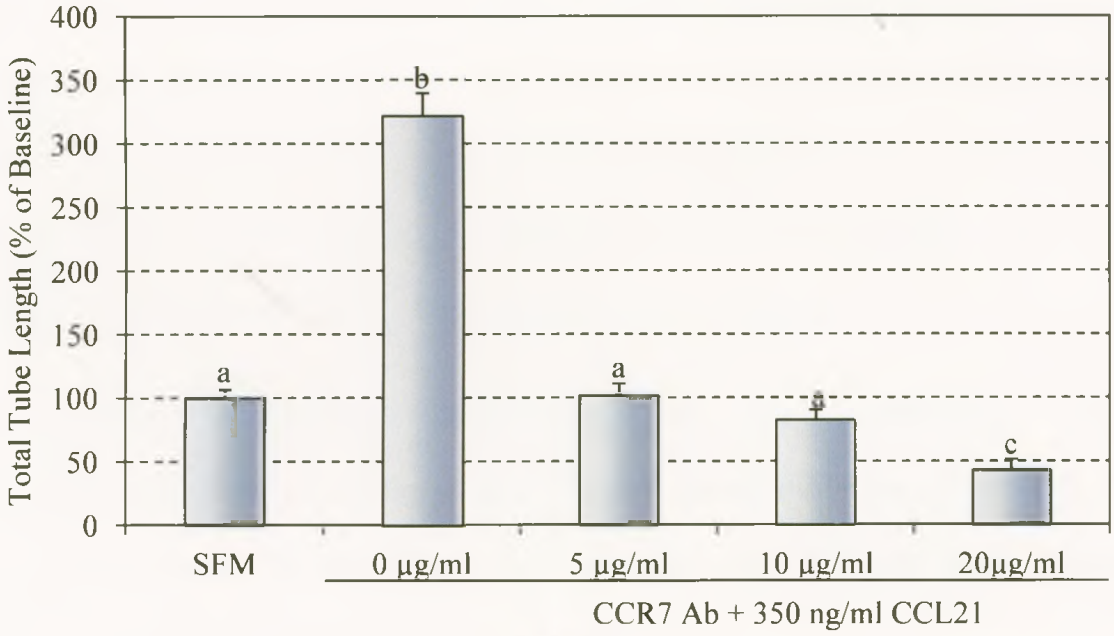

Total Tube Length (% of Baseline)
350
300
250
200
150
100
50
0
a
a
b
c
SFM
100 ng/ml
200 ng/ml
350 ng/ml
CCL21

B)

Figure 27. CCR7 Antibody Inhibits CCL21-Induced Tube Formation by HMVEC-dLy. Cells were treated with varying concentrations of CCR7 antibody (0, 5, 10, and 20 µg/ml) for 2 hours before stimulation with 350 ng/ml CCL21. Cells were seeded on six-well plates coated with Matrigel and incubated for 24 hours. **A**) Representative pictures of tubular network formation were taken after the first four hours of incubation. **B)** Tube formation decreased with increasing concentrations of CCR7 antibody, peaking at 20 µg/ml. Quantification of the length of tubular structures in at least three fields was determined using Image J. Total tube lengths are represented as a mean ± SD for three independent experiments. Statistically significant differences ($p < 0.05$) in the means are indicated by different superscripts.


Baseline
350 ng/ml CCL21
HMVEC-dLy
(10x)
5µg/ml CCR7Ab
+ CCL21
10 µg/ml CCR7Ab
+ CCL21
20 µg/ml CCR7Ab
+ CCL21

A)


Total Tube Length (% of Baseline)
400
350
300
250
200
150
100
50
0
a
b
a
a
c
SFM
0 µg/ml
5 µg/ml
10 µg/ml
20µg/ml
CCR7 Ab + 350 ng/ml CCL21

B)

CHAPTER FOUR:

DISCUSSION

4.1 Thesis Overview

Given the numerous potential inducers of lymphangiogenesis, the immediate intention of this study was to investigate the mechanism underlying the activity of yet another pro-lymphangiogenic factor, CCL21, in mediating VEGF-C secretion. Since past studies in our laboratory have shown that the inhibition of known VEGF-C promoters (COX-2) does not translate into absolute suppression of VEGF-C synthesis, this suggests that alternate/compensatory production mechanisms might exist. While prior studies have established that COX-2 expression by breast cancer cells regulates VEGF-C secretion via EP1/EP4 receptors (Timoshenko et al., 2006) and increases CCR7 expression via EP2/EP4 receptors (Pan et al., 2008), no direct connections were made between CCR7 signalling and VEGF-C expression/secretion.

Based on recent evidence outlining that: 1) the synergy between CCL21/CCR7 and VEGF-C/VEGFR-3 axes represents a major factor in breast cancer spread to distant sites (Shields et al., 2007; Issa et al., 2009); 2) CCR7 overexpression correlates with lymphatic vessel density and lymph node metastasis (Yu et al., 2008); 3) CCL21/CCR7 interaction constitutes a critical event in lymphangiogenesis associated with pancreatic cancer (Zhao et al., 2011), the present study proposed that CCR7 signalling constitutes a regulatory mechanism of VEGF-C mediated breast cancer-induced lymphangiogenesis.

In order to address this hypothesis, the study aimed to determine: 1) whether CCR7 expression plays a regulatory role in VEGF-C secretion by breast cancer cells; 2) the signalling mechanism underlying CCR7-mediated VEGF-C secretion; 3) whether CCL21/CCR7 pair has lymphangiogenic potential *in vitro*.

By employing a two-cell *in vitro* model, the present study established that siRNA-mediated knockdown of CCR7 gene suppressed VEGF-C expression/secretion in MDA-MB-231 breast cancer cells, indicating a direct role of CCR7 in VEGF-C synthesis. Furthermore, since the phosphorylation status of AKT increased after CCR7 activation and PI3K inhibitor effectively reduced VEGF-C protein secretion, it was asserted that PI3K/AKT signalling pathway is the intracellular mechanism of CCR7-mediated VEGF-C synthesis. Finally, the lymphangiogenic potential of CCL21/CCR7 axis on HMVEC-dly was demonstrated through a sequence of 2D assays, exogenous ligand-induced proliferation, migration, and tube formation, aiming to replicate important stages of the lymphangiogenic process.

4.2 Characterization of the CCR7 Chemokine Receptor Expression

Two cell lines were employed as an *in vitro* model for the present study. The MDA-MB-231 cell line, derived from the pleural effusions of a breast cancer patient and characterized by an invasive phenotype, was selected for its ability to express/secrete VEGF-C, thus making it suitable for use in this study. HMVECs-dLy, primary adult human dermal derived lymphatic endothelial cells, were identified as lymphatic cells via flow cytometry, being positive for podoplanin and CD31 but negative for smooth muscle alpha-actin.

In the current research, the expression of CCR7 by MDA-MB-231 and HMVEC-dLy cell lines was analyzed through flow cytometry, Western blot at the protein level, and by real-time PCR at the mRNA level. The corroboration of the results presented in Chapter 3 suggests that both MDA-MB-231 and HMVE-dLy cells express

CCR7 receptor at the protein and mRNA levels. This knowledge was essential for further determination of CCR7 role in the secretion of VEGF-C by breast cancer cells as well as for verification of the lymphangiogenic involvement of CCR7. Both phenomena are regarded as consequences of CCR7 activation following CCL21 binding.

CCR7 is a seven transmembrane-domain G protein coupled receptor with physiological role in immune cell migration, recruitment, and guidance towards draining lymph nodes. Under physiological conditions, CCR7 expression is largely controlled by the homeostatic balance between the cells and their surrounding microenvironment. Under pathological conditions, CCR7 expression was established as consistently upregulated both at the protein and mRNA levels in a distinct and non-random manner in a broad panel of breast cancer cell lines (Muller et al., 2001). More recently, flow cytometry analysis reiterated this finding in the context of fresh primary breast carcinoma cells (Cunningham et al., 2010). While CCR7 expression in various mammary tumor cells may vary, it is known that even low increases in the receptor expression can markedly affect cellular responses to ligand binding (Vines et al., 2002). Since CCR7 was typically found to be highly expressed in metastatic cell lines compared to low metastatic or normal mammary epithelial cells, a logical connection was made with the intense chemotactic and invasive activity involved in breast tumor dissemination.

While a wealth of studies showed that CCR7 is expressed by many types of tumor cells, this is not the case of CCR7 expression by LECs. In fact, the detection of chemokine receptors on human endothelial cells has been controversial (Salcedo et al., 1999) and so far, it was typically confined to CXCL12/CXCR4 chemokine axis involvement in tumor-induced angiogenesis associated with various types of

malignancies (Koshiba et al., 2000; Liu et al., 2008; Chu et al., 2009). The vast majority of studies in this category relied on human umbilical vascular endothelial cells (HUVECs) to perform their angiogenic assays. By contrast, a single study reported CCR7 expression by LECs in the context of breast cancer metastasis (Shields et al., 2007). However, the analysis was, in this case, limited to immunostaining performed on human dermal LECs isolated from neonatal foreskins.

4.3 Characterization of the CCL21 Chemokine Expression and Secretion

The wide physiological distribution of CCL21, combined with its involvement in immune cell colocalization within lymphatics, strongly endorsed CCL21's active role in metastatic spread of CCR7-positive breast tumor cells. This theory became even more outline after Muller et al. (2001) found abundant homeostatic expressions of CCL21 in lymph nodes, which explains the frequent incidence of lymph node metastases in mammary carcinomas.

In the present study, CCL21 expression and secretion by MDA-MB-231 and HMVEC-dLy cells were analyzed at the protein and mRNA levels. Western blot analysis of MDA-MB-231 total cell lysates detected CCL21 at 12 kDa while only traces were observed in HMVEC-dLy. Moreover, CCL21 protein secretion in conditioned media was quantified through ELISA and the same trend was observed. For generation of conditioned media, cells in basal media were placed on a growth factor-reduced Matrigel matrix. In this experiment, Matrigel was used because, unlike collagen, it has a rich

content in sulfated proteoglycans (Kleinman and Martin, 2005) and this can be exploited further for matrix-binding purposes of CCL21, due to its known affinity to proteoglycans.

One important aspect to be emphasized is that CCL21 chemokine ligand is secreted as a small molecular weight protein that is readily immobilized within the extracellular matrix by binding to sulfated proteoglycans (Patel et al., 2001). In fact, the interaction of the chemokines with glycosaminoglycans (GAGs) seems to influence their functionalities in several different ways: i) cell surface retention of chemokines by means of GAGs facilitates generation of high localized concentrations of chemokines with role in directional signalling (Johnson et al., 2005); ii) chemokine oligomerization through GAG binding is important for their *in vivo* activation (Johnson et al., 2004); iii) chemokine/GAG binding determines selective presentation of chemokines to their receptors (Netelenbos et al., 2002); and iv) chemokine/GAG binding enables chemokine protection from enzyme degradation (Sadir et al., 2004).

As such, it was no surprise that the determined bound CCL21 protein fraction was about three fold higher than the soluble fraction. This finding is also consistent with the physiological scenario in which CCL21 is secreted by lymphatics directly into the basement membrane. This data has been confirmed by Shields et al. (2007), who established that tumor cells can generate autologous gradients of CCR7 ligand by secreting it into the extracellular matrix under the influence of slow interstitial flow. Based on this, they have also suggested that CCL21 secretion by tumor cells themselves rather than and/or in addition to their lymphatic production might be targeted therapeutically to prevent the metastatic spread of primary breast tumors.

