The Role of Chemokine Receptors in Breast Cancer Metastasis

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Abstract

Metastasis is a multi-step process during which cancer cells disseminate from the primary tumour and establish secondary tumours in distant sites. The mechanisms for organ-specific metastasis are poorly understood, although recent findings suggest the role of a number of chemokine receptors on various cancer cells such as breast CXCR4 as well as CCR7, are G protein-coupled chemokine receptor (GPCR) that have proven to be of considerable biological significance, since their expression has been shown on various malignant breast cancer cell lines, tumours and metastases. In this study the expression and function of CXCR4 and CCR7 was examined in a range of human breast cancer cell lines covering a spectrum of malignant and non-malignant phenotypes. The data revealed that while surface levels of CXCR4 and CCR7 were uniform across the entire panel of breast cancer cell lines, only highly invasive cells, metastatic in immunocompromised mice, expressed functional chemokine receptors. Moreover, multiple signalling pathways downstream of G proteins in the highly invasive cells were found to be activated, however chemokine treatment failed to activate any of the downstream kinase cascades examined in non-invasive cell lines. For the first time, to the best of our knowledge, chemokine receptor function was demonstrated to be subject to complex and tightly-controlled regulation in epithelial breast cancer cells via differential G protein-receptor complex formation and that this regulation might significantly contribute to the transition from non-metastatic to malignant tumours. Finally, the role of CXCR4 and CCR7 during breast cancer metastasis was verified using a humanised breast cancer metastasis mouse model. By modulating the expression of chemokine receptors using siRNA-mediated knockdown, metastasis of breast cancer cells to the lungs of SCID mice was dramatically inhibited.

In summary, the data point to distinct molecular mechanisms of chemokine receptor activation used by transformed invasive breast epithelial cells which leads to the metastatic spread of these cancer cells to distant sites. Improved understanding of the role of chemokine receptor/ligand interaction in metastasis may lead to novel approaches in the treatment and management of breast cancer as well as other solid tumour malignancies.
Statement of originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis being made available in the University Library.

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Jane Holland, BMPB (Hons)
December 2006
Publications arising from this work

**Journals**


**Conference Proceedings**

The 5th Peter MacCallum Cancer Centre Symposium (2003)

‘**Expression and function of the chemokine receptors CXCR4 and CCR7 on breast cancer cells**’

Poster Presentation

The 42nd Australian Scientific Medical and Research Conference (2003)

‘**SDF-1β and MIP-3β co-operate to promote an invasive phenotype of human breast cancer cells**’

Oral Presentation

The 34th Annual Scientific Meeting of the Australian Society for Immunology: Student Meeting (2004)

‘**Chemokines CXCL12 and CCL19 influence the metastatic pathway in human breast cancer cells**’

Oral Presentation

The 34th Annual Scientific Meeting of the Australian Society for Immunology (2004)

‘**Chemokines CXCL12 and CCL19 influence the metastatic pathway in human breast cancer cells**’

Oral Presentation

The Fluorescence Imagers of South Australia Meeting (FISA) (2005)

‘*Detection of GFP* metastatic breast cancer cells in vivo by fluorescent microscopy*

Oral Presentation- Invited Speaker
The 6th Peter MacCallum Cancer Centre Symposium (2005)
‘CXCL12/CXCR4 Influences the metastatic pathway in human breast cancer cells’
Poster Presentation

The 5th Dubrovnik FEBS Signalling Conference (2006)
‘CXCL12/CXCR4 Influences the metastatic pathway in human breast cancer cells’
Poster Presentation

The Max Delbruck Medical Centre of Research (2006)
Research Head: Walter Birchmeier
‘Chemokines CXCL12 and CCL19 regulate the metastatic pathway in human breast cancer cells’
Oral Presentation- Invited Speaker

The Curie Institute (2006)
Research Head: Frederic Soudou
‘Chemokines CXCL12 and CCL19 regulate the metastatic pathway in human breast cancer cells’
Oral Presentation- Invited Speaker

The Curie Institute (2006)
Research Head: Lionel Larue
‘Chemokines CXCL12 and CCL19 regulate the metastatic pathway in human breast cancer cells’
Oral Presentation- Invited Speaker

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# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CCR</td>
<td>CC chemokine receptor</td>
</tr>
<tr>
<td>CCL</td>
<td>CC chemokine ligand</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CXCR</td>
<td>CXC chemokine receptor</td>
</tr>
<tr>
<td>CXCL</td>
<td>CXC chemokine ligand</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>G418</td>
<td>geneticin</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidise</td>
</tr>
<tr>
<td>hu</td>
<td>human</td>
</tr>
<tr>
<td>IP</td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mu</td>
<td>mouse</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>PA</td>
<td>phosphatidic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered solution</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
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<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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RIPA  radioimmunoprecipitation
RT    room temperature
s     second
SCID  severely compromised immunodeficient mice
SDS   sodium dodecyl sulphate
WB    western blot
Wt    wild-type
CHAPTER 1
INTRODUCTION
1.1 Overview

Breast cancer is the most prevalent malignant disease in women of the Western world. It affects 10% of the female population and the frequency of diagnosed cases is rising each year (1). At this point in time the precise cause of breast cancer is not known largely due to the very complex and heterogeneous nature of this disease. At the molecular level breast cancer is characterised by a vast array of genetic and non-genetic aberrations affecting the regulation of crucial cellular processes. For these reasons breast cancer along with other solid tumour cancers has become increasingly difficult to study. However, in an effort to define these aberrations commonly reported in breast cancers, the study of cancer genetics and tumour cell biology has promoted a greater understanding of the development and progression of breast neoplasms.

In breast cancer patients, death and morbidity is believed to be the result from the metastatic disease state. Once a patient acquires a tumour cell with a metastatic potential, invasive disease stage is generally incurable by conventional therapies. Effectively the process of metastasis involves the dissemination of cancer cells from the primary tumour to specific sites on distant organs. Different molecular mechanisms as well as a host of contributing factors facilitate tumour cells to infiltrate the surrounding tissue, intravasate into the blood and lymph vessels and leave the vascular or lymphatic systems to a new tissue environment where they must be able to survive and proliferate in a microenvironment distinct from the primary tumour.

Chemokines and their receptors have recently been implicated as important factors involved in the metastatic process. Originally they were noted for their ability to direct the migration of leukocytes in inflammatory and immune responses and it is thought that tumour
cells use similar mechanisms to produce organ-specific metastasis. Many types of malignant cells have been reported to express a wide repertoire of chemokine receptors and are capable of responding to chemokines by increased directional migration, and in some cases proliferation and survival (2). The CXCL12/CXCR4 and CCR7/CCL19/CCL21 chemokine/receptor pairs have received a great deal of attention due to the discovery of their potential involvement in promoting metastasis (3-5). However, at the present time the precise molecular mechanisms by which tumour cells utilize chemokines and their receptors to achieve metastasis is not completely understood.

In this study an investigation into the expression and functional relationship of chemokines and their receptors in a variety of human breast cancer cell lines was performed in order to uncover potential mechanisms by which tumour cells may use chemokines and receptors to aid in the metastatic process. The following review summarizes what is known to date about breast cancer and metastasis in terms of epidemiology, clinical features, pathogenesis and contributing factors. In addition examples of common animal models used to study breast cancer are discussed, highlighting their uses and limitations. Finally an introduction to the chemokine family of proteins is provided as well as their involvement in tumour cell biology.
1.2 Breast Cancer

1.2.1. Epidemiology of breast cancer

In a recent estimate of the overall worldwide burden of cancer in the year 2000, more than 10 million new cancer cases were estimated and approximately 6 million cancer deaths reported (6). Breast cancer accounts for about 1 in 10 cancers and is the most frequent cancer affecting women (7). Since 10% of all cancers in the world are breast cancer (only affecting half of the population as breast cancer almost exclusively concerns women), it is now being considered an epidemic. In terms of the absolute number of incidences, breast cancer now ranks first not only in the industrialized world but also in developing countries. The worldwide mortality figure for breast cancer in the year 2002 was approximately 410,000 deaths (8, 9). The incidence and prevalence of breast cancer outweighs any other cancers affecting women (Fig 1.1). However, the frequency of breast cancer varies geographically, with Africa and Asia currently having incidence rates 10 times lower than those of North America and Northern Europe. Over time, clear increases have been seen in the global number of breast cancer cases: from 572,000 in 1980 to 1,050,000 in 2000 (6, 10). The basis for these increases currently unexplained, however possible reasons could be related to various environmental factors. By contrast, in a number of countries in the Western society breast cancer mortality rates are stable and even declining slightly. The basis for this decrease is possibly the result of widespread mammographic screening, more precise and earlier diagnosis along with improvements in treatments including the extensive use of Tamoxifen (8, 11).
1.2.1.1. **Clinical features of breast cancer**

Clinically, breast cancer is recognized as a highly heterogeneous disease characterized by a range of biological, histological and genetic differences (12). Breast tumours are derived from normal epithelial cells populating the inner lining of ductal or lobular structures in the breast that are surrounded by basement membranes (13). Breast cancer is believed to progress through sequential multi-step stages developing from normal cell growth to hyperplasia, through to carcinoma *in situ*, followed by invasive carcinoma and finally culminating in the metastatic disease state (Fig 1.2) (14, 15). Distinct clinical and histological features between invasive and non-invasive carcinomas are frequently observed (16). Of the non-invasive carcinomas, ductal carcinoma *in situ* (DCIS) is believed to be the precursor of invasive breast disease. In DCIS, malignant epithelial cells are confined within the lumens of the mammary ducts without evidence of invasion beyond the basement membrane into the adjacent breast stroma (16-18). Incidence of DCIS has increased significantly during the last decade contributing to almost 20% of all breast cancers diagnosed today (19). The identification of DCIS tumours is important, since if it is a truly pure DCIS, excision should result in cure, as metastatic spread cannot occur.

On the other hand the typical invasive carcinoma, unlike benign lesions, infiltrates surrounding tissues and therefore has a diffused margin. The most common histological type of breast cancers are generally infiltrating invasive ductal carcinomas, which contribute to 75% of all breast carcinomas while invasive lobular carcinomas form about 12% (18, 20). Other invasive subtypes include medullary, papillary and tubular carcinoma, each found in 1-10% of breast cancer patients (21). The recognition of the different types of invasive breast cancer is based on morphological grounds observed and classified over many years. It is now becoming apparent that the morphology can be surprisingly predictive of the underlying
molecular biology of the tumour. For example, in invasive tumours the expression of the adhesion molecule E-cadherin on the cell surface membrane is frequently lost thereby disturbing the normal tight epithelial cell-cell contacts which affects the overall invasiveness of the cancer cell (22).

It must be pointed out that the exact histological stages in the development of breast cancer are to some extent vague and undefined due to the fact that mammary tissue is heterogeneous consisting of different components of epithelial, mesenchymal, endothelial and lymphopoietic derivation (23). The genetic variance of invasive as well as non-invasive breast cancer is reflected by the wide spectrum of histological types and differentiation grades, however it seems that many, if not all invasive carcinomas are preceded by carcinoma in situ (24).

1.2.1.2. Prognostic and predictive breast cancer markers

Prognostic markers can be defined as factors which correlate with patient outcome while predictive factors are used to prospectively select responsiveness or resistance to a specific treatment (25). Both types of markers are used to provide information on the probable behaviour of a tumour and some markers can have both prognostic and predictive value. Research in recent years has identified a large number of potential biologic prognostic markers for breast cancer (Table 1.1). Of the markers listed, urokinase plasminogen activator (uPA) and its inhibitor PA1-1 are perhaps the most promising. Over 20 independent groups have reported that high levels of uPA predict adverse outcome in patients with breast cancer (26). uPA was originally believed to mediate cancer dissemination by promoting degradation of the extracellular matrix (ECM), thus allowing malignant cells to invade locally and eventually spread to distant sites. New data, however, suggest that the uPA system plays a broader role in breast cancer and is involved at multiple stages in the formation and
progression of the disease (25). The data show\(\) that as well as their involvement in breast cancer progression, uPA and PAI-1 are upregulated in most types of cancers (27). Furthermore, levels of uPA and PAI-1 present in primary tumor tissue are associated with poor responses to palliative endocrine therapy and generally correlate with adverse prognosis. These findings suggest that high levels of uPA and/or PAI-1 reflect an aggressive phenotype that may be overcome or suppressed by early systemic therapy in the adjuvant setting.

The most widely used predictive marker in oncology is the oestrogen receptor (ER) which is routinely utilised for selecting hormone responsive breast cancers (28). Although originally introduced to predict response to endocrine ablative therapy for patients with advanced breast cancer, the ER is now more widely used to select patients with early breast cancer likely to respond to the anti-oestrogen, Tamoxifen. In a recent meta-analysis involving over 37,000 women, ER-positive patients were 7-times less likely to develop recurrent disease than ER-negative patients after at least 5 years of adjuvant Tamoxifen treatment (29). The detection of the progesterone receptor (PR) is also used to select hormone-responsive breast cancers. Early work showed that patients with advanced breast cancer were more likely to respond to hormone therapy if their primary cancer expressed both ER and PR compared to those tumours containing ER but lacking PR (30). Knowledge of PR status does not however, appear to enhance the predictive ability of ER in the adjuvant setting (29).

A more recently introduced predictive marker in breast cancer is the \(\text{HER-2}\) oncogene. It is amplified and/or overexpressed in approximately 30% of human breast tumours and the overexpression is associated with increased tumour aggressiveness, increased rates of recurrence, and increased mortality in node-positive patients, while the influence in node-negative patients is more variable (31). \(\text{HER-2}\) is used for selecting patients with advanced
breast cancer for treatment with the therapeutic antibody trastuzumab (Herceptin). In adjuvant treatment, overproduction of HER-2 may also indicate an enhanced sensitivity to high-dose anthracycline-based regimens. On the other hand, in both early and advanced breast cancer, high concentrations of HER-2 appear to correlate with a lower probability of response to hormone therapy. Interpretation of many studies of HER-2, however, are limited by variability in the methods used to detect overexpression, definition of HER-2-positive patients and the fact that most are retrospective analyses.

In summary, the use of selected markers in breast cancer should lead to an improved and cost effective management of these patients that will hopefully lead to enhanced survival and a better quality of life.

1.2.2 Breast cancer tumourgenesis

1.2.2.1. Overview

The major steps of tumour progression can be defined by a number of histopathological criteria: (1) hyperplasia: hyperproliferation of cells; (2) adenoma: encapsulated tumours with relatively normal cells and without infiltration of surrounding tissue; (3) carcinoma: invasive tumours where cells degrade the basal membrane and invade into the surrounding tissue; (4) metastasis: the final stage in tumour progression when tumour cells disseminate either via the hematogenous or the lymphogenic pathway to local lymph nodes and distant organs (32). Studies have shown that when cancer cells reach the final stage of tumour development and solid secondary tumours are established, the chances of long-term survival are heavily reduced (33). Of these cancer patients with tumours that have metastasized, 90% will not survive, making metastasis the most life-threatening aspect of cancer (1, 34). In the early
stages of breast cancer the most common site for metastatic involvement include the regional lymph nodes while in the advanced stages widespread metastatic disease occurs in bones (70%), lungs (66%) and liver (61%) (35). Metastatic tumour cells that have spread to these major organs have the capacity to remain dormant and undetectable for years or decades before developing into clinically relevant lesions. As a result patients who have been successfully treated for their primary tumour still live with the uncertainty of absolute cure or temporary remission (36). The molecular complexity of this cascade and the fact that it is a ‘hidden process’ occurring inside the body, has made it inherently difficult to observe or gain a detailed understanding of the origin and mechanisms of the disease. However, through the development of new technologies, advances in molecular techniques and the availability of metastatic animal models, the understanding of this complex process is slowly unraveling (37-39).

1.2.2.2. Pathogenesis of breast cancer metastasis

The process of metastasis is a highly selective, multi-step process that involves changes in many genes and gene products that are critical for the regulation of numerous cellular functions. The ability to migrate and invade through the basement membrane characterises the transition from a non-invasive to an invasive cancer cell and is a major hallmark of the malignant phenotype (34). Specifically, metastasis of breast cancer cells, like any potentially cancerous cell, is the result of several transformation steps including proliferation, detachment of neoplastic cells, breakdown of the basement membrane, invasion of the ECM, angiogenesis, movement across vascular barriers and eventually proliferation of cancer cells at sites distant from the primary tumour (Fig 1.3) (40). In order for tumour cells to produce clinically detectable lesions, metastatic cells must complete all the steps in this process.
The movement of cancer cells from the primary tumour to specific sites on distant organs almost certainly involves the blood circulatory system, however the lymphatic system has also been shown to play a significant role (41). It was proposed that cancer cells leave the primary tumour through the lymphatic system where they can enter the lymph nodes during early stages of tumour growth (37). The disseminated tumour cells proliferate and form solid metastases in the lymph nodes, whereas tumour cells that spread to distant sites through the blood, die or remain dormant. At later stages, tumour cells spread from the established lymph-node metastases to distant sites where they are able to form solid metastases. As a result, metastasis to other organs is dependent on the presence of lymph-node metastases.

In another model of metastasis, cells are thought to spread through the blood from the primary tumour to distant sites where they progress to ‘full-blown’ metastases without previous entry through the lymph nodes. In patients with breast cancer, this haematogenous dissemination seems to be a very early event in metastatic progression and the presence of these cells in the blood may accurately predict the development of distant metastases. Both models demonstrate that hematogenous dissemination can occur from the overt metastases that eventually develop into the lymph nodes and/or in the distant organs. A genetic comparison of the primary tumour, lymph-node, bone marrow or circulating metastases may help clarify the sequence of events involved in the metastatic spread of cancer cells.

1.2.2.3. **Organ-specific metastasis: ‘seed and soil’**

The pattern of metastasis is believed to be a series of non-random events. In 1889, Stephen Paget put forward the ‘seed and soil’ theory proposing that selected tumour cells (the ‘seed’) have specific affinity for the milieu of certain target organ microenvironment (the ‘soil’) (40). This was supported by the evidence provided from an examination of over 900 autopsies of
patients with different primary tumours. Paget documented a site-specific occurrence of metastases to visceral organs and bone and concluded that metastases formed only when the seed and soil were compatible. Other experimental data supporting the ‘seed and soil’ hypothesis were subsequently provided by studies documenting metastasis to preferential site-specific organs in other cancers such as breast, ovarian and prostate (4, 42-44). Some years later, Paget’s theory was challenged by James Ewing who suggested that metastatic dissemination occurred by purely mechanical factors which were the result of the anatomical structure of the vascular system (45). Ewing’s observations also implicated that tumour cells traverse randomly through the circulation until size restrictions cause them to arrest in capillary bed of organs distant from the primary tumour site. In fact these two theories are not mutually exclusive with current evidence from in vitro and in vivo studies supporting a role for both. In a series of autopsies, Lenord Weiss was able to further document that larger numbers of bone metastases than would be expected based solely on blood-flow patterns were identified for both breast and prostate cancer. By contrast, fewer skin metastases, again based only on blood-flow patterns were found for osteosarcomas, stomach and testicular cancers (41). The results of this study brought validation to the revered ‘seed and soil’ hypothesis first observed by Paget and since then multiple studies have been published providing supporting evidence for Paget’s seed and soil theory.

1.2.3 Factors influencing breast cancer metastasis

To date the precise causes of breast cancer are still not known, although a number of contributing factors have been identified. It is however widely accepted that the common phenotypic abnormality of breast cancer cells is an uncontrolled cell growth. The transformation of a normal to a cancerous cell appears to depend on mutations on genes that control cell cycle progression, thus leading to a loss in the regulation of normal cell growth.
Over the past twenty years a number of genetically altered genes, namely oncogenes, tumour suppressor genes (TSGs) and more recently, metastatic suppressor genes (MSGs), have been identified in breast cancer cells and tissues (Table 1.2). This subchapter presents a summary of the most prominent oncogenes, TSGs and MSGs implicated in breast cancer describing some of their functions in terms of cancer cell biology, tumour development and patient prognosis.

1.2.3.1. Oncogenes

Numerous oncogenes including \(HER-2\) and \(e-Myc\) have been characterised in human cancers, but relatively few have been found to be crucial in the progression of breast cancers. Changes in oncogenes or tumour-promoter genes usually lead to gain-of-function events as a result of gene amplification, point mutation or promoter activation mechanisms (46). Certain oncogenes are known to cause mammary cancer when overexpressed in transgenic mouse models, and specific oncogenes lead to distinct phenotypes in mice (47). Amplification and overexpression of these oncogenes and oncogene products are the major mechanisms through which these genes participate in carcinogenesis.

The \(HER-2\) (human epidermal receptor 2, also referred to as \(HER-2/neu\) or \(erbB-2\)) has proved to be of particular interest in human breast cancer. It encodes a 185kD transmembrane tyrosine kinase growth factor receptor which when activated leads to the activation of multiple signalling transduction pathways that regulate cell proliferation, angiogenesis, altered cell-cell interactions, increased cell motility, metastasis and resistance to apoptosis (48). The \(HER-2\) gene is amplified in 20-30% of invasive breast tumours and has been shown to correlate with aggressive features and poor short term prognosis in cancer patients (49). The knowledge of \(HER-2\) status can be of value in determining choice of therapy since there
is a clear evidence that HER-2 positive tumours show poor response to endocrine therapy (50).

The oncoprotein c-Myc is frequently overexpressed in breast cancer. It encodes a nuclear phosphoprotein which acts as a transcriptional regulator controlling cell proliferation, differentiation and apoptosis (50). Amplification and over-expression of c-Myc is reported to occur in 15-25% of breast tumours and in some cases has been associated with aggressive clinical features and a poor prognosis (30). While many agree that the overexpression of this gene is clearly associated with breast cancer, there continues to be controversy as to whether or not aberrant Myc expression alone is sufficient for breast carcinogenesis.

1.2.3.2. Tumour Suppressor Genes

TSGs, on the other hand, refer to those genes whose loss-of-function results in the promotion of malignancies with mechanisms of tumour-suppressor inactivation exemplified by a loss of heterozygosity (LOH), mutation or methylation (46). TSGs are considered to be negative regulators of growth or have the potential to affect invasion or metastatic potential. Common TSGs reported in breast cancer include p53, BRAC-1 and BRCA-2.

The first TSG linked to hereditary breast cancer was p53 and since the detection of a mutated form of p53 in lung and colon cancers 14 years ago, p53 has perhaps been the most studied TSG (51). p53 codes for a 393 amino acid protein that has a multifunctional transcription factor involved in mediating cell response to various stresses, mainly by inducing or repressing a number of genes involved in cell cycle arrest, senescence, apoptosis, DNA repair, and angiogenesis. According to this important function, p53 activity is controlled in a very complex manner through the dozens of proteins controlling cell cycle progression, DNA
Chapter 1

maintenance and genome integrity, repair after DNA damage and apoptosis (52). p53 is one of the most commonly mutated genes in all human cancers (approximately in 50% of cancers) and in 20-30% of breast cancers (53, 54). While the prognostic and predictive value of p53 is still debated, there is an increasing interest for p53-based therapies.

Only 5–10% of breast cancer cases can be attributed to one of several familial syndromes, the most common being hereditary breast and ovarian cancer caused by mutations of the breast cancer associated gene 1 (BRCA-1) and BRCA-2 tumor-suppressor genes (50, 53). The functions of the BRCA proteins are not fully understood, although it is clear that they play a role in the control of transcription, regulation of the cell cycle and management of DNA damage. The inheritance of a deleterious BRCA mutation is accompanied by a 50–80% risk of developing breast cancer, 60% risk of developing a contralateral breast cancer and 15–25% risk of developing ovarian cancer. The clinical management of BRCA heterozygotes involves several strategies of primary, secondary and tertiary prevention. These include risk-reducing surgery, chemoprevention, lifestyle changes and increased surveillance (30, 55).