On the other hand, by contrast with the surveyed literature (Nagira et al., 1997; Hedrick and Zlotnik, 1997; Gunn et al., 1998) reporting that lymphatic cells are capable of secreting CCL21 both *in vitro* and *in vivo* settings, HMVEC-dLy cells used in the present study were determined as poor producers of CCL21 chemokine. One possible explanation of this result could be the substantial changes in gene expression induced by culture in primary cells that might be the cause of the loss, for instance, of LYVE-1 expression or CCL21 production (Sironi et al., 2006). Cell culture might therefore alter some of the core features of LECs. The other possible explanation of the sparse CCL21 expression could reside in the sensitivity of the method used to detect it.

One option to correct the deficiencies of the currently employed method relies on the analysis of CCL21 expression by LECs in a 3D microenvironment, since its importance on chemokine signalling has been repeatedly emphasized in the past. From an experimental perspective, this would translate into creation of more advanced replicas of the biophysical setting, which should include slow interstitial flow conditions that are regarded as essential for stimulation of the chemokine secretion by LECs.

4.4 The Role of CCL21/CCR7 Pair in Mediating VEGF-C Secretion

In contrast with their well-defined physiological role in the functionality of the immune system, the pathological involvement of the chemokines in tumor progression is much less understood. However, due to recent intense research efforts, it became more and more clear that chemokines are some of the key players involved in tumor-induced angiogenesis, lymphangiogenesis and metastasis.

The present study has put forward the hypothesis that CCL21/CCR7 interaction is in fact responsible for VEGF-C synthesis by CCR7-positive MDA-MB-231 breast cancer cells. For this purpose, CCR7 expression was temporarily inhibited with siRNA targeting the CCR7 gene in MDA-MB-231 cells. The effectiveness of the temporary siRNA expression was assessed by means of conventional real-time PCR, quantitative real-time PCR, and Western blot. Important decreases in CCR7 mRNA and protein expression levels were observed in CCR7 gene silenced in MDA-MB-231 cells when compared to control siRNA.

Furthermore, the impact of transient CCR7-gene silencing on VEGF-C mRNA expression and intra/extracellular protein expression/secretion by MDA-MB-231 cells was assessed 48 hours post nucleofection. Since real-time PCR, quantitative real-time PCR, Western blot, and ELISA analyses performed have confirmed that the knockdown of the CCR7 gene translates into decreased amounts of the lymphangiogenic factor VEGF-C at all investigated levels, it can be affirmed that CCL21/CCR7 interaction might constitute one of the mechanisms responsible for VEGF-C production in the analyzed breast cancer cell line model.

When it comes to contribution of the CCL21/CCR7 axis in the development of mammary malignancy, there are at least two areas in which this pair has shown to be actively involved, often through VEGF-C-mediated signalling: lymph nodes metastasis and immune response modulation (Shields et al., 2007; Shields et al. 2007b; Yu et al., 2008; Pan et al., 2008; Shields et al., 2010). On the other hand, while there has been an impressive body of research verifying VEGF-C involvement in lymphangiogenesis and/or lymph node metastasis (Skobe et al., 2001; Karpanen et al., 2001; Mandriota et al.,

2001; Nakamura et al., 2005; Timoshenko et al., 2006; Hirakawa et al., 2007; Yu et al., 2007; Guo et al., 2009), only few prior studies emphasized VEGF-C/CCR7 correlations with lymphangiogenesis (Yan et al., 2004; Deguchi et al., 2010). However, they were performed in a clinical context, for a different type of cancer, and without attempting to uncover the molecular mechanisms underlying their connection.

4.5 Signalling Mechanism of the CCR7-Mediated VEGF-C Secretion

Since a certain correlation between CCR7 activation and VEGF-C secretion has been established, the next question to be answered by the present investigation was related to the underlying mechanism responsible for CCR7-mediated VEGF-C synthesis by MDA-MB-231 cells. The hypothesis that PI3K/AKT signalling pathway could be in fact responsible for CCR7-mediated VEGF-C secretion was addressed.

While this assumption is absolutely new in the context of CCR7 chemokine receptor, similar molecular mechanisms have confirmed to be true for insulin-like growth factor-I receptor (IGFIR)-mediated secretion of VEGF-C in lung carcinoma (Tang et al., 2003). In general, CCL21/CCR7 binding has shown to be responsible for increased migration, invasion, and proliferation of tumor cells through different intracellular signalling pathways.

Many prior studies seem to imply that a direct consequence of CCL21 binding is constituted by the downstream activation of the G protein complex that will induce – via $G\beta\gamma$ subunits released – activation of the PI3K/AKT pathway (Stephens et al., 1994).

This particular intracellular signalling pathway has been associated in the past with several types of genetic deregulations that are frequently present in a wide majority of human malignancies like inhibition of intracellular pro-apoptotic/upregulation of pro-survival signals (Vivanco and Sawyers, 2002; Gershtein et al., 2007). Similar mechanisms have also been related to the involvement of PI3K/AKT pathway in lymphangiogenesis, in a VEGF-C/VEGFR-3 mediated manner (Makinen et al., 2001). Moreover, Pan et al. (2008) reported that the PKA/AKT-dependent signalling was involved in the induction of CCR7 expression by COX-2 in breast cancer cells. However, they did not test whether PI3K/AKT activation which is characteristic to EP4 receptor (Fujino et al., 2003) was involved in CCR7 upregulation.

In order to test the working hypothesis of the current objective, the effect of CCR7 stimulation on AKT phosphorylation was assessed through Western blot performed on serum starved MDA-MB-231 cells at different time intervals. While the increase in phosphorylation status of AKT was evident at all time points – and practically proved that AKT is activated following CCL21 binding – the weak phosphorylation response observed for untreated cells suggests that this signalling pathway might be constitutively activated. This phenomenon might be interpreted as a consequence of the autocrine loop that is perpetuated by the tumor cell-secreted chemokine ligand CCL21 or other receptor-ligand interactions. Conversely, to verify whether CCL21-induced AKT phosphorylation was mediated by CCR7, this receptor was blocked with increasing concentrations of neutralizing antibody. The analysis of densitometry data has indicated that the most effective inhibition of AKT phosphorylation occurred for the highest

amount of CCR7 MAb tested. Taken together, these results show that PI3K/AKT is indeed activated by CCL21/CCR7 binding.

Finally, the functional role of AKT signalling in induction of VEGF-C secretion was investigated. For this purpose, following a two step treatment of MDA-MB-231 cells with: i) PI3 kinase inhibitor at increasing concentrations; and ii) CCL21 chemokine ligand, the VEGF-C protein secretion in conditioned media was quantified through ELISA. Since the addition of PI3K inhibitor led to significant decreases in VEGF-C secretion, it can be asserted that CCL21/CCR7 interaction induces VEGF-C secretion by MDA-MB-231 cells via PI3K/AKT intracellular signalling pathway.

4.6 The Role of CCL21/CCR7 Pair in the Induction of LECs Proliferation, Migration, and Tubular Network Formation

As surveyed literature suggests, CCL21/CCR7 pair has multiple roles in tumor development and progression (Takeuchi et al., 2004; Redondo-Munoz et al., 2008; Sun et al., 2009; Shields et al., 2007; Shields et al., 2010). However, while the complete palette of functionalities assumed by this chemokine axis in breast cancer is yet to be deciphered, one of the newer theories links CCL21/CCR7 interaction with angiogenesis and lymphangiogenesis in pancreatic cancer (Zhao et al., 2011).

Given that the pro-lymphangiogenic effect of VEGF-C in breast cancer is well acknowledged (Skobe et al., 2001; Nakamura et al., 2005; Timoshenko et al., 2006; Hirakawa et al., 2007; Zhang et al., 2008), it is reasonable to believe that CCR7-mediated secretion of VEGF-C is responsible for the formation of new lymphatic vessels.

However, in addition to the indirect pro-lymphangiogenic role assumed by CCL21 chemokine via VEGF-C secretion, the current study proposed that the lymphatic development is also caused by the direct binding between CCL21 ligand and CCR7, expressed on the surface of LECs.

As mentioned before, lymphangiogenesis is a complex and multistep process. Since none of the existent *in vitro* assays can replicate all of its steps in a combined manner, lymphangiogenesis is generally tested *in vitro* through separate assays, each attempting to mimic different stages of the lymphangiogenic process (Bruyere and Noel, 2010). Within the limited scope of the current study, LEC "genesis" was simulated through a succession of assays replicating the following lymphangiogenic steps: i) proliferation; ii) migration; and iii) formation of tubular-like structures.

In order to quantify the effect of CCL21 on HMVEC-dLy proliferation, serum-starved cells were treated for 24 hours with various concentrations of chemokine ligand. The proportion of nuclei that incorporated BrdU increased after CCL21 treatment by at least 70% with respect to the control group. Conversely, when CCR7 signalling was blocked with increasing concentrations of CCR7 antibodies, LEC proliferation was overall decreased and peaked for 10 μg/ml CCR7 antibody, when it reached the serum-free level. Based on these two experiments, it can be inferred that CCR7 activation through CCL21 binding constitutes an effective signalling pathway for LEC proliferation.

Further, to test the effect of CCL21/CCR7 interaction on HMVEC-dLy migration, a Boyden chamber assay was devised. HMVEC-dLy cells responded to the chemokinetic effect of CCL21 and migrated towards the transwell membrane.

Conversely, when CCR7 receptor was blocked with various concentrations of CCR7 antibody, LECs migration was significantly inhibited. Interestingly, neither CCL21 stimulation nor CCR7 receptor blocking induced dose-dependent effects on HMVEC-dLy migration, a phenomenon that could be explained perhaps through chemokine receptor saturation followed by its internalization and cell desensitization (Zimmermanm et al., 1999), although the extent of this effect is somewhat unclear in case of CCR7-positive immune cells (Bardi et al., 2001; Kohout et al., 2004).

Finally, the effect of CCL21/CCR7 binding on HMVEC-dLy morphogenesis was analyzed. For this, serum-free HMVEC-dLy cells were treated with increasing concentrations of CCL21 and incubated for 24 hours onto growth factor reduced Matrigel. Image-based quantification of the tubular network lengths revealed that CCL21 ligand stimulates tube formation. However, the results also showed that tubulogenesis is actually a constitutive process for HMVECs-dLy when placed on Matrigel matrix, since they are able to form small lengths of tubular network even in the absence of external stimulation with CCL21 ligand. Conversely, blocking of the CCR7 receptor has inhibited in tubulogenesis, which means that HMVEC-dLy morphogenesis is indeed mediated by CCR7 activation by its cognate ligand CCL21. Based on these results, it can be inferred that within the framework of the investigated *in vitro* model, CCL21 assumed a direct pro-lymphangiogenic role that is complemented by its indirect action via VEGF-C.

4.7 Biological Implications

Despite the intense research efforts that have occurred over the decade that has elapsed since the positive identification of the lymphatic markers, the role of lymphangiogenesis in the complex pathology of tumoral processes in general, and in that of breast cancer in particular, has just begun to emerge. According to the newer studies, lymphatics have started to be reclassified as active, rather than passive conduits in cancer, since they are able to fine tune the balance between peripheral tolerance and immunity with strong implications on immune host responses to tumor invasion (Lund and Swartz, 2010).

However, when it comes to the molecular regulators of the lymphangiogenic process, the overall picture is far from being complete and tends to permanently broaden with many other types of interactions often placed outside of the conventional VEGF family of the pro-lymphangiogenic factors (Norrmen et al., 2011). Among them, the interplay between CCL21/CCR7 chemokine axis and VEGF-C has been recently underscored as being central to the metastatic dissemination via lymphatics (Shields et al., 2007; Issa et al., 2009). In this regard, the present study has added a new piece to this "puzzle", by emphasizing that a closed loop/circular communication exists between CCL21/CCR7 and VEGF-C/VEGFR-3 axes in a sense that not only the paracrine activity of VEGF-C promotes CCL21 secretion by LECs (Shields et al., 2009), but also CCL21 stimulates VEGF-C synthesis via PI3K/AKT intracellular signalling, at least in the analyzed *in vitro* model. Since this bidirectional crosstalk between the aforementioned axes has a proved effect on lymphangiogenesis, this study adds several new elements to

the multifaceted role of CCL21/CCR7 chemokine pair in mammary malignancy (Figure 28).

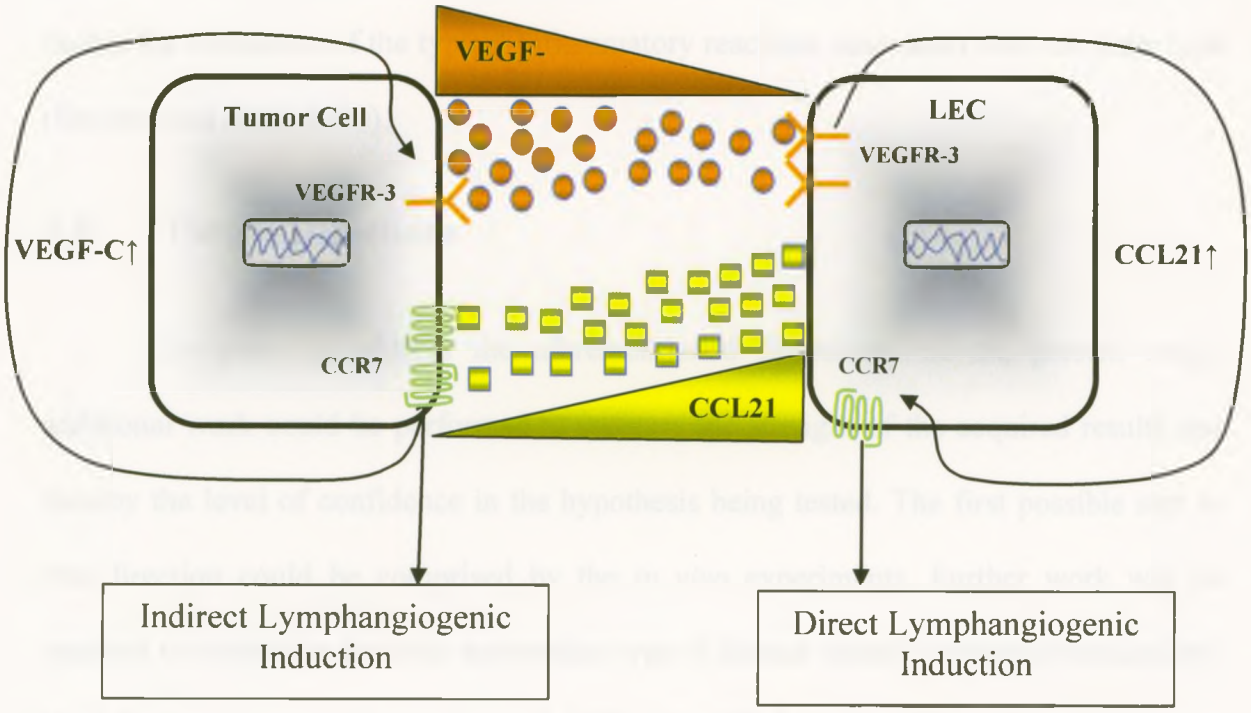
Given the severity of the implications of pathological lymphatic vasculogenesis, (Sleeman et al., 2009; Tammela and Alitalo, 2010; Schulte-Merker et al., 2011), any efforts directed towards inhibition of its occurrence are fully justifiable. Chemokines are holding much promise in this regard and a better understanding of their participation in tumor biology will be undoubtedly beneficial for their further exploitation as potent cancer therapy candidates, especially due to the pleoitropism of chemokine receptors throughout the human body.

Since the effectiveness of blocking the CCL21 chemokine signalling has already been scrutinized from a therapeutic perspective (Lanati et al., 2010), the value of the present CCL21/CCR7-related results is apparent.

4.8 Possible Limitations of the Experimental Design

Perhaps the most important limitation of the proposed research approach resides in the exclusive use of the *in vitro* techniques. However, this *in vitro* step is practically indispensable for incipient examination and understanding of the underlying molecular mechanisms related to CCL21/CCR7 chemokine pair functionality and its influence on breast cancer-induced lymphangiogenesis.

Figure 28. Schematic Model of the Crosstalk between VEGF-C/VEGFR-3 and CCL21/CCR7 Axes. These two axes have been shown to influence breast cancer progression by distinct, but complementary mechanisms: 1) VEGF-C produced by breast cancer cells acts in an autocrine manner to increase tumor cells invasiveness by increasing their proteolytic activity and motility; 2) VEGF-C also acts in a paracrine manner to increase lymphatic endothelial cells secretion of CCL21; 3) the paracrine secretion of CCL21 guides the CCR7-expressing tumor cells towards draining lymphatics; and 4) CCL21/CCR7 interaction increases VEGF-C production by tumor cells, thus amplifies their lymphangiogenic potential. In fact, CCL21/CCR7 chemokine pair has been found to regulate lymphangiogenesis in two different ways: i) directly by stimulation of the pro-lymphangiogenic traits of LECs; and ii) indirectly through increasing the secretion of the lymphangiogenic factor VEGF-C by breast cancer cells.


VEGF-
Tumor Cell
VEGFR-3
VEGF-C↑
CCR7
LEC
VEGFR-3
CCL21↑
CCR7
CCL21
Indirect Lymphangiogenic Induction
Direct Lymphangiogenic Induction

Another possible limitation of the study design is related to the employment of 2D only cultures in lymphangiogenesis assays. By contrast with 2D cultures, that are required to address separately the steps involved in lymphatic vasculogenesis, a more enhanced type of 3D culture could have been employed. The 3D LEC culture can be regarded as an intermediate/complementary step between *in vitro* and *in vivo* assays that enable the avoidance of the typical inflammatory reactions associated with the latter type (Bruyere and Noel, 2010).

4.9 Future Directions

In order to address the aforementioned limitations of the present study, additional work could be performed to increase the strength of the acquired results and thereby the level of confidence in the hypothesis being tested. The first possible step in this direction could be comprised by the *in vivo* experiments. Further work will be required to determine the most appropriate type of animal model for these investigations. Once the most adequate animal model will be identified, the specific working hypothesis to be tested is that CCL21/CCR7 axis acts as a promoter of tumor-associated growth and lymphangiogenesis *in vivo*. For this purpose, tumor cells will be injected in the experimental animals and the inhibitory effects of CCR7 antagonists on tumor growth and lymphangiogenesis will be assessed through: i) tumor weight measurements, ii) lymphatic endothelial markers, and iii) lymphatic endothelial microvessel density.

Another possible extension of the present work could aim to analyze CCR7, CCL21, VEGF-C, and lymphangiogenic markers expression in a selective panel of human breast cancer tissues collected at various phases of development of the primary

mammary malignancy. The principal objective of this study would be the investigation of possible correlations between these molecules and clinical stage/grade of breast tumor, respectively.

CHAPTER FIVE:

SUMMARY AND CONCLUSIONS

5.1 Summary

Based on the results presented in Chapter 3, the following remarks can be made:

- CCR7 chemokine receptor is expressed at the protein and mRNA levels by the two cell model analyzed *in vitro* (MBA-MD-231 and HMVEC-dLy);
- CCL21 chemokine is expressed at the protein and mRNA levels by the analyzed tumor cells;
- CCL21 secretion by MDA-MB-231 is substantially higher in 2D-matrix conditions than in 2D culture conditions;
- transient transfection with siRNA targeting human CCR7 chemokine receptor results in reduced VEGF-C expressions at the mRNA and protein levels in MDA-MB-231 cells;
- knockdown of CCR7 chemokine receptor leads to a decrease in VEGF-C protein secretion by MDA-MB-231 cells;
- CCL21chemokine stimulates the phosphorylation of AKT at Ser 473 in MDA-MB-231 cells;
- AKT phosphorylation was mediated by CCR7 activation since CCR7 antibody blocks CCL21-induced phosphorylation of AKT in MDA-MB-231 cells;
- activation of the PI3K/AKT signalling pathway is involved in the regulation of CCR7-mediated VEGF-C secretion;
- CCL21/CCR7 pair promotes proliferation and migration of HMVEC-dLy;

- CCL21/CCR7 pair stimulates the formation of tubular network structures by HMVEC-dLy.

5.2 Conclusions

This study reports the influence of CCL21 chemokine and its receptor on the secretion of VEGF-C and elucidates the correlation between the CCL21/CCR7 axis and lymphangiogenesis. Moreover, these results support the role of the PI3K/AKT signalling pathway in CCR7 mediated VEGF-C secretion. It can also be reasoned that CCR7 positive tumor cells are capable of secreting endogenous ligand and thereby propagating autocrine CCR7 activation.

The expression of functional CCR7 chemokine receptor by HMVEC-dLy suggests that lymphatic endothelial cells are capable to respond to the chemokine gradients. In fact, the results found support a positive role of the CCL21/CCR7 chemokine pair in lymphatic vessel formation, including the ability to induce lymphatic endothelial cells proliferation, migration and to stimulate tube formation. Based on these findings, it can be asserted that in the investigated model, CCL21 is capable to assume a direct pro-lymphangiogenic role.

To conclude, corroboration of data presented in this study indicates that VEGF-C and CCL21 display a significant crosstalk. In fact, CCL21/CCR7 chemokine pair has been found to regulate lymphangiogenesis in two different ways: i) directly by stimulation of the pro-lymphangiogenic traits of LECs; and ii) indirectly through increasing the secretion of the lymphangiogenic factor VEGF-C by breast cancer cells.

REFERENCES

Agarwal, B., Saxena, R., Morimiya, A., Mehrotra, S. and Badve, S. (2005) Lymphangiogenesis does not occur in breast cancer. *Am J Surg Pathol*, **29**:1449-1455.

Akekawatchai, C., Holland, J.D., Kochetkova, M., Wallace, J.C. and McColl, S.R. (2005) Transactivation of CXCR4 by the insulin-like growth factor-1 receptor (IGF-1R) in human MDA-MB-231 breast cancer epithelial cells. *J Biol Chem*, **280**:39701-39708.

Ali, S. and Lazennec, G. (2007) Chemokines: novel targets for breast cancer metastasis. *Cancer Metastasis Rev*, **26**:401-420.

Alitalo, K., Tammela, T. and Petrova, T.V. (2005) Lymphangiogenesis in development and human disease. *Nature*, **438**:946-953.

Allavena, P., Germano, G., Marchesi, F. and Mantovani, A. (2011) Chemokines in cancer related inflammation. *Exp Cell Res*, **317**:664-673.