1.2.3.3. Metastasis suppressor genes

Metastasis suppressor genes (MSG) are a biologically diverse novel class of genes shown to prevent the process of metastasis without drastically affecting the growth of the primary tumour (56, 57). It is commonly accepted that the metastatic disease process requires completion of many steps, so the elimination of only one step in the cascade will terminate the process. Thus the restoration of MSG expression in metastatically competent tumour cells to the extent that it can interrupt a step of the metastatic cascade might prove to be of clinical benefit in patients in which the metastatic process has not been completed. To date in breast
cancer, there have been a few candidates that fit into this category; they include NM23, BRMS-1, KiSS-1, E-cadherin, maspin and KAI1 (57).

1.2.3.4. Other molecular Factors

Many other molecular factors are believed to influence the ability of tumour cells to enter the metastatic pathway. They include growth factors, adhesion molecules, proteolytic enzymes and chemokines (58, 59). A common role demonstrated for these factors is to assist tumour cells to complete specific steps in the metastatic cascade. For example it is believed that the detachment of cancer cells from the primary tumour requires the loss or dysfunction of homotypic cell-cell adhesion, in which epithelial tissue is mediated largely by the cadherin family of cell adhesion molecules, E-cadherin in particular (60). At the same time the ability of tumour cells to proteolytically degrade components of the ECM and basement membrane to invade or metastasize depend on the increased expression and activity of metalloproteinases (MMPs) (61, 62). More recently it has been suggested that tumour cells use chemoattractant signals presented by chemokines to home to certain organs to produce metastases (4, 40, 45). However the precise manner in which many of these mediators influence metastasis still remains to be established with mechanistic studies ongoing.

1.3 Mouse Models to Study Breast Cancer

For numerous reasons, the molecular analysis of metastasis in vitro can only provide a partial understanding of the complex events involved in tumourgenesis; furthermore studies conducted in patients can only be performed retrospectively. As a result mouse tumour models were developed and have been instrumental in furthering the understanding of the multifaceted events involved in cancer progression (63-65). To date there are many methods
available to study metastasis in mice, each with their own limitations and advantages in modelling human breast cancer (66). The most commonly used systems include transgenic mice, xenograft or syngeneic transplantations of tumour cells and chemically-induced carcinogenesis models (32). Collectively these models have led to the identification of many molecular aberrations in cancer cells that can contribute to their ability to metastasize. However it should be stressed that the fundamental differences in the anatomy, physiology and biochemistry of mice compared to humans exist, raising the question of accuracy in comparing mouse and human cancers (66, 67). Also it is important to note that no one model can represent all of the different stages or forms of human breast cancer. Nevertheless these models do represent an important and valuable source of novel insights into the molecular principles of multistage carcinogenesis (67).

1.3.1. Transgenic mouse models

The generation of rodent models with 'genetically engineered' gene mutations (transgenic, knockout) has been the most useful in terms of studying and understanding the development of breast carcinomas. In order to mimic human breast cancer many transgenes have been generated by manipulation of the mouse genome through gain of function or knockout techniques of critical components in oncogenic pathways (68, 69). The most common models used for breast cancer studies cover a wide range of various targets such as growth factors, receptors, cell cycle regulators, signal transduction mediators, cellular differentiation effectors, oncogenes and tumour suppressor genes (68, 70). Specific promoters can be used to drive the expression of transgenes in the mammary epithelium with many known oncogenes expressed under their control to initiate or modulate breast carcinogenesis in mice. For example in the MMTV-Neu and MMTV-PyMT transgenic mice, the expression of the oncogene is driven by the mouse mammary tumour virus promoter; MMTV (64).
The first reported transgenic mouse model of breast cancer was the MMTV-myc model in which overexpression of the c-Myc transcription factor in the mammary gland resulted in spontaneous mammary adenocarcinoma (71). Since then, more than 100 transgenic models (mainly murine) have been generated for the study of mammary gland biology and breast cancer therapy and prevention. The most commonly used transgenic mouse models that develop metastatic mammary cancer include the HER-2/Neu, polyoma middle T antigen (PyMT), simian virus 40 (SV40) T antigen, Ha-Ras, Wnt-1, TGF-α, and c-Myc.

1.3.1.1. MMTV-Neu

As discussed previously the amplification of the HER-2/Neu oncogene is associated with 20 to 30% of human breast cancers and is correlated with aggressive tumour behaviour and poor prognosis. The precise mechanism that HER-2 is amplified in breast tumour cells is unknown; therefore mouse models of breast cancer initiated by HER-2 have proven useful for investigating the process of tumourigenesis. For example, transgenic mice in which either wild-type Neu (c-Neu) or activated Neu (NeuNT) is constitutively overexpressed in the mammary gland develop invasive mammary adenocarcinomas (72). However, the extent to which established Neu-induced tumours and metastatic lesions remain dependent upon this oncogeneic pathway for maintenance of the transformed state is unknown.

Doxycycline-dependent expression of HER-2/Neu in mammary epithelial cells of transgenic mice also results in invasive mammary carcinoma and extensive metastasis, yet the tumours regress with the loss of HER-2/Neu expression upon the withdrawal of doxycycline. However, most mice exhibit recurrences of the tumours (73). These recurrent tumours exhibit epithelial-mesenchymal transition (EMT), which seems to be mediated by the upregulated expression of
the transcriptional repressor Snail, a molecular process shown to have a high prognostic value in predicting human breast cancer recurrence (74).

1.3.1.2.  

**MMTV-PyMT**

Mammary gland-specific expression of PyMT under the control of the MMTV promoter/enhancer in transgenic mice (MMTV-PyMT) results in widespread transformation of the mammary epithelium and in the development of multifocal mammary adenocarcinomas and metastatic lesions in the lymph nodes and in the lungs (75). Tumor formation and progression in these mice is characterized by four stages: hyperplasia, adenoma/mammary intra-epithelial neoplasia, and early and late carcinoma. The close similarity of this model to human breast cancer is also exemplified by the fact that in these mice a gradual loss of steroid hormone receptors (estrogen and progesterone) and β1-integrin is associated with overexpression of HER-2 and cyclin D1 in late-stage metastatic cancer (76). The MMTV-PyMT mouse model of breast cancer is furthermore characterized by short latency, high penetrance, and a high incidence of lung metastasis occurring independently of pregnancy and with a reproducible kinetics of progression.

1.3.1.3.  

**Inducible and conditional knockout systems**

Investigating the functional role of distinct genes during the multiple stages of breast carcinogenesis requires the ability to modulate their function in time and space. Inducible transgene expression can be obtained by the use of the bacteria-derived tetracycline-inducible system permitting the switching on or off (Tet-On/Tet-Off system) of a gene of interest in a tissue- and time-specific manner (77). In contrast, mice are modified by the genetic ablation of a gene of interest in an inducible manner to generate conditional knockouts with the use of
the Cre/loxP phage recombinase system, as an example (78). To inactivate a gene at a certain time point in mammary epithelial cells, recombinase activity can be controlled by the expression of a tamoxifen-inducible version of Cre (MMTV-ERTM-Cre) or by using the tetracycline-inducible system to drive Cre expression (79).

The use of conditional knockout is employed in situations where the specific knockdown of genes is embryonic lethal. For example in the case of BRAC-1 this difficulty has been overcome using a Cre-loxP approach (80). The knockout mice are generated by crossing a BRAC-1-conditional allele with mice carrying MMTV-Cre or WAP-Cre transgenes that are expressed predominantly in the mammary epithelium. This allows Cre-mediated recombination of BRAC-1, primarily in the mouse mammary epithelium.

Taken together, these examples indicate that transgenic mouse models of breast cancer metastasis are essential in understanding the role of several molecules modulating key steps during malignant progression.

1.3.2. Implantation/transplantation models

There are various ways to mimic breast cancer growth and metastasis using tumour transplantation experiments. Most models use established cancer cell lines and spontaneously or genetically engineered immortalized normal epithelial cells. The site of cancer cell injection, together with the specific choice of breast cancer cell line used, largely defines primary and secondary metastatic growth. Orthotopic implantation (implantation of tumour cells into the same organ or tissue as their site of origin) into the mammary fat pad is commonly selected since the formation of primary tumours and the subsequent formation of metastasis in part resembles the multiple stages involved in the development of human breast
cancer (81). Orthotopic implantation (implantation of cancer cells into the same anatomical site as the primary tumour) into the mammary fat pad is commonly selected since the formation of tumours and the subsequent formation of metastases partly resembles the multi-step progression of breast cancer in human patients. In contrast, intravenous (i.v.) tail vein injection results mainly in lung metastasis, whereas portal vein injection promotes colonization of cancer cells to the liver and intracardiac infusion gives rise to a broad target organ spectrum, which includes bone. In some cases subcutaneous injection of breast cancer cells is performed but this microenvironment is often too different from breast tissue and hence orthotopic implantation is preferred.

1.3.2.1. Syngeneic transplantation

The transplantation of cancer cells from one mouse into another mouse with identical genetic backgrounds (syngeneic transplantation) bypasses potential immunologic host-versus-graft reaction and concomitantly allows the investigation of the contribution of an intact immune system to malignant tumour progression (82). Syngeneic mouse models have been employed to establish organ-specific metastasis by several rounds of transplantation/metastasis formation and the selection of metastatic cell lines in vivo (82). For example, 4T1 cells, which were originally derived from a spontaneous mouse mammary tumour of a BALB/c mouse, grow rapidly when injected into the mammary fat pad of a syngeneic animal and are able to metastasize to lungs, liver, bone, and brain (83). Derived subclones of 4T1 cells, which exhibit various degrees of metastatic ability, have been employed recently to generate distinct gene expression signatures for each stage of tumour progression, namely primary tumour formation, lymph node colonization, metastatic outgrowth in the lymph node, and distant organ metastasis (84). This and other syngeneic mouse models have been successfully
employed for the testing of experimental drugs designed to interfere with tumor malignancy (82).

1.3.2.2. **Xenograft transplantation**

To investigate the growth and metastasis of human breast cancer cells *in vivo*, xenograft transplantation experiments are performed in immunocompromised mice (85). Mice with immune defects ranging from depleted T-cells (nude mice) to absent T, B (SCID), and NK cells (SCID-Beige) are used as hosts (32, 66, 86-88). As with the syngeneic model, human breast cancer cells can be injected subcutaneously, intravenously, intracardiacly, or orthotopically. For example, MDA-MB-231 cells, an oestrogen-independent breast cancer cell line derived from the pleural effusion of a cancer patient, is able to colonize bone, liver, lung, adrenal glands, ovary, and brain after intravenous injection (89, 90). The implantation of established cell lines derived from human breast cancer is relatively simple and allows the genetic or pharmacological manipulation of the implanted cells (88). However, there are clear limitations of xenograft models with a major issue being immune responses, which have a key role during tumor development, are impaired in immunocompromised mice. In addition, many human cells are not fully adapted to grow in a murine environment and therefore cannot be transplanted into immunosuppressed animals. This raises the possibility that xenograft models may be unrepresentative of human breast cancers.

1.3.3. **Chemical carcinogenesis**

Mammary carcinomas have been induced by both chemical xenobiotics and physical agents in rodents (91-93). The most widely used chemically-induced models of breast cancer include the polycyclic aromatic hydrocarbon dimethylbenzanthracene (DMBA) or the directly acting
alkylating agent, N-methyl-nitrosourea (NMU) (32). These carcinogen-induced tumours arise from terminal end buds (94), an analogous structure to the terminal ductal lobular unit in humans, which is also the proposed site of origin DCIS (95). Substantial evidence suggests that this animal model, to a degree, mimics human breast cancer (91, 96, 97). Comparative studies have also shown that chemically-induced mammary carcinomas develop similar to their human counterparts and have altered expression of TGF-β, HER-2 and cyclin D1, the genes continually affected in human breast cancers (96, 98).