Andre, F., Cabioglu, N., Assi, H., Sabourin, J.C., Delaloge, S., Sahin, A., Broglio, K., Spano, J.P., Combadiere, C., Bucana, C., Soria, J.C. and Cristofanilli, M. (2006) Expression of chemokine receptors predicts the site of metastatic relapse in patients with axillary node positive primary breast cancer. *Ann Oncol*, **17**:945-951.

Arai, H. and Charo, I.F. (1996) Differential regulation of G-protein-mediated signaling by chemokine receptors. *J Biol Chem*, **271**:21814-21819.

Azenshtein, E., Meshel, T., Shina, S., Barak, N., Keydar, I. and Ben-Baruch, A. (2005) The angiogenic factors CXCL8 and VEGF in breast cancer: regulation by an array of pro-malignancy factors. *Cancer Lett*, **217**:73-86.

Azzali, G. (2007) Tumor cell transendothelial passage in the absorbing lymphatic vessel of transgenic adenocarcinoma mouse prostate. *Am J Pathol*, **170**:334-346.

Baggiolini, M. and Moser, B. (1997) Blocking chemokine receptors. *J Exp Med*, **186**:1189-1191.

Balkwill, F. (2004) Cancer and the chemokine network. *Nat Rev Cancer*, **4**:540-550.

Balkwill, F. and Mantovani, A. (2001) Inflammation and cancer: back to Virchow? *Lancet*, **357**:539-545.

Baluk, P., Fuxe, J., Hashizume, H., Romano, T., Lashnits, E., Butz, S., Vestweber, D., Corada, M., Molendini, C., Dejana, E. and McDonald, D. (2007) Functionally specialized junctions between endothelial cells of lymphatic vessels. *The Journal of experimental medicine*, **204**:2349-2362.

Banerji, S., Ni, J., Wang, S.X., Clasper, S., Su, J., Tammi, R., Jones, M. and Jackson, D.G. (1999) LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol*, **144**:789-801.

Banziger-Tobler, N.E., Halin, C., Kajiya, K., and Detmar, M. (2008) Growth hormone promotes lymphangiogenesis. *Am. J.Pathol*, **173**: 586–597.

Barbero, S., Bonavia, R., Bajetto, A., Porcile, C., Pirani, P., Ravetti, J.L., Zona, G.L., Spaziante, R., Florio, T. and Schettini, G. (2003) Stromal cell-derived factor 1alpha stimulates human glioblastoma cell growth through the activation of both extracellular

signal-regulated kinases 1/2 and Akt. *Cancer Res*, **63**:1969-1974.

Barbieri, F., Bajetto, A. and Florio, T. (2010) Role of chemokine network in the development and progression of ovarian cancer: a potential novel pharmacological target. *J Oncol*, **2010**:426956.

Bardi, G., Lipp, M., Baggiolini, M. and Loetscher, P. (2001) The T cell chemokine receptor CCR7 is internalized on stimulation with ELC, but not with SLC. *European Journal of Immunology*, **31**:3291-3297.

Basolo, F., Conaldi, P.G., Fiore, L., Calvo, S. and Toniolo, A. (1993) Normal breast epithelial cells produce interleukins 6 and 8 together with tumor-necrosis factor: defective IL6 expression in mammary carcinoma. *Int J Cancer*, **55**:926-930.

Bazigou, E., Xie, S., Chen, C., Weston, A., Miura, N., Sorokin, L., Adams, R., Muro, A.F., Sheppard, D. and Makinen, T. (2009) Integrin-alpha9 is required for fibronectin matrix assembly during lymphatic valve morphogenesis. *Dev Cell*, **17**:175-186.

Ben-Baruch, A. (2006) The multifaceted roles of chemokines in malignancy. *Cancer Metastasis Rev*, **25**, 357–371.

Ben-Baruch, A. (2008) Organ selectivity in metastasis: regulation by chemokines and their receptors. *Clin Exp Metastasis*, **25**:345-356.

Berndt, S., Bruyere, F., Jost, M., and Noel, A. (2008) In vitro and in vivo models of angiogenesis to dissect MMP functions. *In The Cancer Degradome-Proteases and Cancer Biology*, pp: 303–322.

Breiteneder-Geleff, S., Soleiman, A., Kowalski, H., Horvat, R., Amann, G., Kriehuber, E., Diem, K., Weninger, W., Tschachler, E., Alitalo, K. and Kerjaschki, D. (1999) Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. *Am J Pathol*, **154**:385-394.

Bruyere, F. and Noel, A. (2010) Lymphangiogenesis: in vitro and in vivo models. *FASEB J*, **24**:8-21.

Buettner, M., Meyer, B., Schreck, S. and Niedobitek, G. (2007) Expression of RANTES and MCP-1 in epithelial cells is regulated via LMP1 and CD40. *Int J Cancer*, **121**:2703-2710.

Buonamici, S., Trimarchi, T., Ruocco, M.G., Reavie, L., Cathelin, S., Mar, B.G., Klinakis, A., Lukyanov, Y., Tseng, J.-C., Sen, F., Gehrie, E., Li, M., Newcomb, E., Zavadil, J., Meruelo, D., Lipp, M., Ibrahim, S., Efstratiadis, A., Zagzag, D., Bromberg, J.S., Dustin, M.L. and Aifantis, I. (2009) CCR7 signalling as an essential regulator of CNS infiltration in T-cell leukaemia. *Nature*, **459**:1000-1004.

Cabioglu, N., Gong, Y., Islam, R., Broglio, K.R., Sneige, N., Sahin, A., Gonzalez-Angulo, A.M., Morandi, P., Bucana, C., Hortobagyi, G.N. and Cristofanilli, M. (2007) Expression of growth factor and chemokine receptors: new insights in the biology of inflammatory breast cancer. *Ann Oncol*, **18**:1021-1029.

Cabioglu, N., Sahin, A.A., Morandi, P., Meric-Bernstam, F., Islam, R., Lin, H.Y., Bucana, C.D., Gonzalez-Angulo, A.M., Hortobagyi, G.N. and Cristofanilli, M. (2009) Chemokine

receptors in advanced breast cancer: differential expression in metastatic disease sites with diagnostic and therapeutic implications. *Ann Oncol*, **20**:1013-1019.

Cabioglu, N., Summy, J., Miller, C., Parikh, N.U., Sahin, A.A., Tuzlali, S., Pumiglia, K., Gallick, G.E. and Price, J.E. (2005a) CXCL-12/stromal cell-derived factor-1alpha transactivates HER2-neu in breast cancer cells by a novel pathway involving Src kinase activation. *Cancer Res*, **65**:6493-6497.

Cabioglu, N., Yazici, M.S., Arun, B., Broglio, K.R., Hortobagyi, G.N., Price, J.E. and Sahin, A. (2005b) CCR7 and CXCR4 as Novel Biomarkers Predicting Axillary Lymph Node Metastasis in T1 Breast Cancer. *Clinical Cancer Research*, **11**:5686-5693.

Chu, C.Y., Cha, S.T., Lin, W.C., Lu, P.H., Tan, C.T., Chang, C.C., Lin, B.R., Jee, S.H. and Kuo, M.L. (2009) Stromal cell-derived factor-1alpha (SDF-1alpha/CXCL12)-enhanced angiogenesis of human basal cell carcinoma cells involves ERK1/2-NF-kappaB/interleukin-6 pathway. *Carcinogenesis*, **30**:205-213.

Cohen-Kaplan, V., Naroditsky, I., Zetser, A., Ilan, N., Vlodavsky, I. and Doweck, I. (2008) Heparanase induces VEGF C and facilitates tumor lymphangiogenesis. *Int J Cancer*, **123**:2566-2573.

Comerford, I., Litchfield, W., Harata-Lee, Y., Nibbs, R.J. and McColl, S.R. (2007) Regulation of chemotactic networks by 'atypical' receptors. *Bioessays*, **29**:237-247.

Cueni, L.N., and Detmar, M. (2009) Galectin-8 interacts with podoplanin and modulates lymphatic endothelial cell functions. *Exp. Cell. Res*, **315**: 1715–1723.

Cunningham, H.D., Shannon, L.A., Calloway, P.A., Fassold, B.C., Dunwiddie, I., Vielhauer, G., Zhang, M. and Vines, C.M. (2010) Expression of the C-C chemokine receptor 7 mediates metastasis of breast cancer to the lymph nodes in mice. *Transl Oncol*, **3**:354-361.

Da, M.X., Wu, Z. and Tian, H.W. (2008) Tumor lymphangiogenesis and lymphangiogenic growth factors. *Arch Med Res*, **39**:365-372.

Dadras, S.S., Paul, T., Bertoncini, J., Brown, L.F., Muzikansky, A., Jackson, D.G., Ellwanger, U., Garbe, C., Mihm, M.C. and Detmar, M. (2003) Tumor lymphangiogenesis: a novel prognostic indicator for cutaneous melanoma metastasis and survival. *Am J Pathol*, **162**:1951-1960.

Das, S. and Skobe, M. (2008) Lymphatic vessel activation in cancer. *Ann N Y Acad Sci*, **1131**:235-241.

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

Deguchi, K., Ichikawa, D., Soga, K., Watanabe, K., Kosuga, T., Takeshita, H., Konishi, H., Morimura, R., Tsujiura, M., Komatsu, S., Shiozaki, A., Okamoto, K., Fujiwara, H. and Otsuji, E. (2010) Clinical significance of vascular endothelial growth factors C and D and chemokine receptor CCR7 in gastric cancer. *Anticancer Res*, **30**:2361-2366.

Dejana, E., Orsenigo, F., Molendini, C., Baluk, P. and McDonald, D. (2009) Organization and signaling of endothelial cell-to-cell junctions in various regions of the blood and

lymphatic vascular trees. *Cell and Tissue Research*, **335**:17-25.

Devalaraja, M.N. and Richmond, A. (1999) Multiple chemotactic factors: fine control or redundancy? *Trends Pharmacol Sci*, **20**:151-156.

DeVries, M.E., Kelvin, A.A., Xu, L., Ran, L., Robinson, J. and Kelvin, D.J. (2006) Defining the origins and evolution of the chemokine/chemokine receptor system. *J Immunol*, **176**:401-415.

Ding, Y., Shimada, Y., Maeda, M., Kawabe, A., Kaganoi, J., Komoto, I., Hashimoto, Y., Miyake, M., Hashida, H. and Imamura, M. (2003) Association of CC chemokine receptor 7 with lymph node metastasis of esophageal squamous cell carcinoma. *Clin Cancer Res*, **9**:3406-3412.

Dubinett, S.M., Lee, J.M., Sharma, S. and Mule, J.J. (2010) Chemokines: can effector cells be redirected to the site of the tumor? *Cancer J*, **16**:325-335.

Eccles, S., Paon, L. and Sleeman, J. (2007) Lymphatic metastasis in breast cancer: importance and new insights into cellular and molecular mechanisms. *Clin Exp Metastasis*, **24**:619-636.

Fang, L. and Hwang, S.T. (2009) Roles for CCR7 in Cancer Biology. In *Chemokine Receptors in Cancer*. pp. 93-108.

Feng, L.Y., Ou, Z.L., Wu, F.Y., Shen, Z.Z. and Shao, Z.M. (2009) Involvement of a novel chemokine decoy receptor CCX-CKR in breast cancer growth, metastasis and patient survival. *Clin Cancer Res*, **15**:2962-2970.

Ferlay, J., Shin, H.R., Bray, F., Forman, D., Mathers, C. and Parkin, D.M. (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer*, **127**:2893-2917.

Fernandis, A.Z., Prasad, A., Band, H., Klosel, R. and Ganju, R.K. (2004) Regulation of CXCR4-mediated chemotaxis and chemoinvasion of breast cancer cells. *Oncogene*, **23**:157-167.

Finak, G., Bertos, N., Pepin, F., Sadekova, S., Souleimanova, M., Zhao, H., Chen, H., Omeroglu, G., Meterissian, S., Omeroglu, A., Hallett, M. and Park, M. (2008) Stromal gene expression predicts clinical outcome in breast cancer. *Nat Med*, **14**:518-527.

Forster, R., Davalos-Misslitz, A.C. and Rot, A. (2008) CCR7 and its ligands: balancing immunity and tolerance. *Nat Rev Immunol*, **8**:362-371.

Forster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Muller, I., Wolf, E. and Lipp, M. (1999) CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell*, **99**:23-33.

Freund, A., Jolivel, V., Durand, S., Kersual, N., Chalbos, D., Chavey, C., Vignon, F. and Lazennec, G. (2004) Mechanisms underlying differential expression of interleukin-8 in breast cancer cells. *Oncogene*, **23**:6105-6114.

Garin, A. and Proudfoot, A.E.I. (2011) Chemokines as targets for therapy. *Experimental Cell Research*, **317**:602-612.

Gershtein, E.S., Scherbakov, A.M., Shatskaya, V.A., Kushlinsky, N.E. and Krasil'nikov, M.A. (2007) Phosphatidylinositol 3-kinase/AKT signalling pathway components in human breast cancer: clinicopathological correlations. *Anticancer Res*, **27**:1777-1782.

Giraudo, E., Inoue, M., Hanahan, D. (2004) An amino-bisphosphonate targets MP-9-expressing macrophages and angiogenesis to impair cervical carcinogenesis. *J. Clin. Invest.*, **114**:623–633.

Goda, S., Inoue, H., Umehara, H., Miyaji, M., Nagano, Y., Harakawa, N., Imai, H., Lee, P., Macarthy, J.B., Ikeo, T., Domae, N., Shimizu, Y. and Iida, J. (2006) Matrix metalloproteinase-1 produced by human CXCL12-stimulated natural killer cells. *Am J Pathol*, **169**:445-458.

Goldberg-Bittman, L., Neumark, E., Sagi-Assif, O., Azenshtein, E., Meshel, T., Witz, I.P. and Ben-Baruch, A. (2004) The expression of the chemokine receptor CXCR3 and its ligand, CXCL10, in human breast adenocarcinoma cell lines. *Immunol Lett*, **92**:171-178.

Gosling, J., Dairaghi, D.J., Wang, Y., Hanley, M., Talbot, D., Miao, Z. and Schall, T.J. (2000) Cutting edge: identification of a novel chemokine receptor that binds dendritic cell- and T cell-active chemokines including ELC, SLC, and TECK. *J Immunol*, **164**:2851-2856.

Grimshaw, M.J. (2005) Endothelins in breast tumour cell invasion. *Cancer Lett*, **222**:129-138.

Gunningham, S.P., Currie, M.J., Han, C., Robinson, B.A., Scott, P.A., Harris, A.L. and Fox, S.B. (2000) The short form of the alternatively spliced flt-4 but not its ligand vascular endothelial growth factor C is related to lymph node metastasis in human breast cancers. *Clin Cancer Res*, **6**:4278-4286.

Guo, X., Chen, Y., Xu, Z., Qian, Y. and Yu, X. (2009) Prognostic significance of VEGF-C expression in correlation with COX-2, lymphatic microvessel density, and clinicopathologic characteristics in human non-small cell lung cancer. *Acta Biochim Biophys Sin (Shanghai)*, **41**:217-222.

Hamrah, P., Chen, L., Zhang, Q. and Dana, M.R. (2003) Novel expression of vascular endothelial growth factor receptor (VEGFR)-3 and VEGF-C on corneal dendritic cells. *Am J Pathol*, **163**:57-68.

Hanahan, D., Weinberg, R.A. (2000) The hallmarks of cancer. *Cell*, **100**:57–70.

He, L., Wang, J. and Shan, Y. (2005) [Expression and significance of PTEN and Survivin between the primary foci and the lymph node metastases foci of laryngeal squamous cell carcinoma]. *Lin Chuang Er Bi Yan Hou Ke Za Zhi*, **19**:412-414, 416.

He, Y., Rajantie, I., Ilmonen, M., Makinen, T., Karkkainen, M.J., Haiko, P., Salven, P. and Alitalo, K. (2004) Preexisting lymphatic endothelium but not endothelial progenitor cells are essential for tumor lymphangiogenesis and lymphatic metastasis. *Cancer Res*, **64**:3737-3740.

He, Y., Rajantie, I., Pajusola, K., Jeltsch, M., Holopainen, T., Yla-Herttuala, S., Harding, T., Jooss, K., Takahashi, T. and Alitalo, K. (2005) Vascular Endothelial Cell Growth

Factor Receptor 3–Mediated Activation of Lymphatic Endothelium Is Crucial for Tumor Cell Entry and Spread via Lymphatic Vessels. *Cancer Research*, **65**:4739-4746.

Hedrick, J.A., and Zlotnik, A. (1997). Identification and characterization of a novel beta chemokine containing six conserved cysteines. *J Immunol*, **159**:1589–1593.

Helmlinger, G., Netti, P.A., Lichtenbeld, H.C., Melder, R.J. and Jain, R.K. (1997) Solid stress inhibits the growth of multicellular tumor spheroids. *Nat Biotechnol*, **15**:778-783.

Hembruff, S.L. and Cheng, N. (2009) Chemokine signaling in cancer: Implications on the tumor microenvironment and therapeutic targeting. *Cancer Ther*, 7:254-267.

Hirakawa, S., Brown, L.F., Kodama, S., Paavonen, K., Alitalo, K. and Detmar, M. (2007) VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites. *Blood*, **109**:1010-1017.

Holland, J.D., Kochetkova, M., Akekawatchai, C., Dottore, M., Lopez, A. and McColl, S.R. (2006) Differential functional activation of chemokine receptor CXCR4 is mediated by G proteins in breast cancer cells. *Cancer Res*, **66**:4117-4124.

Huang, D., Shi, F.D., Jung, S., Pien, G.C., Wang, J., Salazar-Mather, T.P., He, T.T., Weaver, J.T., Ljunggren, H.G., Biron, C.A., Littman, D.R. and Ransohoff, R.M. (2006) The neuronal chemokine CX3CL1/fractalkine selectively recruits NK cells that modify experimental autoimmune encephalomyelitis within the central nervous system. *FASEB J*, **20**:896-905.

Hughes, M.R., Anderson, N., Maltby, S., Wong, J., Berberovic, Z., Birkenmeier, C.S., Haddon, D.J., Garcha, K., Flenniken, A., Osborne, L.R., Adamson, S.L., Rossant, J., Peters, L.L., Minden, M.D., Paulson, R.F., Wang, C., Barber, D.L., McNagny, K.M. and Stanford, W.L. (2010) A novel ENU-generated truncation mutation lacking the spectrin-binding and C-terminal regulatory domains of Ank1 models severe hemolytic hereditary spherocytosis. *Exp Hematol*, **39**:305-320, 320 e301-302.

Issa, A., Le, T.X., Shoushtari, A.N., Shields, J.D. and Swartz, M.A. (2009) Vascular endothelial growth factor-C and C-C chemokine receptor 7 in tumor cell-lymphatic cross-talk promote invasive phenotype. *Cancer Res*, **69**:349-357.

Jackson, D.G. (2003) The lymphatics revisited: new perspectives from the hyaluronan receptor LYVE-1. *Trends Cardiovasc Med*, **13**:1-7.

Jeltsch, M., Kaipainen, A., Joukov, V., Meng, X., Lakso, M., Rauvala, H., Swartz, M., Fukumura, D., Jain, R.K. and Alitalo, K. (1997) Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science*, **276**:1423-1425.

Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E. and Forman, D. (2011) Global cancer statistics. *CA Cancer J Clin*, **61**:69-90.

Ji, R.C. (2005) Characteristics of lymphatic endothelial cells in physiological and pathological conditions. *Histol Histopathol*, **20**:155-175.

Ji, R.C. (2006) Lymphatic endothelial cells, tumor lymphangiogenesis and metastasis: New insights into intratumoral and peritumoral lymphatics. *Cancer Metastasis Rev*, **25**:677-694.

Johnson, Z., Kosco-Vilbois, M.H., Herren, S., Cirillo, R., Muzio, V., Zaratin, P., Carbonatto, M., Mack, M., Smailbegovic, A., Rose, M., Lever, R., Page, C., Wells, T.N. and Proudfoot, A.E. (2004) Interference with heparin binding and oligomerization creates a novel anti-inflammatory strategy targeting the chemokine system. *J Immunol*, **173**:5776-5785.

Johnson, W.E., Caterson, B., Eisenstein, S.M. and Roberts, S. (2005) Human intervertebral disc aggrecan inhibits endothelial cell adhesion and cell migration in vitro. *Spine (Phila Pa 1976)*, **30**:1139-1147.

Jurisic, G. and Detmar, M. (2009) Lymphatic endothelium in health and disease. *Cell Tissue Res*, **335**:97-108.

Kaplan, R.N., Rafii, S. and Lyden, D. (2006) Preparing the "soil": the premetastatic niche. *Cancer Res*, **66**:11089-11093.

Karnoub, A.E. and Weinberg, R.A. (2006) Chemokine networks and breast cancer metastasis. *Breast Dis*, **26**:75-85.

Karpanen, T., Egeblad, M., Karkkainen, M.J., Kubo, H., Yla-Herttuala, S., Jaattela, M. and Alitalo, K. (2001) Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer Res*, **61**:1786-1790.

Keeley, E. C., Mehrad, B., Strieter, R. M. (2008) Chemokines as mediators of neovascularization. *Arterioscler. Thromb. Vasc. Biol.*, **28**: 1928–1936.

Kerjaschki, D. (2005) The crucial role of macrophages in lymphangiogenesis. *J Clin Invest*, **115**:2316-2319.

Kim, M., Koh, Y.J., Kim, K.E., Koh, B.I., Nam, D.H., Alitalo, K., Kim, I. and Koh, G.Y. (2010) CXCR4 signaling regulates metastasis of chemoresistant melanoma cells by a lymphatic metastatic niche. *Cancer Res*, **70**:10411-10421.

Kim, J.W., Ferris, R.L. and Whiteside, T.L. (2005) Chemokine C receptor 7 expression and protection of circulating CD8+ T lymphocytes from apoptosis. *Clin Cancer Res*, **11**:7901-7910.

Kleinman, H.K. and Martin, G.R. (2005) Matrigel: basement membrane matrix with biological activity. *Semin Cancer Biol*, **15**:378-386.

Kochetkova, M., Kumar, S. and McColl, S.R. (2009) Chemokine receptors CXCR4 and CCR7 promote metastasis by preventing anoikis in cancer cells. *Cell Death Differ*, **16**:664-673.