The use of experimentally-induced mammary tumours provides information that cannot be readily available in human populations. They offer dose-response modelling, exposure assessment and risk characterization, which are the required criteria for quantifying the estimated risk of cancer development associated with toxic chemical exposure (96). These models have been proven to be very successful in replicating, to a degree, oncogenic activation reported for human cancers. Furthermore, they have been shown to produce changes at the histological level which closely resemble human cancers in both aetiology and biology (99).

1.4 Chemokines and Tumour Cell Biology

1.4.1. Overview

Chemokines were originally noted for their ability to stimulate directional migration of nearly all classes of leukocytes. For example, neutrophils migrate in response to chemokines such as CXCL8, eosinophils to CCL11 and T-cells to a whole host of chemokines such as CXCL12, CCL19, CCL21 and CCL18 (100). It is now widely recognized that most cell types, not just immune cells, express chemokines and their receptors which therefore function in a wide
variety of biological processes (101-103). Furthermore the expression of chemokine expression has been reported in a variety of human tumours and have been identified as playing a critical role in several key steps in tumourgenesis and/or metastasis (Table 1.3) (104-107). The physiological role of leukocyte homing to inflammatory sites is known in considerable detail and similar mechanisms of site-directed migration have been proposed for tumour cell migration (108). As further studies are performed, it is becoming evident that chemokines may in fact be involved at virtually every step of tumour development, influencing tumour transformation, survival, growth, progression, angiogenesis, invasion and metastasis, making the chemokine family of proteins a key contributing factor during cancer development.

1.4.2. The Chemokine Family

1.4.2.1. Chemokine structure

The chemokine family of proteins represents the largest known group of chemoattractant cytokines with over 40 human genes identified to date (109). These single polypeptides are rich in highly basic amino acids, low in molecular-weight (8-14 kDa) and despite the large size of the family, chemokines are remarkably homologous (110, 111). Chemokines characteristically contain conserved N-terminal cysteine residues forming essential disulfide bonds (112). The arrangement of these conserved cysteines provides the basis of chemokine nomenclature and classification also giving rise to four subfamilies: the CC, CXC, C and CX3C groups (Fig 1.4). The CC and the CXC chemokines, in which the first two cysteines are adjacent or separated by one amino acid respectively, make up the majority of the chemokine family. The two minor subfamilies include the C chemokines that contain only two cysteines in their primary amino acid structure and the CX3C chemokine fractalkine that
possesses a unique primary amino acid sequence containing three intervening amino acids between the first two cysteines. The CXC family can be further divided on the basis of the presence of a glutamine-leucine-arginine (ELR) motif located upstream of the CXC sequence (102).

1.4.2.2. Chemokine function

Chemokines are expressed by a wide variety of cell types of both haematopoietic and non-haematopoietic origin (113) and therefore are involved in a wide range of biological processes (101, 109, 114). However, for the most part chemokines can be classified into two main groups based on their function and pattern of expression: inflammatory (alternatively called inducible) and homeostatic (alternatively called constitutive) (Table 1.4) (115-117). The majority of chemokines are inflammatory chemokines, which are upregulated or induced as part of the immune response. Their main function is to direct the recruitment of specialized effector cell populations to sites of infection/inflammation (117, 118). Inflammatory chemokines are produced by cells of many different tissues including migrating leukocytes in response to bacterial toxins and inflammatory cytokines such as tumour necrosis factor (TNF) and interferons (IFNs) (109, 118) and therefore have been implicated in a variety of diseases (109, 119, 120). As an example, chemokines have been reported to be mediators of inflammatory tissue destruction in rheumatoid arthritis. CCL2, CCL3, and CCL5 levels were shown to be increased in arthritic joints with their level of expression coinciding with the severity in patients with rheumatoid arthritis (119).

On the other hand, homeostatic chemokines are produced constitutively in defined areas of primary and secondary lymphoid tissues such as the bone marrow, thymus, spleen and lymph nodes (116, 121) as well as in non-lymphoid tissues such as mucosa and skin (116). They are
believed to drive the basal trafficking of leukocytes throughout the body and localisation within these tissues therefore proving to be critical factors in the maintenance of homeostatic immunosurveillance (122, 123). CXCL12 (SDF-1) displays a broad pattern of expression throughout the body and is responsible for B-cell lymphopoiesis, bone marrow myelopoiesis and cardiac ventricular septum formation (124). CXCR4 is also a major receptor for strains of HIV-1 that arise during progression of immunodeficiency and AIDS.

More recently, the distinction between these different classes of chemokines has become less clear, with a new group emerging that are capable of being homeostatic and/or inflammatory depending on the pathological or physiological signals. Included in this third division of chemokines is CCL20 (MIP-3α), which is expressed constitutively in secondary lymphoid organs, but is also induced in peripheral tissues, such as the central nervous system (125). In addition, CXCL9 (Mig) and CXCL10 (IP-10) are constitutively expressed in the colon (126), but under inflammatory conditions are induced in the skin only (44, 127). Similarly, a number of chemokines are upregulated at peripheral sites of inflammation, thereby classifying them as inflammatory, however they can also be constitutively expressed in secondary lymphoid tissue. As for the case of eotaxin, its expression is induced in the lung under inflammatory conditions such as OVA-induced eosinophilia (128), it is also constitutively expressed in secondary lymphoid tissues such as the thymus and lymph nodes (129).
1.4.3. **Chemokine receptors**

1.4.3.1. **Structure and specificity**

Chemokines attract target cells by binding to chemokine receptors expressed on their surface. As there are numerous chemokines, there are also numerous chemokine receptors subdivided into four main subfamilies CCR, CXCR, XCR and CX3CR which is also based on the structure of their ligands (130). Eighteen chemokine receptors have been cloned so far including, 6 receptors for CXC chemokines, 11 receptors for CC chemokines, and 1 receptor each for the C and CX3C chemokines (114). A distinguishing feature of chemokine receptors is the remarkable degree of promiscuity with regard to ligand binding (101), however some chemokine receptors bind to only one chemokine, such as the CXCL12/CXCR4 chemokine/receptor pair. Chemokine receptors generally interact with members of only one chemokine sub-family (and do so with high affinity) although exceptions do exist. For example, the CXC chemokines CXCL9 (Mig), CXCL10 (IP-10) and I- CXCL11 (TAC) have been shown to bind to CCR3, but act as antagonists rather than agonists (131). Also the Duffy antigen receptor for chemokines (DARC), an apparently non-functional chemokine binding protein expressed on erythrocytes and endothelial cells, has been shown to bind chemokines from both the CXC and CC sub-families with similar affinity (132).

Given the role of chemokines in host defense, it is not surprising that chemokine receptors are also gateways for the entry of at least two important human pathogens into target cells. First, CCR5 and CXCR4 act as co-receptors, in conjunction with CD4, for the cellular entry of human immunodeficiency virus (HIV) and accordingly, are currently considered excellent potential anti-HIV targets (133, 134). Second, the promiscuous chemokine binding protein DARC is the surface receptor used by *Plasmodium vivax*, a causative agent of malaria, and assist the virus to gain entry into erythrocytes (135).
1.4.3.2. **Chemokine receptor signalling**

The specific effects of chemokines are mediated through their seven-transmembrane spanning heterotrimeric guanine nucleotide-binding proteins (G-proteins)-coupled receptors, referred to as GPCRs. Upon chemokine binding, various conformational changes on the receptor lead to the exchange of GDP to GTP on the G-α subunit and the dissociation of GTP-bound α and βγ subunit, giving rise to an activated conformational chemokine receptor state (Fig 1.5) (136). The G-protein subunits are free to initiate a cascade of intracellular signalling events that translates into a range of biological effects including cell migration, rearrangement of cytoskeletal compartments, mobilisation of calcium and gene transcription demonstrated for leukocytes (Fig 1.6) (137). Two major signal transduction enzymes activated include phospholipase C (PLCβ2 and β3), specific for phosphatidylinositol and a phosphatidylinositol-3OH-kinase (PI-3K). The PLCβ cleaves phosphatidylinositol-(4,5)-bisphosphate yielding two secondary messengers inositol (1,4,5) tris-phosphate (IP₃) and diacylglycerol (DAG). IP₃ induces the release of Ca²⁺ from intracellular stores leading to transient increase in the concentration of free calcium (138) while DAG is able to activate several isoforms of protein kinase C (PKC). Activation signals following stimulation by chemokines also involves the initiation of serines kinases protein kinase various protein tyrosine kinase (PTK)-dependent cascades, which includes the activation of a family of focal adhesion kinase (FAK) proteins, p125FAK and Pyk2. This leads to the reorganisation of various cytoskeletal proteins including paxilllin as well as the activation of small GTPases such as Rho and Ras.

Signalling via the previously mentioned kinases has been implicated in cellular proliferation, survival, adhesion and motility (139) although it is still unclear whether chemokine stimulation is able to trigger all these effects directly. In leukocytes the signalling pathways triggered by chemokines in vitro are characteristically brief often ending within a few minutes
although it is not known whether this is a universal effect across other cell types (140). Despite the growing information available and interest in chemokine receptor signalling, the complex rules that govern leukocyte signal transduction pathways still remain unclear. Moreover, even less is known about the chemokine-mediated pathways activated in other cell types, such as cancer or epithelial cells. As a result many more studies are being performed in order to extend the knowledge on chemokine signalling with respect to other cell systems.

1.4.4. **Roles of chemokines in tumour transformation**

There is increasing evidence that chemokines are directly involved in the neoplastic transformation of a variety of tumour cells (106). DNA oligonucleotide microarray experiments revealed that CXCR4 expression was correlated to the transforming ability of the oncoprotein RET/PTC in human thyroid cancer cell lines (141). CXCR4 expression was not present in normal thyroid cells implicating a role for CXCR4 in the transformation of thyroid follicular cells. Several other chemokines including CXCL1 (Groα/MGSAα) and inflammatory mediator CXCL8, bind promiscuously and not only to their natural receptor, CXCR2, but also to the GPCRs encoded by tumourigenic viruses such as Kaposi’s sarcoma-associated herpes virus-8 (HHV-8) (106). Transgenic expression of the HHV-8-encoded receptor results in the development of angio-proliferative lesions resembling Kaposi’s sarcoma in mice (142), strongly indicating that excessive signaling through the CXCR2-like G-proteins promotes oncogenic cellular transformation. A different study conducted by Burger et al. demonstrated that a point mutation of CXCR2 in transfected NIH 3T3 cells, a fibroblastic cell line derived from mouse embryos, leads to constitutive signalling of the receptor and causes transformation similar to HHV-8 transfected cells (143). These studies along with others suggest that CXC chemokines (CXCL8 and CXCL1) are capable of...
continually stimulating certain cells expressing the CXCR2 receptor to ultimately promote oncogenic transformation.

1.4.5. 

Chemokines in growth and angiogenesis/angiostasis

Chemokines have also been shown to affect tumour growth via direct and indirect mechanisms that promote or inhibit angiogenesis. CXCL8 was the first chemokine identified to stimulate endothelial cell chemotaxis, proliferation and in vivo angiogenesis in a variety of human tumours (106). Luan et al. tested the biological consequence of overexpressing CXCL1, CXCL2, CXCL3 chemokines following their transfection of non-tumourigenic immortalised mouse melanocytes (144). This resulted in the formation of highly vascular tumours in nude mice and antibodies to these chemokines reduced the formation of tumours in the SCID mice and blocked the angiogenic response to conditioned medium from tumourigenic transfectants. In another study using a tumour xenograft model, CXCL8 was shown to be a transcriptional target of Ras signalling, an oncogene whose role has been well established in promoting cellular transformation. The Ras-dependent CXCL8 secretion was demonstrated to be essential in the initiation of tumour-associated inflammation and neovascularisation (145).