Kohout, T.A., Nicholas, S.L., Perry, S.J., Reinhart, G., Junger, S. and Struthers, R.S. (2004) Differential desensitization, receptor phosphorylation, beta-arrestin recruitment, and ERK1/2 activation by the two endogenous ligands for the CC chemokine receptor 7. *J Biol Chem*, **279**:23214-23222.

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

Kuang, Y., Wu, Y., Jiang, H. and Wu, D. (1996) Selective G protein coupling by C-C chemokine receptors. *J Biol Chem*, **271**:3975-3978.

Kulbe, H., Hagemann, T., Szlosarek, P.W., Balkwill, F.R. and Wilson, J.L. (2005) The inflammatory cytokine tumor necrosis factor-alpha regulates chemokine receptor expression on ovarian cancer cells. *Cancer Res*, **65**:10355-10362.

Laakkonen, P., Waltari, M., Holopainen, T., Takahashi, T., Pytowski, B., Steiner, P., Hicklin, D., Persaud, K., Tonra, J.R., Witte, L. and Alitalo, K. (2007) Vascular endothelial growth factor receptor 3 is involved in tumor angiogenesis and growth. *Cancer Res*, **67**:593-599.

Lanati, S., Dunn, D.B., Roussigné, M., Emmett, M.S., Carriere, V., Jullien, D., Budge, J., Fryer, J., Erard, M., Cailler, F., Girard, J.-P. and Bates, D.O. (2010) Chemotrap-1: An Engineered Soluble Receptor That Blocks Chemokine-Induced Migration of Metastatic Cancer Cells In vivo. *Cancer Research*, **70**:8138-8148.

Laurent, T.C. and Fraser, J.R. (1992) Hyaluronan. *FASEB J*, **6**:2397-2404.

Lazennec, G. and Richmond, A. (2010) Chemokines and chemokine receptors: new insights into cancer-related inflammation. *Trends Mol Med*, **16**:133-144.

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

Leu, A.J., Berk, D.A., Lymboussaki, A., Alitalo, K. and Jain, R.K. (2000) Absence of functional lymphatics within a murine sarcoma: a molecular and functional evaluation. *Cancer Res*, **60**:4324-4327.

Lewis, C.E., Pollard, J.W. (2006) Distinct role of macrophages in different tumor microenvironments. *Cancer Res*, **66**:605–12.

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

Liang, Z., Brooks, J., Willard, M., Liang, K., Yoon, Y., Kang, S. and Shim, H. (2007) CXCR4/CXCL12 axis promotes VEGF-mediated tumor angiogenesis through Akt signaling pathway. *Biochem Biophys Res Commun*, **359**:716-722.

Liersch, R., Biermann, C., Mesters, R.M. and Berdel, W.E. (2010) Lymphangiogenesis in cancer: current perspectives. *Recent Results Cancer Res*, **180**:115-135.

Liu, H., Pan, Z., Li, A., Fu, S., Lei, Y., Sun, H., Wu, M. and Zhou, W. (2008) Roles of chemokine receptor 4 (CXCR4) and chemokine ligand 12 (CXCL12) in metastasis of hepatocellular carcinoma cells. *Cell Mol Immunol*, **5**:373-378.

Liu, Y., Ji, R., Li, J., Gu, Q., Zhao, X., Sun, T., Wang, J., Du, Q. and Sun, B. (2010) Correlation effect of EGFR and CXCR4 and CCR7 chemokine receptors in predicting breast cancer metastasis and prognosis. *J Exp Clin Cancer Res*, **29**:16.

Locati, M., Otero, K., Schioppa, T., Signorelli, P., Perrier, P., Baviera, S., Sozzani, S. and Mantovani, A. (2002) The chemokine system: tuning and shaping by regulation of receptor expression and coupling in polarized responses. *Allergy*, **57**:972-982.

Locati, M., Torre, Y.M., Galliera, E., Bonecchi, R., Bodduluri, H., Vago, G., Vecchi, A. and Mantovani, A. (2005) Silent chemoattractant receptors: D6 as a decoy and scavenger receptor for inflammatory CC chemokines. *Cytokine Growth Factor Rev*, **16**:679-686.

Lund, A.W. and Swartz, M.A. (2010) Role of lymphatic vessels in tumor immunity: passive conduits or active participants? *J Mammary Gland Biol Neoplasia*, **15**:341-352.

Ma, J., Wang, Q., Fei, T., Han, J.D., Chen, Y.G. (2007) MCP-1 mediates TGF-{beta}-induced angiogenesis by stimulating vascular smooth muscle cell migration. *Blood*, **1**:109(3):987-94.

Maby-El Hajjami, H. and Petrova, T.V. (2008) Developmental and pathological lymphangiogenesis: from models to human disease. *Histochem Cell Biol*, **130**:1063-1078.

Maheshwari, A., Christensen, R.D. and Calhoun, D.A. (2003) ELR+ CXC chemokines in human milk. *Cytokine*, **24**:91-102.

Makinen, T., Veikkola, T., Mustjoki, S., Karpanen, T., Catimel, B., Nice, E.C., Wise, L., Mercer, A., Kowalski, H., Kerjaschki, D., Stacker, S.A., Achen, M.G. and Alitalo, K. (2001) Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. *EMBO J*, **20**:4762-4773.

Mandriota, S.J., Jussila, L., Jeltsch, M., Compagni, A., Baetens, D., Prevo, R., Banerji, S., Huarte, J., Montesano, R., Jackson, D.G., Orci, L., Alitalo, K., Christofori, G. and Pepper, M.S. (2001) Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J*, **20**:672-682.

Mantovani, A. (1999) The chemokine system: redundancy for robust outputs. *Immunol Today*, **20**:254-257.

Mantovani, A., Bonecchi, R. and Locati, M. (2006) Tuning inflammation and immunity by chemokine sequestration: decoys and more. *Nat Rev Immunol*, **6**:907-918.

Mantovani, A., Savino, B., Locati, M., Zammataro, L., Allavena, P. and Bonecchi, R. (2010) The chemokine system in cancer biology and therapy. *Cytokine Growth Factor Rev*, **21**:27-39.

Mantovani, A., Schioppa, T., Porta, C., Allavena, P., Sica, A. (2006) Role of tumor-associated macrophages in tumor progression and invasion. *Cancer Metastasis Rev*, **25**:315–22.

Maroni, P., Bendinelli, P., Matteucci, E. and Desiderio, M.A. (2007) HGF induces CXCR4 and CXCL12-mediated tumor invasion through Ets1 and NF-kappaB. *Carcinogenesis*, **28**:267-279.

Matsui, J., Funahashi, Y., Uenaka, T., Watanabe, T., Tsuruoka, A. and Asada, M. (2008) Multi-kinase inhibitor E7080 suppresses lymph node and lung metastases of human mammary breast tumor MDA-MB-231 via inhibition of vascular endothelial growth factor-receptor (VEGF-R) 2 and VEGF-R3 kinase. *Clin Cancer Res*, **14**:5459-5465.

Matteucci, E., Ridolfi, E., Maroni, P., Bendinelli, P. and Desiderio, M.A. (2007) c-Src/histone deacetylase 3 interaction is crucial for hepatocyte growth factor dependent decrease of CXCR4 expression in highly invasive breast tumor cells. *Mol Cancer Res*, **5**:833-845.

Mburu, Y.K., Wang, J., Wood, M.A., Walker, W.H. and Ferris, R.L. (2006) CCR7 mediates inflammation-associated tumor progression. *Immunol Res*, **36**:61-72.

Mehrad, B., Keane, M.P. and Strieter, R.M. (2007) Chemokines as mediators of angiogenesis. *Thromb Haemost*, **97**:755-762.

Mishra, P., Banerjee, D. and Ben-Baruch, A. (2010) Chemokines at the crossroads of tumor-fibroblast interactions that promote malignancy. *J Leukoc Biol*, **89**:31-39.

Mori, T., Kim, J., Yamano, T., Takeuchi, H., Huang, S., Umetani, N., Koyanagi, K. and Hoon, D.S. (2005) Epigenetic up-regulation of C-C chemokine receptor 7 and C-X-C chemokine receptor 4 expression in melanoma cells. *Cancer Res*, **65**:1800-1807.

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

Murphy, P.M., Baggiolini, M., Charo, I.F., Hebert, C.A., Horuk, R., Matsushima, K., Miller, L.H., Oppenheim, J.J. and Power, C.A. (2000) International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev*, **52**:145-176.

Nagira, M., Imai, T., Hieshima, K., Kusuda,J., Ridanpaa, M., Takagi, S., Nishimura, J., Kakizaki,M., Nomiyama,H., and Yoshie, O. (1997). Molecular cloning of a novel human CC chemokine secondary lymphoid-tissue chemokine that is a potent chemoattractant for lymphocytes and mapped to chromosome 9p13. *J Biol Chem*, **272**:19518–19524.

Nakamura, Y., Yasuoka, H., Tsujimoto, M., Imabun, S., Nakahara, M., Nakao, K., Nakamura, M., Mori, I. and Kakudo, K. (2005) Lymph vessel density correlates with nodal status, VEGF-C expression, and prognosis in breast cancer. *Breast Cancer Res Treat*, **91**:125-132.

Neptune, E.R. and Bourne, H.R. (1997) Receptors induce chemotaxis by releasing the betagamma subunit of Gi, not by activating Gq or Gs. *Proc Natl Acad Sci U S A*, **94**:14489-14494.

Netelenbos, T., Zuijderduijn, S., Van Den Born, J., Kessler, F.L., Zweegman, S., Huijgens, P.C. and Drager, A.M. (2002) Proteoglycans guide SDF-1-induced migration of hematopoietic progenitor cells. *J Leukoc Biol*, **72**:353-362.

Nilsson, I., Bahram, F., Li, X., Gualandi, L., Koch, S., Jarvius, M., Soderberg, O., Anisimov, A., Kholova, I., Pytowski, B., Baldwin, M., Yla-Herttuala, S., Alitalo, K., Kreuger, J. and Claesson-Welsh, L. VEGF receptor 2/-3 heterodimers detected in situ by proximity ligation on angiogenic sprouts. *EMBO J*, **29**:1377-1388.

Norrmen, C., Tammela, T., Petrova, T.V. and Alitalo, K. (2011) Biological basis of

therapeutic lymphangiogenesis. *Circulation*, **123**:1335-1351.

Olsson, A.K., Dimberg, A., Kreuger, J. and Claesson-Welsh, L. (2006) VEGF receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol*, **7**:359-371.

Paavonen, K., Puolakkainen, P., Jussila, L., Jahkola, T. and Alitalo, K. (2000) Vascular endothelial growth factor receptor-3 in lymphangiogenesis in wound healing. *Am J Pathol*, **156**:1499-1504.

Padera, T.P., Kadambi, A., di Tomaso, E., Carreira, C.M., Brown, E.B., Boucher, Y., Choi, N.C., Mathisen, D., Wain, J., Mark, E.J., Munn, L.L. and Jain, R.K. (2002) Lymphatic metastasis in the absence of functional intratumor lymphatics. *Science*, **296**:1883-1886.

Paget, S. (1889) The distribution of secondary growths in cancer of the breast. *Lancet*, **1**:571–573.

Pajusola, K., Aprelikova, O., Pelicci, G., Weich, H., Claesson-Welsh, L. and Alitalo, K. (1994) Signalling properties of FLT4, a proteolytically processed receptor tyrosine kinase related to two VEGF receptors. *Oncogene*, **9**:3545-3555.