In contrast, angiostatic chemokines have been shown to affect the overall growth of tumours, with CXCL10 the most well-studied. In SCID mice, the production of CXCL10 inversely correlated with the tumourgenesis of lung cancer (106, 146). Intratumoural injection of CXCL10 attenuated the growth and neovascularisation of tumours while the functional depletion of CXCL10 by systemic administration of neutralizing antibodies augmented tumour growth and neovascularisation (107). Early studies have demonstrated that CXCL4 (platelet factor 4; PF4) has angiostatic properties and is capable of inhibiting endothelial cell
proliferation, angiogenesis in chick chorioallantoic membrane assays and tumour growth in immunodeficient mice (147).

Collectively these studies demonstrate that both stimulators and inhibitors of angiogenesis exist in the chemokine family. Furthermore it is predicted that chemokines form a balanced network of angiogenic and angiostatic regulators that are disrupted in cancer. The balance of chemokines produced by a tumour and its stroma may also determine the degree of angiogenesis surrounding the tumour and thus, the consequent invasiveness of the tumour (148).

1.4.6. **Chemokines and tumour-leukocyte interactions**

Tumours consist not only of cancer cells but are surrounded by various types of stromal cells such as fibroblasts or endothelial cells. Moreover, tumours have the ability to recruit inflammatory cells such as neutrophils, macrophages and lymphocytes, which are known to secrete a variety of biologically active molecules including cytokines, chemokines, proteases and lipid mediators. Collectively tumour cells, stromal cells as well as inflammatory cells contribute to the chemokine milieu at the tumour site and can regulate the influx of leukocytes that may be subsequently attracted to the tumour.

The ability of the immune system to inhibit the growth of tumours has been suggested to be related to the expression of tumour-associated chemokines (120, 149). In breast carcinomas different types of leukocytes, such as primary T-cells and monocytes, have been reported to infiltrate the tumour site to regulate inflammation (150, 151). These inflammatory cells are responsible for the production of CCL5 (RANTES) and studies have shown that the level of CCL5 expression correlates with the extent of macrophage infiltration and lymph node
metastasis (107). In a mouse model, long-term administration of CCL5 antagonist, Met-CCL5, significantly reduced the subcutaneous growth of CCL5-producing syngeneic mouse breast cancer cells by inhibiting the infiltration of leukocytes to the tumour (107). In esophageal carcinoma, CCL2 (MCP-1) expression was positively correlated with the level of macrophage infiltration, tumour angiogenesis and invasion (152). While in ovarian cancers, tumour cells were shown to produce inflammatory chemokines such as CCL2 (153) and CCL5 (154), which have been found localized to the epithelial areas within the tumours (153). The level of CCL2 expression correlated with large numbers of tumour-associated lymphocytes and macrophages (TAMs). Of note the intratumoural macrophages potentially release a variety of factors including metalloproteinases (MMPs), which degrade the extracellular matrix to increase the overall invasiveness of the cancer.

Some tumour cells not only regulate their chemokine expression to help recruit inflammatory cells, but can use these factors to further tumour growth and progression. Melanoma is perhaps a good example in which chemokines (CXCL1, CXCL2, CXCL3 and CXCL8) have been shown to exert autocrine control over neoplastic cell proliferation (155). Blocking CXCL1 or the CXCR2 receptor attenuated melanoma cell proliferation in vitro (156), whereas overexpression of CXCL1, CXCL2 or CXCL3 in a variety of tumour-derived cell lines enhanced colony-forming activity and tumourigenicity in nude mice (157). Other CXCR2 ligands have been identified as having autocrine roles in the growth of pancreatic, head and neck, and non-small-cell lung carcinoma (158, 159), whereas in mouse models, CXCL5 (ENA-78) has been proven to affect tumour growth, vascularity and apoptosis (159).
It is now appreciated that the chemokine-receptor system can be altered dramatically in neoplastic tissue with chemokines inducing a direct effect on stromal and neoplastic cells as well as contributing to the regulation of leukocyte recruitment to the site of the tumour.

1.4.7. Chemokines and tumour invasion/metastasis

1.4.7.1. CXCR4/CXCL12 and cancer metastasis

CXCR4 is by far the most common chemokine receptor overexpressed in human cancers. More than 25 different human malignancies, including breast cancer, ovarian cancer, melanoma, and prostate cancer, express CXCR4 (100). Although CXCR4 has been detected in a broad range of malignant cells the expression appears to be low or absent in many normal tissues, including breast (4) and ovary (42). Its ligand, CXCL12 is constitutively produced in the lung, liver, bone marrow and lymph nodes, and is also expressed at lower levels in the brain (4), all the common sites representing metastasis in many cancers. This strongly suggests that the CXCL12/CXCR4 interaction is of particular importance to the metastatic pathway of many cancers.

The evidence of specific homing in breast cancer came first from experiments conducted by Muller and colleagues (4). Functional in vitro experiments revealed that particular mammary carcinoma cell lines were capable of chemotactic migration, invasion and actin polymerisation in response to CXCL12. Furthermore, the treatment of CXCR4-expressing breast cancer cells with neutralizing anti-CXCR4 antibody reduced metastasis to lungs in an i.v. injection and orthotopic implantation model. The contributing role of CXCR4 in lung metastasis has also been supported in a number of other observations in pancreatic cancer cells (160) and in murine melanoma cells (161).
CXCR4 activation by CXCL12 may act in multiple ways to increase the metastatic properties, growth, and/or survival of cancer cells. For example the activation of CXCR4 has been shown to rapidly increase the affinity of β1 integrin on B16 cells for vascular cell adhesion molecule-1 (VCAM-1). Under shear stress conditions, overexpression of CXCR4 in B16 cells resulted in greater than tenfold increase in adhesion to tumour necrosis factor (TNF) stimulated lung endothelial cells, which expressed VCAM-1 (162). These results suggest that endothelial cell-derived CXCL12 may trigger the arrest of circulating cancer cells on endothelial cells by potentially activating adhesion molecules in a manner analogous to the process described in leukocytes. Furthermore, CXCR4 is likely to be regulated by a number of other cellular pathways influencing metastatic behaviour. It has been reported that CXCR4 up-regulation can result from HER-2 receptor tyrosine kinase-mediated signalling through inhibition of ligand-induced CXCR4 degradation (163). Furthermore, kisspeptin-10, a product of the KISS1 metastatic suppressor gene, has recently been shown to inhibit CXCL12-stimulated chemotaxis (164). It was demonstrated that kisspeptin-10 binds to GPR54, a G\(_{q}\)-coupled receptor and was shown to inhibit CXCR4 activation, calcium flux and Akt phosphorylation in HeLa cells \textit{in vitro} (164). This study suggests a relationship between different classes of G protein-coupled receptors that may impact on the functional ability of tumour-associated chemokine receptors to facilitate metastasis, although \textit{in vivo} experiments will be required to validate this.

1.4.7.2. \textit{CCR7/CCL19/CCL21 and regional lymph node metastasis}

The most common site of metastasis for solid and hematopoietic cancers are thought to be the regional draining lymph nodes (LN) (165). Indeed, the extent of LN involvement is an important prognostic indicator in many cancers, including melanoma (166). The initiation of
lymph node metastasis is believed to occur when tumour cells invade the lymphatics, or alternatively, are attracted there first by specific chemokines. Evidence for the latter possibility is supported by the finding that CCR7-expressing B16 murine melanoma cells metastasize 700-fold more efficiently to the draining LN following injection of tumour cells in the footpad than B16 murine melanoma cells which do not express this receptor (167). It has also been shown that CCR7 and its ligand CCL21 are necessary for efficient migration of mature, peripheral dendritic cells to the lymphatic vessels and, subsequently, to the draining LN (168). Lymphatic endothelial cells and secondary lymphoid organs are both rich sources of CCL21 (169). Interestingly, intravenous injection of CCR7-transduced B16 cells did not result in enhanced accumulation of the tumour cells in the lungs or peripheral LNs compared to mock-transduced cells, suggesting that CCR7 expression specifically enhanced LN metastasis but had little effect on pulmonary metastasis. However this effect may be tumour type-specific.

In a recent clinical study by Mashion et al., immunohistochemical staining revealed that CCR7-positive gastric carcinoma cells could be detected in 42 of 64 (66%) cases with significant differences in both lymph node metastasis and lymphatic invasion between CCR7-positive and -negative cases (5). Also patients with CCR7-positive tumors had a significantly poorer prognosis than those with CCR7-negative tumors. Furthermore, gastric carcinoma cell lines were tested for CCR7 expression/function and 4 of 6 displayed functional CCR7 expression as determined by calcium mobilization, actin polymerization and invasion/migration assays. Similar observations were made in esophageal squamous cell carcinoma (170) and non-small lung carcinoma (171) patients. Thus, cancer cells, including melanoma, appear to utilize CCR7 in a fashion similar to dendritic cells to facilitate entry into the lymphatic system and subsequent retention of tumour cells in secondary lymphoid organs.
1.4.7.3. CCR10/CCL27 and cancer survival in the skin

The skin is a common metastatic site for melanoma cancer patients (172). CCR10 has been implicated as a skin-specific chemokine, which is expressed abundantly on melanoma cells only and undetectable in other tissue. Its ligand CCL27 (CTACK) mediates function via CCR10, which is expressed by skin-homing memory T cells and by activated melanocytes to mediate chemotactic responses of skin-homing T cells (173). CCL27-CCR10 interactions may be involved in cutaneous lymphocyte immunosurveillance and appear to be involved in T lymphocyte-dependent inflammatory processes in skin (174). In vivo, neutralizing anti-CCL27 antibodies coinjected with CCR10-B16 melanoma cells were able to block tumour formation (175). This effect suggested that skin-derived CCL27 plays an important part in tumour formation, through the activation of CCR10.

1.4.8. Therapeutic implications

A large number of clinical studies have shown that selected chemokines and their receptors are expressed and up-regulated in a number of human cancers including those of breast, lung, prostate, colon and melanoma. It is now obvious that chemokine receptors such as CXCR4, CCR7 and CCR10 play a significant role in cancer metastasis. It is also becoming increasingly clear that chemokines and their receptors may facilitate tumour cell dissemination as well as have an effect at many of the key steps of the metastatic pathway, including adherence of tumour cells to the endothelium, extravasation from blood vessels, metastasis, colonization, angiogenesis, proliferation and survival.

Inhibition of chemokine receptors with antibodies or specific small molecule inhibitors in multiple tumour models (primary and metastatic) have demonstrated that these receptors do contribute to the processes of tumourgenesis and metastasis. Although experimental data...
suggest that chemokines and their receptors may be involved specifically in the arrest and migration of tumour cells out of the blood vessels at distant sites, it is not clear that inhibition of this step is feasible for intervention, as many micrometastases have occurred already prior to diagnosis of primary tumours, which often can be removed surgically. It is the suppression of already existing micrometastases or the inhibition of growth of life-threatening secondary tumours that will be the major target for anti-tumour therapies. As an example, antagonists for PF-4, an anti-angiogenic chemokine, to block angiogenesis, which is a critical step in the conversion of micrometastases to macroscopic tumours, has proved to be a therapeutic target of interest since the inhibitor significantly blocks the growth of established intracranial glioma in nude and syngeneic mice and improves survival (176). Guleng et al. demonstrated that neutralization of CXCR4 with blocking antibodies resulted in the delay of tumour formation by CXCR4-positive Colon 38 tumour cells (177).

As discussed earlier, there has been an accumulation of evidence pointing to tumour-associated chemokines in the regulation of inflammation and thus it is not surprising that chemokines may be excellent agents in cancer immunotherapy. Since they function physiologically as immunostimulatory molecules (promoting chemotaxis and the effector function of leukocytes subpopulations), they can be used to enhance anti-tumour immunity in the host and can be angiostatic and thus inhibit tumour growth. As a result several approaches have been used to deliver chemokines directly to the tumour environment. For example, the injection of recombinant chemokine peptides into tumour sites have been shown to exert a strong anti-tumour affect. CCL21 injected into the local tumour environment was capable of completely eradicating alveolar carcinoma and Lewis Lung Carcinoma (178).
In addition chemokines and their receptors are important in carcinogenesis and metastasis as summarized above. Disrupting steps in the metastatic cascade may also prove useful for treating different cancers. However, given the complex nature of tumour progression and metastasis formation as well as the high degree of heterogeneity within cancer, it is not likely that any single inhibitor or functional modulator of chemokines or their receptors will become the absolute cure for cancer. It is more likely that when used as adjuvant therapy with other treatments, chemokine-receptor-based agents will contribute significantly to the control of metastasis such that it may lead to many cancers becoming dormant and clinically manageable.
1.5 The Research Project

The involvement of chemokines in breast cancer metastasis was first reported in the landmark study by Muller et al. They demonstrated that the chemokine receptors CXCR4 and CCR7 were expressed at high levels in malignant breast cancer cells, breast cancer tissue and metastases and their respective ligands were expressed in organs associated with breast cancer metastasis. Muller’s study and subsequent reports, provided convincing data concerning the involvement of specific chemokine receptors in particular human cancers, however, failed to address the underlying molecular mechanisms cancer cells use chemokines and their receptors to govern metastasis. Therefore the purpose of this study was to examine the expression and functional the role of chemokines implicated in breast cancer metastasis utilising in vitro and in vivo analysis. The understanding of chemokine-mediated mechanisms orchestrated by cancer cells during metastasis may ultimately provide novel targets for therapeutic intervention and/or early detection and prevention of this severe female malignancy.
1.6 Hypothesis and Aims of the Study

The central hypothesis of the research project is:

"Chemokines and their receptors influence the metastatic ability of breast cancer cells".

In order to test this hypothesis the following aims were addressed:

To characterise the specific expression and functional relationship of chemokine receptors in human breast cancer epithelial cells.

To identify specific chemokine-mediated signalling pathways activated in breast cancer cells.

To further examine the role of chemokine receptors in an experimental mouse model of breast cancer metastasis.

These aims are addressed sequentially in each of the chapters presented in this thesis.
Table 1.1. Examples of biological markers that have been shown to be of prognostic value in breast cancer.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Process involved in</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER-2, p53</td>
<td>Oncogenesis</td>
</tr>
<tr>
<td>VEGF</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td>uPA, PAI-1, CB, CD, CL, cadherin E</td>
<td>Metastasis</td>
</tr>
</tbody>
</table>

Table 1.2. Oncogenes and TSG, their functions and targeted therapies implicated in breast cancer.

<table>
<thead>
<tr>
<th>Oncogene</th>
<th>Function</th>
<th>Targeted therapy</th>
<th>Current clinical trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER-2</td>
<td>Tyrosine receptor</td>
<td>Anti-HER-2 antibodies (trastuzumab, pertuzumab)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kinase inhibitors</td>
<td>(CI-1003, EKB-569, lapatinib)</td>
<td></td>
</tr>
<tr>
<td>Ras</td>
<td>G-protein</td>
<td>Farnesyl transferase inhibitors</td>
<td></td>
</tr>
<tr>
<td>PI3K</td>
<td>Kinase</td>
<td>Rapamycin/rapamycin analogues</td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>Cell-cycle mediator</td>
<td>Flavopiridolm</td>
<td></td>
</tr>
<tr>
<td>c-myc</td>
<td>Transcription factor</td>
<td>Antisense</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>Induces cell-cycle arrest, cell-cycle</td>
<td>Phase II p53 peptide vaccine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>checkpoint activation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P27</td>
<td>Inhibit cyclin-dependent protein kinases;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>arrest cell cycle in G1 phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRCA-1</td>
<td>Regulates DNA transcription; acts to</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>repair damaged DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRCA-2</td>
<td>Repairs damaged DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTEN</td>
<td>Negative regulator of Akt kinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb</td>
<td>Repressor of cell cycle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSG</td>
<td>Function</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maspin</td>
<td>Promotes metastasis, angiogenesis, and</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>apoptosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KISS</td>
<td>Promotes metastasis</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1.3. A summary of chemokine receptors expressed in human cancers.

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Receptors expressed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>CXCR4, CCR7</td>
<td>(4)</td>
</tr>
<tr>
<td>Ovarian</td>
<td>CXCR4</td>
<td>(42, 153, 154)</td>
</tr>
<tr>
<td>Prostate</td>
<td>CXCR4</td>
<td>(179)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>CXCR4</td>
<td>(160)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>CXCR4, CCR10, CCR7, CCR9</td>
<td>(180)</td>
</tr>
<tr>
<td>Oesophageal</td>
<td>CXCR4</td>
<td>12, 13</td>
</tr>
<tr>
<td>Lung (NSCLC)</td>
<td>CXCR4, CCR7</td>
<td>(181)</td>
</tr>
<tr>
<td>Head and Neck</td>
<td>CXCR4, CCR7, CXCR5</td>
<td>16, 17</td>
</tr>
<tr>
<td>Bladder</td>
<td>CXCR4</td>
<td>(182)</td>
</tr>
<tr>
<td>Colorectal</td>
<td>CXCR4, CCR7</td>
<td>(183, 184)</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>CXCR4</td>
<td>(185)</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>CXCR4</td>
<td>(124, 186)</td>
</tr>
<tr>
<td>Stomach cancer</td>
<td>CCR7</td>
<td>(5)</td>
</tr>
</tbody>
</table>
Table 1.4. Functional classification of chemokines into inflammatory and homeostatic groupings.

<table>
<thead>
<tr>
<th>Inflammatory chemokines</th>
<th>Homeostatic chemokines</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CC chemokines</strong></td>
<td></td>
</tr>
<tr>
<td>CCL1 / I-309</td>
<td>CCL18 / DC-CK1</td>
</tr>
<tr>
<td>CCL2 / MCP-1</td>
<td>CCL19 / ELC</td>
</tr>
<tr>
<td>CCL3 / MIP-1α</td>
<td>CCL21 / SLC</td>
</tr>
<tr>
<td>CCL4 / MIP-1β</td>
<td>CCL25 / TECK</td>
</tr>
<tr>
<td>CCL5 / RANTES</td>
<td>CCL28 / MEC</td>
</tr>
<tr>
<td>CCL7 / MCP-3</td>
<td></td>
</tr>
<tr>
<td>CCL8 / MCP-2</td>
<td></td>
</tr>
<tr>
<td>CCL11 / eotaxin-1</td>
<td></td>
</tr>
<tr>
<td>CCL12 / MCP-5</td>
<td></td>
</tr>
<tr>
<td>CCL13 / MCP-4</td>
<td></td>
</tr>
<tr>
<td>CCL17 / TARC</td>
<td></td>
</tr>
<tr>
<td>CCL20 / MIP-3α</td>
<td>CCL20 / MIP-3α</td>
</tr>
<tr>
<td>CCL22 / MDC</td>
<td></td>
</tr>
<tr>
<td>CCL24 / eotaxin-2</td>
<td></td>
</tr>
<tr>
<td>CCL26 / eotaxin-3</td>
<td></td>
</tr>
<tr>
<td>CCL27 / CTACK</td>
<td></td>
</tr>
<tr>
<td><strong>CXC chemokines</strong></td>
<td>CCL12 / SDF-1</td>
</tr>
<tr>
<td>CXCL1 / GROα</td>
<td>CXCL13 / BLC</td>
</tr>
<tr>
<td>CXCL2 / GROβ</td>
<td></td>
</tr>
<tr>
<td>CXCL3 / GROγ</td>
<td></td>
</tr>
<tr>
<td>CXCL5 / ENA-78</td>
<td></td>
</tr>
<tr>
<td>CXCL6 / GCP-2</td>
<td></td>
</tr>
<tr>
<td>CXCL7 / NAP-2</td>
<td></td>
</tr>
<tr>
<td>CXCL8 / IL-8</td>
<td></td>
</tr>
<tr>
<td>CXCL9 / Mig</td>
<td></td>
</tr>
<tr>
<td>CXCL10 / IP-10</td>
<td></td>
</tr>
<tr>
<td>CXCL11 / L-TAC</td>
<td></td>
</tr>
<tr>
<td>CXCL16</td>
<td></td>
</tr>
<tr>
<td><strong>C and CX3C chemokines</strong></td>
<td></td>
</tr>
<tr>
<td>XCL1 / lymphotactin</td>
<td></td>
</tr>
<tr>
<td>CX3CL1 / fractalkine</td>
<td></td>
</tr>
</tbody>
</table>
Fig 1.1. *Estimated numbers of breast cancer incidences and prevalence in females for the year 2002.* The most prevalent cancers with a five-year survival rate are shown for women worldwide, along with the number of new cases annually. In terms of prevalence, breast (17.9%), colorectal (11.5%), and prostate (9.6%) cancers are the most common cancers. Adapted from (1).
Females

- Breast: 4408
- Colon/Rectum: 1315
- Prostate: 1162
- Stomach: 522
- Cervix uteri: 494
- Lung: 423
- Bladder: 250
- Oral cavity: 99
- Corpus uteri: 775
- Non-Hodgkin lymphoma: 726

Thousand

Prevalence
Incidence
Fig 1.2. *Clinical and pathological progression of mammary tumourgenesis.* The natural history of breast cancer is believed to progress through a series of defined clinical and histological changes. Atypical hyperproliferation usually presents as an initial event and progresses through to an *in situ* then to invasive carcinoma and finally to the metastatic disease.
Atypical ductal Hyperplasia (ADH) → Ductal carcinoma in situ (DCIS) → Invasive ductal carcinoma (IDC)

Normal

Metastasis
Fig 1.3. *The multi-step progression of breast cancer metastasis.* The progression of metastasis involves a series of sequential multi-step events that result in the movement of cancer cells from the primary tumour to specific organs. Initially cancerous cells undergo clonal proliferation at the site of the primary tumour. Cancer cells acquire the ability to detach and released from the primary tumour by the breakdown the basement membrane for invasion into the extracellular matrix (ECM). Promotion of new-blood vessel formation or angiogenesis assists the cancer cells to intravasate into vascular or lymphatic vessels where they then arrest in distant capillary beds. Cancer cells adhere to vascular endothelial cells and then extravasate out of the blood vessels into a new tissue environment. At this stage tumour cells must be able to survive and proliferate in response to a different microenvironment distant from the primary tumour to form metastatic lesions.
Primary malignant neoplasm → Angiogenesis → Intravasation → Embolism → Arrest in distant capillary beds → Extravasation → Adherence to endothelial cells

- Metastasis
- Tumour cell proliferation
- Response to microenvironment
Fig 1.4.  *Structural classification of the chemokine family.* Chemokines can be classed into two major (CXC and CC) and two minor (C and CX3C) sub-families based on a cysteine-containing motif. Members of the CXC sub-family have an intervening amino acid between the first two cysteines, while members of the CC sub-family are characterised by the juxtaposition of the first two cysteines and the C sub-family members lack two (the first and third) of the four conserved cysteine residues. The CX3C chemokine group have three amino acid residues between the first and second cysteines.