Pan, M.R., Hou, M.F., Chang, H.C. and Hung, W.C. (2008) Cyclooxygenase-2 up-regulates CCR7 via EP2/EP4 receptor signaling pathways to enhance lymphatic invasion of breast cancer cells. *J Biol Chem*, **283**:11155-11163.

Parkin DM, W.S., Ferlay J, Storm H (2008) Cancer incidence in five continents. Volume IX. *IARC Sci Publ*, 1-837.

Patel, D.D., Koopmann, W., Imai, T., Whichard, L.P., Yoshie, O. and Krangel, M.S. (2001) Chemokines Have Diverse Abilities to Form Solid Phase Gradients. *Clinical Immunology*, **99**:43-52.

Peiper, S.C., Wang, Z.X., Neote, K., Martin, A.W., Showell, H.J., Conklyn, M.J., Ogborne, K., Hadley, T.J., Lu, Z.H., Hesselgesser, J. and Horuk, R. (1995) The Duffy antigen/receptor for chemokines (DARC) is expressed in endothelial cells of Duffy negative individuals who lack the erythrocyte receptor. *J Exp Med*, **181**:1311-1317.

Pepper, M.S. (2001) Lymphangiogenesis and tumor metastasis: myth or reality? *Clin Cancer Res*, **7**:462-468.

Porcile, C., Bajetto, A., Barbero, S., Pirani, P. and Schettini, G. (2004) CXCR4 activation induces epidermal growth factor receptor transactivation in an ovarian cancer cell line. *Ann N Y Acad Sci*, **1030**:162-169.

Porcile, C., Bajetto, A., Barbieri, F., Barbero, S., Bonavia, R., Biglieri, M., Pirani, P., Florio, T. and Schettini, G. (2005) Stromal cell-derived factor-1alpha (SDF-1alpha/CXCL12) stimulates ovarian cancer cell growth through the EGF receptor transactivation. *Exp Cell Res*, **308**:241-253.

Porter, D.A., Krop, I.E., Nasser, S., Sgroi, D., Kaelin, C.M., Marks, J.R., Riggins, G. and Polyak, K. (2001) A SAGE (serial analysis of gene expression) view of breast tumor progression. *Cancer Res*, **61**:5697-5702.

Prasad, A., Fernandis, A.Z., Rao, Y. and Ganju, R.K. (2004) Slit protein-mediated

inhibition of CXCR4-induced chemotactic and chemoinvasive signaling pathways in breast cancer cells. *J Biol Chem*, **279**:9115-9124.

Raman, D., Baugher, P.J., Thu, Y.M. and Richmond, A. (2007) Role of chemokines in tumor growth. *Cancer Lett*, **256**:137-165.

Raman, D., Sobolik-Delmaire, T. and Richmond, A. (2011) Chemokines in health and disease. *Exp Cell Res*, **317**:575-589.

Ran, S., Volk, L., Hall, K. and Flister, M.J. (2009) Lymphangiogenesis and lymphatic metastasis in breast cancer. *Pathophysiology*.

Redondo-Munoz, J., Jose Terol, M., Garcia-Marco, J.A. and Garcia-Pardo, A. (2008) Matrix metalloproteinase-9 is up-regulated by CCL21/CCR7 interaction via extracellular signal-regulated kinase-1/2 signaling and is involved in CCL21-driven B-cell chronic lymphocytic leukemia cell invasion and migration. *Blood*, **111**:383-386.

Ribatti, D., Mangialardi, G., Vacca, A. (2006) Stephen Paget and the 'seed and soil' theory of metastatic dissemination. *Clin Exp Med*, **6**:145–149.

Rot, A. and von Andrian, U.H. (2004) Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. *Annu Rev Immunol*, **22**:891-928.

Rubin, J.B. (2009) Chemokine signaling in cancer: one hump or two? *Semin Cancer Biol*, **19**:116-122.

Saaristo, A., Tammela, T., Farkkila, A., Karkkainen, M., Suominen, E., Yla-Herttuala, S. and Alitalo, K. (2006) Vascular endothelial growth factor-C accelerates diabetic wound healing. *Am J Pathol*, **169**:1080-1087.

Sabin, F.R. (1909) The lymphatic system in human embryos, with a consideration of the morphology of the system as a whole. *Am J Anat*, **9**:43-91

Sadir, R., Imberty, A., Baleux, F. and Lortat-Jacob, H. (2004) Heparan sulfate/heparin oligosaccharides protect stromal cell-derived factor-1 (SDF-1)/CXCL12 against proteolysis induced by CD26/dipeptidyl peptidase IV. *J Biol Chem*, **279**:43854-43860.

Saeki, H., Moore, A.M., Brown, M.J. and Hwang, S.T. (1999) Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *J Immunol*, **162**:2472-2475.

Saharinen, P., Tammela, T., Karkkainen, M.J. and Alitalo, K. (2004) Lymphatic vasculature: development, molecular regulation and role in tumor metastasis and inflammation. *Trends Immunol*, **25**:387-395.

Salcedo, R., Wasserman, K., Young, H.A., Grimm, M.C., Howard, O.M., Anver, M.R., Kleinman, H.K., Murphy, W.J. and Oppenheim, J.J. (1999) Vascular endothelial growth factor and basic fibroblast growth factor induce expression of CXCR4 on human endothelial cells: In vivo neovascularization induced by stromal-derived factor-1alpha. *Am J Pathol*, **154**:1125-1135.

Sanchez-Sanchez, N., Riol-Blanco, L., de la Rosa, G., Puig-Kroger, A., Garcia-Bordas, J.,

Martin, D., Longo, N., Cuadrado, A., Cabanas, C., Corbi, A.L., Sanchez-Mateos, P. and Rodriguez-Fernandez, J.L. (2004) Chemokine receptor CCR7 induces intracellular signaling that inhibits apoptosis of mature dendritic cells. *Blood*, **104**:619-625.

Sanchez-Sanchez, N., Riol-Blanco, L. and Rodriguez-Fernandez, J.L. (2006) The multiple personalities of the chemokine receptor CCR7 in dendritic cells. *J Immunol*, **176**:5153-5159.

Sancho, M., Vieira, J.M., Casalou, C., Mesquita, M., Pereira, T., Cavaco, B.M., Dias, S. and Leite, V. (2006) Expression and function of the chemokine receptor CCR7 in thyroid carcinomas. *J Endocrinol*, **191**:229-238.

Schacht, V., Ramirez, M.I., Hong, Y.K., Hirakawa, S., Feng, D., Harvey, N., Williams, M., Dvorak, A.M., Dvorak, H.F., Oliver, G. and Detmar, M. (2003) T1alpha/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. *EMBO J*, **22**:3546-3556.

Schoppmann, S.F., Birner, P., Stockl, J., Kalt, R., Ullrich, R., Caucig, C., Kriehuber, E., Nagy, K., Alitalo, K. and Kerjaschki, D. (2002) Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. *Am J Pathol*, **161**:947-956.

Schulte-Merker, S., Sabine, A. and Petrova, T.V. (2011) Lymphatic vascular morphogenesis in development, physiology, and disease. *J Cell Biol*, **193**:607-618.

Shields, J.D., Fleury, M.E., Yong, C., Tomei, A.A., Randolph, G.J. and Swartz, M.A. (2007) Autologous chemotaxis as a mechanism of tumor cell homing to lymphatics via interstitial flow and autocrine CCR7 signaling. *Cancer Cell*, **11**:526-538.

Shields, J.D., Emmett, M.S., Dunn, D.B., Joory, K.D., Sage, L.M., Rigby, H., Mortimer, P.S., Orlando, A., Levick, J.R. and Bates, D.O. (2007b) Chemokine-mediated migration of melanoma cells towards lymphatics--a mechanism contributing to metastasis. Oncogene, 26:2997-3005.

Shields, J.D., Kourtis, I.C., Tomei, A.A., Roberts, J.M. and Swartz, M.A. (2010) Induction of Lymphoidlike Stroma and Immune Escape by Tumors That Express the Chemokine CCL21. *Science*.

Shim, H., Lau, S.K., Devi, S., Yoon, Y., Cho, H.T. and Liang, Z. (2006) Lower expression of CXCR4 in lymph node metastases than in primary breast cancers: Potential regulation by ligand-dependent degradation and HIF-1[alpha]. *Biochemical and Biophysical Research Communications*, **346**:252-258.

Singh, B., Berry, J.A., Vincent, L.E. and Lucci, A. (2006) Involvement of IL-8 in COX-2-mediated bone metastases from breast cancer. *J Surg Res*, **134**:44-51.

Sironi, M., Conti, A., Bernasconi, S., Fra, A.M., Pasqualini, F., Nebuloni, M., Lauri, E., De Bortoli, M., Mantovani, A., Dejana, E. and Vecchi, A. (2006) Generation and characterization of a mouse lymphatic endothelial cell line. *Cell Tissue Res*, **325**:91-100.

Skobe, M., Hawighorst, T., Jackson, D.G., Prevo, R., Janes, L., Velasco, P., Riccardi, L., Alitalo, K., Claffey, K. and Detmar, M. (2001) Induction of tumor lymphangiogenesis by

VEGF-C promotes breast cancer metastasis. *Nat Med*, 7:192-198.

Sleeman, J., Schmid, A. and Thiele, W. (2009) Tumor lymphatics. *Semin Cancer Biol*.

Sleeman, J.P. and Cremers, N. (2007) New concepts in breast cancer metastasis: tumor initiating cells and the microenvironment. *Clin Exp Metastasis*, **24**:707-715.

Sleeman, J.P., Krishnan, J., Kirkin, V. and Baumann, P. (2001) Markers for the lymphatic endothelium: in search of the holy grail? *Microsc Res Tech*, **55**:61-69.

Sozzani, S., Rusnati, M., Riboldi, E., Mitola, S. and Presta, M. (2007) Dendritic cell-endothelial cell cross-talk in angiogenesis. *Trends Immunol*, **28**:385-392.

Stacker, S.A., Caesar, C., Baldwin, M.E., Thornton, G.E., Williams, R.A., Prevo, R., Jackson, D.G., Nishikawa, S., Kubo, H. and Achen, M.G. (2001) VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. *Nat Med*, 7:186-191.

Strieter, R.M., Burdick, M.D., Mestas, J., Gomperts, B., Keane, M.P. and Belperio, J.A. (2006) Cancer CXC chemokine networks and tumour angiogenesis. *Eur J Cancer*, **42**:768-778.

Su, J.L., Yen, C.J., Chen, P.S., Chuang, S.E., Hong, C.C., Kuo, I.H., Chen, H.Y., Hung, M.C. and Kuo, M.L. (2007) The role of the VEGF-C/VEGFR-3 axis in cancer progression. *Br J Cancer*, **96**:541-545.

Sun, R.H., Wang, G.B., Li, J. and Cui, J. (2009) Role of CCL21/CCR7 in invasion of colorectal carcinoma cell line SW480. *Ai Zheng*, **28**:708-713.

Sundar, S.S. and Ganesan, T.S. (2007) Role of lymphangiogenesis in cancer. *J Clin Oncol*, **25**:4298-4307.

Takeuchi, H., Fujimoto, A., Tanaka, M., Yamano, T., Hsueh, E. and Hoon, D.S. (2004) CCL21 chemokine regulates chemokine receptor CCR7 bearing malignant melanoma cells. *Clin Cancer Res*, **10**:2351-2358.