C, systeine; X an amino acid other than cysteine.
Fig 1.5. *Overview of the G-protein cycle.* A. In the resting state, G proteins are heterotrimers of GDP-bound $\alpha$ (blue) and $\beta\gamma$ (green) subunits. B. Chemokine interaction with the receptor results in a conformational change leading to G-protein binding and, subsequently, GDP release. The dissociation GDP-bound $\alpha$ and $\beta\gamma$ subunit enables the subunits to go on to activate a variety of downstream effector protein. The signal is terminated upon the hydrolysis of GTP to GDP by G$\alpha$. 
Fig 1.6. *Chemokine receptor signalling.* The interaction between a chemokine with its G-protein-coupled receptor activates a cascade of several intracellular signalling pathways. Activation of these pathways regulates and range of cellular events such as the reorganization of cytoskeletal compartments, locomotion and chemotaxis as well as the modulation of gene translation. The scheme presented is not an exhaustive or complete depiction of all the signaling pathways induced by chemokines and their receptors rather a summary of the major pathways activated.
CHEMOTAXIS MODULATION OF GENE TRANSLATION

GYTOSKELETON REARRANGEMENT
CHAPTER 2

MATERIAL & METHODS
2.1. General Reagents and Antibodies

2.1.1. General chemicals

The following chemicals were obtained from Sigma Australia (Castle Hill, NSW, Australia): polyoxyethylene sorbitan monolaurate (Tween-20), Triton-X 100, diethylpyrocarbonate (DEPC) and DL-Dithiothreitol (DTT)

The following chemicals were obtained from BDH Chemicals (Kilsyth, VIC, Australia): sodium chloride (NaCl), disodium hydrogen orthophosphate (Na₂HPO₄), ethylenediaminetetra-acetic acid (EDTA), potassium dihydrogen orthophosphate (KH₂PO₄), D-glucose, HEPES, calcium chloride (CaCl₂), paraformaldehyde (PFA), sodium hydroxide (NaOH), ethanol, chloroform, propan-2-ol (isopropanol), glacial acetic acid and hydrochloric acid (HCl).

The following chemicals were obtained from Ajax Chemicals (Auburn, NSW): potassium chloride (KCl), magnesium chloride (MgCl₂), sodium azide (NaN₃) sodium carbonate (Na₂CO₃), sodium hydrogen carbonate (NaHCO₃), glycerol and acetone.

Tris hydrochloride (Tris-HCl) was obtained from ICN Biomedicals Australasia (Seven Hills, NSW) and agarose was obtained from Progen Biotechnik (Heidelberg, Germany).

The sources of specialised reagents are stated at the relevant place in the text.
2.1.2. **Chemokine peptides**

Synthetic peptides used in this study were obtained from the Biomedical Research Centre, University of British Columbia, Vancouver, Canada.

2.1.3. **Antibodies and conjugates**

Primary antibodies used in Flow cytometry and Western blot analysis are listed in Table 2.1. Secondary detection reagents are listed in Table 2.2.

2.1.4. **Oligonucleotides**

Primers used for PCR amplification and reverse transcription are listed in Table 2.3. All primers were purchased from GeneWorks (Adelaide, SA, Australia) and were of sequencing/PCR purity. Primers were received in a lyophilised form, diluted in sterile Milli-Q water and stored at -20°C.

2.1.5. **General Solutions**

2.1.5.1. **DEPC-treated water**

DEPC was diluted to 0.1% (vol/vol) in Milli-Q water, incubated overnight at RT and then autoclaved.

2.1.5.2. **TAE**

1x TAE consisted of 40 mM M Tris-acetate, 0.02 M Na acetate, 1 mM EDTA (pH 7.4).
2.1.5.3. *Hank’s Balanced Salt Solution (HBSS)*

The following reagents were dissolved in Milli-Q water to generate 10 X stocks: 80 g/L NaCl, 4 g/L KCl, 0.32 g/L Na₂HPO₄, 0.6 g/L KH₂PO₄ and 10 g/L D-glucose and the solution was sterilised by autoclaving. When used in calcium mobilization assays (Section 2.4.3.), the solution was diluted to 1 X in Milli-Q water, and HEPES buffer (pH 7.4) and CaCl₂ were added to a final concentration of 0.01 M and 1.6 mM, respectively.

2.1.5.4. *Staining buffer for Flow cytometry*

PBS was mixed with 1% heat-inactivated human serum and 0.04% NaN₃ (both w/v) and stored at 4°C.

2.1.5.5. *3.7% PFA*

3.7% PFA (w/v) was prepared by dissolving paraformaldehyde (BDH Laboratory Supplies, Poole, UK) in PBS (pH 7.4) at 55°C with stirring for approximately 30 min. PFA was stored at 4°C for up to one month.

2.1.5.6. *PBS/Tween*

Tween-20 was added to PBS to a final concentration of 0.2% (w/v) and the solution mixed thoroughly.

2.1.5.7. *Triton-X100 lysis buffer*

Triton-X100 lysis buffer was prepared with 1% Triton X-100, 20 mM HEPES, 150 mM NaCl, 1.5 MgCl₂, 1 mM EGTA and 10% glycerol. 10 mM sodium vanadate, 10 mM sodium
fluoride, 10 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM protease inhibitor (PI) cocktail (Sigma-Aldrich) were added immediately before use.

2.1.5.8. **RIPA buffer**

The modified (radioimmunoprecipitation assay) RIPA lysis buffer was made 1% NP-40, 150 mM NaCl, 50 mM Tris-HCL, 10% glycerol, 10 mM sodium vanadate, 10 mM sodium fluoride, 10 mM PMSF and 10 mM PI.

2.1.5.9. **Low salt wash buffer**

The low salt wash buffer for immunoprecipitation was made using 20 mM of Tris adjusted to pH 7.5 with HCL.

2.1.5.10. **SDS Reducing buffer (Sample Buffer)**

Two-times sample buffer was prepared by combining 50 mM Tris HCL (pH 6.8), 50 mM DL-Dithiothreitol (DTT), 1% SDS, 0.005% bromophenol blue, 10% glycerol.
2.2 Cell culture

2.2.1 Culture media

2.2.1.1 Serum

Foetal calf serum (FCS) was obtained from JRH Biosciences Ltd (Hampshire, UK) and was heat-inactivated by incubation at 55°C for 1 hour. Human AB serum was obtained from the Red Cross (Adelaide, SA, Australia); serum from 6 different donors was pooled and then heat-inactivated at 55°C for 1 hour.

2.2.1.2 RPMI Complete

The culture medium referred to throughout the text as RPMI Complete contains the following: RPMI supplemented with 10mM HEPES, 100 units/mL of penicillin/streptomycin and 10% heat-inactivated FCS, all obtained from the Infectious Diseases Laboratories Media Production Unit (IMVS), SA.

2.2.1.3 DMEM Complete

The culture medium referred to throughout the text as DMEM Complete contains the following: DMEM supplemented with 20 mmol/L HEPES, 100 units/mL of penicillin/streptomycin and 10% heat-inactivated FCS, all obtained from the IMVS.

2.2.1.4 F12:DMEM Complete

The culture medium referred to throughout the text as F12:DMEM Complete contains the following: A 1:1 mixture of DMEM and Ham’s F-12 medium (Invitrogen Life Technologies,
supplemented with 5% FCS, 100 units/mL of penicillin/streptomycin, 20 mmol/L HEPES (all obtained from the IMVS) SA, Australia, 10 μg/mL insulin (Sigma), 1.4 μmol/L cortisol (Sigma) and 20 ng/mL epidermal growth factor (Sigma).

2.2.2. Cancer cell lines and maintenance

Human breast non-metastatic cell lines MDA-MB-453, MDA-MB-134, MDA-MB-468 and MCF10A, metastatic cell lines MDA-MB-231, BT-549, as well as the malignant leukemia cell line, Jurkat T cell, and the human embryonic kidney cell line HEK 293T were all obtained from the American Type Culture Collection (ATCC; Manassa, VA). The cell lines were passaged according to ATCC instructions and grown at 37°C in 5% CO₂ atmosphere in the appropriate medium summarized in Table 2.4.

2.3 Molecular Techniques

2.3.1. RNA extraction

Total cellular RNA was isolated from breast cancer cell lines using Trizol (Life Technologies, Gilbertsville, PA, USA) as described by the manufacturers instructions. In brief, the cell/Trizol mixture was incubated at RT for 5 min prior to the addition of 200 μL chloroform. After vigorous shaking, the mixture was incubated for a further 2-3 min at RT and centrifuged at 12 000 x g for 15 minutes at 4°C. The upper aqueous phase was transferred to a clean reaction tube and the extraction of remaining RNA from the lower phase was repeated with 400 μl of DEPC-treated water. RNA from both extractions was precipitated separately by the addition of 500 μl of isopropanol, followed by a 10-minute incubation at RT. The precipitate was then centrifuged at 12 000 x g for 10 minutes at 4°C. The pellet was washed in 70%
ethanol and air-dried for approximately 15 minutes. RNA was dissolved in 20 μL of DEPC-treated water and incubated at 55°C for 10 minutes to ensure complete resuspension. RNA purity was determined by measuring optical density at 260 nm and 280 nm, and calculated using the following formula:

\[
\text{purity} = \frac{\text{OD}_{260}}{\text{OD}_{280}}.
\]

The concentration of RNA was calculated using the following formula:

\[
\text{concentration (μg/μL)} = \text{OD}_{260} \times \text{dilution factor} \times 0.04.
\]

### 2.3.2. cDNA synthesis from RNA

Generation of first strand cDNA from RNA was conducted using Superscript II reverse-transcriptase (Invitrogen) and the associated buffer and dithiothreitol (DTT) reagent as provided (Life Technologies). Each reaction was set up as follows: 1 μL of random hexamer primers (500 μg/mL; Amersham Pharmacia Biotech) was combined with 2.5 μg of total RNA and the volume adjusted to 12 μL with DEPC-treated water. The mixture was heated to 70°C for 10 minutes, then immediately cooled to 4°C and the following reagents added: 4 μL of first strand buffer, 2 μL of DTT and 1 μL of 10 mM dNTP mix (10 mM each dATP, dTTP, dCTP, dGTP diluted in DEPC-treated water; Amersham Pharmacia Biotech). The contents of the tube were mixed gently, centrifuged briefly and incubated for 2 minutes at 42°C. Finally, 1 μl of Superscript II (200 U/μL) was added and the reaction allowed to proceed at 42°C for 50 minutes before being terminated by heating to 70°C for 15 minutes. The cDNA products were stored at −20°C until further use.
2.3.3 Polymerase Chain Reaction (PCR)

PCR reactions for CXCR4, CCR7 and G-protein subunit amplification were performed using AmpliTaq Gold polymerase (Perkin Elmer Life Sciences, Boston, MA) and the supplied buffer with MgCl₂. The following 25 μL reaction was set up for each template with each primer set: 1.25 μL of cDNA template was added to a 0.2 mL reaction tube and heat-denatured at 95°C for 10 minutes. The temperature was then reduced to 4°C and the following reagents added: 5 μL of each oligonucleotide primer at 5 pmol/μL (Table 2.4), 2.5 μL of 10X reaction buffer, 1.25 μL of 25 mM MgCl₂, 0.5 μL of 10 mM dNTP mix (10 mM each dATP, dTTP, dCTP, dGTP diluted in sterile Milli-Q water), 9.25 μL of sterile Milli-Q water and 0.25 μL of AmpliTaq Gold polymerase (Perkin Elmer). Reactions were cycled in a hot-bonnet thermal cycler as follows: (Step 1) 95°C 10 min; (Step 2) 95°C 30 s; (Step 3) 55°C 1 min; (Step 4) 72°C 1 min, with steps 2-4 repeated for the required number of cycles. After completion of cycling, an extension step of 5 min at 72°C was performed. All reactions were then held at 4°C until analyzed.

2.3.4 Agarose Gel Electrophoresis

Two percent (w/v) agarose gels were prepared from electrophoresis grade agarose dissolved in 1 X TAE (Section 2.1.5.2) by boiling. Gels were run in TAE at ~100 mA in a horizontal gel apparatus. Following electrophoresis, gels were stained with ethidium bromide (diluted to 1X concentration in TAE; Molecular Probes) for 5-10 minutes and visualized and analysed using a Molecular Imager FX and Quantity One software package.
2.3.5 **Whole Cell Lysis of Breast Cancer Cells**

1 x 10^6 cells were lysed at 4 °C for 20 min in 500 μL Triton-X100 lysis buffer (Section 2.1.5.7). The lysates were centrifuged at 14 000 rpm at 4 °C for 10 min to remove insoluble materials, and the supernatants were collected. Total protein concentration was determined using the BCA assay (Section 2.3.6).