Tammela, T. and Alitalo, K. (2010) Lymphangiogenesis: Molecular mechanisms and future promise. *Cell*, **140**:460-476.

Tammela, T., Petrova, T.V. and Alitalo, K. (2005) Molecular lymphangiogenesis: new players. *Trends Cell Biol*, **15**:434-441.

Tammela, T., Saaristo, A., Holopainen, T., Lyytikka, J., Kotronen, A., Pitkonen, M., Abo-Ramadan, U., Yla-Herttuala, S., Petrova, T.V. and Alitalo, K. (2007) Therapeutic differentiation and maturation of lymphatic vessels after lymph node dissection and transplantation. *Nat Med*, **13**:1458-1466.

Tang, Y., Zhang, D., Fallavollita, L. and Brodt, P. (2003) Vascular endothelial growth factor C expression and lymph node metastasis are regulated by the type I insulin-like growth factor receptor. *Cancer Res*, **63**:1166-1171.

Thiele, W. and Sleeman, J.P. (2006) Tumor-induced lymphangiogenesis: a target for cancer therapy? *J Biotechnol*, **124**:224-241.

Timoshenko, A.V., Chakraborty, C., Wagner, G.F. and Lala, P.K. (2006) COX-2-mediated stimulation of the lymphangiogenic factor VEGF-C in human breast cancer. *Br J Cancer*, **94**:1154-1163.

Timoshenko, A.V., Rastogi, S. and Lala, P.K. (2007) Migration-promoting role of VEGF-C and VEGF-C binding receptors in human breast cancer cells. *Br J Cancer*, **97**:1090-1098.

Tobler, N.E. and Detmar, M. (2006) Tumor and lymph node lymphangiogenesis--impact on cancer metastasis. *J Leukoc Biol*, **80**:691-696.

Ueda, M., Terai, Y., Kumagai, K., Ueki, K., Yamaguchi, H., Akise, D. and Ueki, M. (2001) Vascular endothelial growth factor C gene expression is closely related to invasion phenotype in gynecological tumor cells. *Gynecol Oncol*, **82**:162-166.

Ueno, T., Toi, M., Saji, H., Muta, M., Bando, H., Kuroi, K., Koike, M., Inadera, H. and Matsushima, K. (2000) Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. *Clin Cancer Res*, **6**:3282-3289.

Van der Auwera, I., Vermeulen, P., Van Marck, E. and Dirix, L. (2004) Lack of lymphangiogenesis during breast carcinogenesis. *J Clin Pathol*, **57**:1342-1343.

van der Schaft, D.W., Pauwels, P., Hulsmans, S., Zimmermann, M., van de Poll-Franse, L.V. and Griffioen, A.W. (2007) Absence of lymphangiogenesis in ductal breast cancer at the primary tumor site. *Cancer Lett*, **254**:128-136.

Veikkola, T., Jussila, L., Makinen, T., Karpanen, T., Jeltsch, M., Petrova, T.V., Kubo, H., Thurston, G., McDonald, D.M., Achen, M.G., Stacker, S.A. and Alitalo, K. (2001) Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J*, **20**:1223-1231.

Vila-Coro, A.J., Rodriguez-Frade, J.M., Martin De Ana, A., Moreno-Ortiz, M.C., Martinez, A.C. and Mellado, M. (1999) The chemokine SDF-1alpha triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway. *FASEB J*, **13**:1699-1710.

Vines, C.M., Xue, M., Maestas, D.C., Cimino, D.F. and Prossnitz, E.R. (2002) Regulation of N-formyl peptide-mediated degranulation by receptor phosphorylation. *J Immunol*, **169**:6760-6766.

Vivanco, I. and Sawyers, C.L. (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer*, **2**:489-501.

Vleugel, M.M., Bos, R., van der Groep, P., Greijer, A.E., Shvarts, A., Stel, H.V., van der Wall, E. and van Diest, P.J. (2004) Lack of lymphangiogenesis during breast carcinogenesis. *J Clin Pathol*, **57**:746-751.

Wang, J., Seethala, R.R., Zhang, Q., Gooding, W., van Waes, C., Hasegawa, H. and Ferris, R.L. (2008) Autocrine and paracrine chemokine receptor 7 activation in head and neck cancer: implications for therapy. *J Natl Cancer Inst*, **100**:502-512.

Wang, J., Zhang, X., Thomas, S.M., Grandis, J.R., Wells, A., Chen, Z.G. and Ferris, R.L. (2005) Chemokine receptor 7 activates phosphoinositide-3 kinase-mediated invasive and

prosurvival pathways in head and neck cancer cells independent of EGFR. *Oncogene*, **24**:5897-5904.

Waugh, D.J. and Wilson, C. (2008) The interleukin-8 pathway in cancer. *Clin Cancer Res*, **14**:6735-6741.

Wente, M.N., Mayer, C., Gaida, M.M., Michalski, C.W., Giese, T., Bergmann, F., Giese, N.A., Buchler, M.W. and Friess, H. (2008) CXCL14 expression and potential function in pancreatic cancer. *Cancer Lett*, **259**:209-217.

WHO (World Health Organization), World Cancer Report 2008, WHO Press:Geneva, Switzerland

Wick, N., Haluza, D., Gurnhofer, E., Raab, I., Kasimir, M.T., Prinz, M., Steiner, C.W., Reinisch, C., Howorka, A., Giovanoli, P., Buchsbaum, S., Krieger, S., Tschachler, E., Petzelbauer, P. and Kerjaschki, D. (2008) Lymphatic precollectors contain a novel, specialized subpopulation of podoplanin low, CCL27-expressing lymphatic endothelial cells. *Am J Pathol*, **173**:1202-1209.

Williams, C.S., Leek, R.D., Robson, A.M., Banerji, S., Prevo, R., Harris, A.L. and Jackson, D.G. (2003) Absence of lymphangiogenesis and intratumoural lymph vessels in human metastatic breast cancer. *J Pathol*, **200**:195-206.

Wilson, J.L., Burchell, J. and Grimshaw, M.J. (2006) Endothelins induce CCR7 expression by breast tumor cells via endothelin receptor A and hypoxia-inducible factor-1. *Cancer Res*, **66**:11802-11807.

Witte, M.H., Dellinger, M.T., McDonald, D.M., Nathanson, S.D., Boccardo, F.M., Campisi, C.C.C., Sleeman, J.P. and Gershenwald, J.E. (2011) Lymphangiogenesis and hemangiogenesis: Potential targets for therapy. *Journal of Surgical Oncology*, **103**:489-500.

Worbs, T. and Forster, R. (2007) A key role for CCR7 in establishing central and peripheral tolerance. *Trends Immunol*, **28**:274-280.

Wu, F.Y., Ou, Z.L., Feng, L.Y., Luo, J.M., Wang, L.P., Shen, Z.Z. and Shao, Z.M. (2008) Chemokine decoy receptor d6 plays a negative role in human breast cancer. *Mol Cancer Res*, **6**:1276-1288.

Yan, C., Zhu, Z.G., Yu, Y.Y., Ji, J., Zhang, Y., Ji, Y.B., Yan, M., Chen, J., Liu, B.Y., Yin, H.R. and Lin, Y.Z. (2004) Expression of vascular endothelial growth factor C and chemokine receptor CCR7 in gastric carcinoma and their values in predicting lymph node metastasis. *World J Gastroenterol*, **10**:783-790.

Yu, M., Tang, Z., Alousi, S., Berk, R.S., Miller, F. and Kosir, M.A. (2007) Expression patterns of lymphangiogenic and angiogenic factors in a model of breast ductal carcinoma in situ. *Am J Surg*, **194**:594-599.

Yu, S., Duan, J., Zhou, Z., Pang, Q., Wuyang, J., Liu, T., He, X., Xinfa, L. and Chen, Y. (2008) A critical role of CCR7 in invasiveness and metastasis of SW620 colon cancer cell in vitro and in vivo. *Cancer Biol Ther*, **7**:1037-1043.

Zhang, X.H., Huang, D.P., Guo, G.L., Chen, G.R., Zhang, H.X., Wan, L. and Chen, S.Y.

(2008) Coexpression of VEGF-C and COX-2 and its association with lymphangiogenesis in human breast cancer. *BMC Cancer*, **8**:4.

Zhang, C., Hao, L., Wang, L., Xiao, Y., Ge, H., Zhu, Z., Luo, Y. and Zhang, Y. (2011) Elevated IGFIR expression regulating VEGF and VEGF-C predicts lymph node metastasis in human colorectal cancer. *BMC Cancer*, **10**:184.

Zhao, B., Cui, K., Wang, C.-L., Wang, A.-L., Zhang, B., Zhou, W.-Y., Zhao, W.-H. and Li, S. (2011) The chemotactic interaction between CCL21 and its receptor, CCR7, facilitates the progression of pancreatic cancer via induction of angiogenesis and lymphangiogenesis. *Journal of Hepato-Biliary-Pancreatic Sciences*, 1-8.

Zimmermann, N., Conkright, J.J. and Rothenberg, M.E. (1999) CC chemokine receptor-3 undergoes prolonged ligand-induced internalization. *J Biol Chem*, **274**:12611-12618.

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

Zlotnik, A., Yoshie, O. and Nomiyama, H. (2006) The chemokine and chemokine receptor superfamilies and their molecular evolution. *Genome Biol*, **7**:243.

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Publications

Journal:

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Abstracts:

Tutunea-Fatan E., Majumder M., Lala P.K., 2011, "A Novel Lymphangiogenic Role of the CCL21/CCR7 Chemokine Axis in an *In Vitro* Breast Cancer Model," *1st Canadian Cancer Research Conference*, Nov. 2011,Tor onto, Canada

Tutunea-Fatan E., Majumder M., Lala P.K., 2011, "CCR7 Signalling Is a Regulator of VEGF-C Secretion in Breast Cancer Cells", *8th Oncology Research and Education Day*, Jun. 2011, London, Canada Abstract No. 67

Tutunea-Fatan E., Majumder M., Lala P.K., 2011, "The role of CCL21/CCR7 axis in breast cancer induced lymphangiogenesis," *102nd Annual Meeting of American Association for Cancer Research (AACR)*, Apr. 2011, Orlando, USA, Abstract No. 5152

Majumder M., Postovit L.M., Tutunea-Fatan E., Torres-Garcia E.J., Lala P.K., 2011, "Cyclooxygenase-2 promotes breast cancer progression by induction and sustenance of tumor-initiating cells," *AACR 1st Special Conference on Stem Cells, Development, and Cancer*, Mar. 2011, Vancouver, Canada, Abstract No. 248370_3

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Posters:

"A Novel Lymphangiogenic Role of the CCL21/CCR7 Chemokine Axis in an *In Vitro* Breast Cancer Model," presented at the *Murray Barr Research Day*, Oct. 2011, London, Canada

"CCR7 Signalling Is a Regulator of VEGF-C Secretion in Breast Cancer Cells," presented at the *8th Oncology Annual Research & Education Day,* Jun. 2011, London, Canada

"The Role of CCL21/CCR7 Axis in Breast Cancer Induced Lymphangiogenesis," presented at the *102nd Annual Meeting of American Association for Cancer Research (AACR)*, Apr. 2011, Orlando, USA

"The Role of CCL21/CCR7 Axis in Lymphatic Metastasis of Breast Cancer," presented at the *Murray Barr Research Day*, Oct. 2010, London, Canada