2.3.6 **Protein Concentration Determination**

The protein concentration of lysates was determined using a bichenchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) as recommended by the manufacturer. Protein standards of bovine serum albumin (Grade V, Sigma) ranging from 0-2000 ug/mL were used to produce a standard curve. The samples were diluted (1 in 10) and 10 μL were assayed in a 96-well flat bottom tray. BCA reagent was added to each of the samples and the plate was incubated at 37°C for 30 min. Absorbance was measured at 570 nm using a microplate reader (Amersham Biotrack reader II).

2.3.7 **SDS PAGE Analysis and Western Blot**

Fifty μg of whole cell lysate preparations were denatured by boiling for 5 min in sample buffer (Section 2.1.5.10). The samples were loaded on 4% stacking and 12% running polyacrylamide gel and run for 2 h at 150 V. The proteins were transferred onto PVDF membrane (Hybond™ P, Amersham Biosciences) by wet transfer (Bio-Rad) for 2 h at 100 V. The membranes were blocked with 1% blocking solution (Roche Applied Science) and incubated with primary antibodies (Table 2.1) followed by horseradish peroxidase-conjugated secondary antibodies (Table 2.2) at the dilutions indicated. Protein bands were visualized by enhanced chemiluminescence (Amersham Biosciences).
2.3.8 Co-Immunoprecipitation using Magnetic Beads

For co-immunoprecipitations, 1 mg of protein lysates was incubated with 1 µg of antibody at 4 °C overnight. Immunocomplexes were precipitated with 20 µL protein G-coated microbeads at 4 °C for 1 h and then separated on a magnetic column (both from Miltenyi Biotech, Bergisch Gladbach, Germany). The columns were washed four times with 200 µL lysis buffer supplemented with inhibitors (2 mM Na3VO4, 50 mM NaF, 10 mM phenylmethylsulfonyl fluoride, and 1:100 protease inhibitor) followed by a final wash with 100 µL of low salt wash buffer (20 mM of Tris-HCL, pH 7.5). The bound protein complexes were eluted with pre-heated sample buffer, separated by SDS-PAGE and subjected to Western blot (Section 2.3.7).

2.3.9 Retroviral infection of GFP in breast cancer cell lines

Retroviral supernatants were produced using 293T packaging cells which were transfected with 10 µg of specific or control expression vectors, 8 µg of pVPack-VSV-G and 8 µg of pVPack-GP (Stratagene La Jolla, CA), using 60 µL of Lipofectamine 2000 reagent (Invitrogen, Life Technologies, Inc.) in 100-mm tissue culture dishes in Opti-MEM medium (Invitrogen, Life Technologies, Inc.) without foetal calf serum and without antibiotics, essentially as recommended by the supplier. The medium was replaced 16 h later, and virus-containing supernatants were harvested at 48 h post-transfection. Supernatants were filtered through a 0.45-µm Minisart syringe filter (Sartorius AG, Gottingen, Germany), and polybrene (Sigma) was added to a final concentration of 8 µg/mL.

For infection, MDA-MB-231 cells were plated in a 60-mm tissue culture dish at ~40% confluence, and 24 h later the cell medium was removed before 5 mL of specific or control viral supernatants were added. The supernatant was replaced by cell growth medium after 6 h
of infection. The infected cells were then incubated for an additional 24 h at 37 °C before being plated at 1:20 dilution for the selection of individual clones in puromycin (5 ng/mL)-containing media. After 1 week, individual clones were selected and expanded for further analysis.

2.3.10 Retroviral-mediated siRNA knockdown

The siRNA retroviral expression vector was constructed by subcloning the human H1 gene promoter into the self-inactivating pMSCV plasmid. The resultant vector was digested with BglII and HindIII, and the annealed oligos were inserted to produce siRNA-expressing construct. The 21-nucleotide CXCR4 target sites at position 470–490 of human CXCR4 cDNA sequence was selected while the target sites 332 to 351 bases of the CCR7 open reading frame was chosen (Table 2.5). The previously described oligonucleotides containing specific target sequences for Renilla luciferase were used to produce the expression vector for the negative control (187).

2.4 In vitro assays

2.4.1. Flow cytometric analysis

5 x 10^6 cells/mL suspended in staining buffer (Section 2.1.5.4) was fixed in 3.7% PFA (Section 2.1.5.5) in PBS at room temperature for 10 min. The Fc receptors were blocked with purified human IgG (Sigma Chemical Co, St Louis, MO, USA) (10 μg per 10^6 of cells) at room temperature for 30 min. The blocked cell suspension (50 μL) was aliquoted to round bottom tubes and incubated with specific or isotype control antibodies at 4 °C for 30 min. For CXCR4 detection, the cells were stained with fluorescein isothiocyanate-conjugated anti-
mouse detection antibodies and for CCR7 detection, cells were stained with phycoerythrin-conjugated anti-mouse detection antibodies. The labelled cells were washed with staining buffer followed by PBS with 0.01% NaN₃. Cells were processed on a Becton Dickinson FACScan and data analysed using CellQuest Pro software (BD Biosciences). Positive events were defined on the basis of histogram markers, which were set according to the level of background staining observed using isotype-matched control antibodies. All percentage values presented in the data have been corrected for background staining, by subtracting the percentage of events defined as positive by the markers in relevant control samples (generally <1% for monoclonal antibodies).

2.4.2. Radiolabelled Binding Assay (RIA)

Two μg of CXCL12 was labelled with ¹²⁵I using the iodine monochloride method as described (188). After separation of iodinated CXCL12 from iodide ions on a Sephadex G25 PD-10 column RIA was performed as described (188). Briefly, 4 × 10⁶ cells of each cell line were incubated in suspension with concentrations of ¹²⁵I-CXCL12, ranging from 10 pM to 10 nM with or without 100-fold excess of unlabelled CXCL12. Following 2 h incubation with gentle shaking at 4°C, each cell suspension was centrifuged through 200 μL of foetal calf serum in order to remove free ¹²⁵I-CXCL12, and the level of radioactivity in cell pellets was determined using a γ-counter. Specific binding was determined by subtracting cpm obtained in the presence of excess unlabelled CXCL12 from total cpm. Scatchard transformation of saturation binding curves was performed using Prism 4 software (GraphPad).
2.4.3. **Calcium Mobilisation**

1 × 10⁵ breast cancer cells in 1 × HBSS (Section 2.1.5.3) were pre-incubated for 15 mins with 2μM of Fura-2AM at 37°C (Molecular Probes, Eugene, OR), which was then followed by two washes with HBSS. Cells were treated with either CXCL12 (300 ng/mL), CCL19 (750 ng/mL), phosphatidic acid (PA; 300ng/mL) or PBS and the fluorescence was monitored for 100 s. The changes in intracellular calcium were quantified using a luminescence spectrometer (SLM 8000-Aminco-Bowman Series 2, Urbana, IL) and the data represented as a fold increase relative to PBS treated cells. Cells were lysed with 10 μL 10% Triton X-100 to obtain a maximum value of free calcium. To obtain a minimum value of free calcium, 75 μL 100 mM EGTA and 10 μL 2M NaOH was added to the cuvette to chelate free calcium ions. In order to calculate calcium ion mobilisation in units of nM, experimental values were entered into the following formula:

\[ Y = 224 \times \frac{(Y - Y_{\text{min}})}{(Y_{\text{max}} - Y)} \]

where \( Y_{\text{min}} \) was the value obtained after addition of NaOH and EGTA, \( Y_{\text{max}} \) was the value obtained after addition of 10% Triton X-100 and \( Y \) was the value obtained from the experimental sample.

2.4.4. **Actin Polymerisation Assay**

For quantification of actin polymerisation, breast cancer cells were serum starved for 2 h, and then suspended to 1 × 10⁴/mL in PBS and treated with CXCL12 (100ng/mL) or CCL19 (750 ng/mL) for 15 min at 37°C in FACS tubes. The cells were pelleted and washed twice in PBS, fixed in 0.5 mL of 3.7% PFA and permeabilised in 200 μL PBS with 0.1% Triton-X-100 at 4°C. Fixed cells were stained with NBD-phallacidin (Molecular Probes Eugene, OR) for 30 min in the dark at 4°C, washed twice in PBS and resuspended in 300 μL in PBS to be analysed using a FACScan (BD Australia; emission wavelength 536 nm). The mean fluorescent intensities (MFIs) were recorded for both unstimulated and stimulated cells and
the data was presented as the difference in intracellular F-actin (polymerised actin) relative to unstimulated cells as a %, (control = basal level of F-actin).

For qualitative assessment, cells were grown on glass cover-slips and stained with phallacidin as described above. Cover-slips were mounted on glass slides with Vecta-shield mounting liquid (Vector Laboratories Burlingame, CA, UK) containing 4',6-diamidino-2-phenylindole (DAPI) for nuclear counter-staining and visualized by fluorescent microscopy.

2.4.5. Phosphorylation-Protein Detection

To determine the activation states of various signalling intermediates, breast cancer cell lines were serum-starved for 2 h and then left untreated or stimulated with various doses of CXCL12 (5-500 ng/mL), CCL19 (5-750 ng/mL) in PBS. The cells were washed twice with PBS and lysed in the modified RIPA buffer (Section 2.2.5.8) at 4°C. The lysates were then used in a customized Bio-Plex 6-Plex phosphoprotein Assay Kit (Bio-Rad, # X70000054E) to detect the levels of six phosphoproteins p-ERK1/2, p-IκBα, p-JNK, p-p38MAPK, p-GSK-3α/β and p-Akt. The assay was performed according to the manufacturer’s protocol. Briefly, 200 µg of cell lysate was incubated overnight at 4°C with 50 µL phosphoprotein 6-plex coupled beads (0.25 µL/well + wash buffer) in a 96-well filter plate that was placed on a platform shaker at 300 rpm. The plate was washed 3 times in wash buffer to remove unbound proteins. Twenty five µL of diluted biotinylated detection antibodies (0.5 µL/well + detection antibody diluent) were added to the reaction for 30 min, resulting in formation of sandwiches of antibodies around the target proteins. The plate was washed three times in wash buffer and 50 µL of diluted streptavidin-phycoerythrin (0.5 µL/well + wash buffer) was added for 10 min to bind to the biotinylated detection antibodies on the bead surface. Finally the plate was...
rinsed three times with resuspension buffer, resuspended in 125 µL/well using the same buffer and kept in the dark until further processing. The level of phosphoproteins present in the lysates was acquired and analysed using the Bio-Plex Suspension Array System (Luminex 100 system) from Bio-Rad Laboratories. The data recorded was processed and expressed as a fold-activation relative to the amount of phosphoproteins present in untreated cells.

2.4.6. Adenylate Cyclase Inhibition Assay (cAMP Assay)

To assay the cAMP production, breast cells were grown as a confluent monolayer in a 96-well tissue culture dish. Before stimulation, cells were incubated with 10 mM IBMX (Alexis Biochemicals, Lausen Switzerland) for 30 min to inhibit phosphodiesterase activity. Cells were then treated with 1 µM forskolin (FSK; Sigma-Aldrich, Castle Hill, Australia) alone to induce cAMP production, or with CXCL12 (100 ng/mL), CCL19 (750 ng/mL) or PA (100 ng/mL) for 30 min. Cells were washed two times in ice-cold HBSS/IBMX and lysed and the level of cAMP in cell lysates was assayed using a competitive enzyme immunoassay (R&D Systems, MN) as recommended by the supplier. In brief, 100 µL of lysates were added to a 96-well plate pre-coated with a goat anti-rabbit polyclonal antibody. Next 50 µL of cAMP conjugated to alkaline phosphatase was added to each well followed by 50 µL of cAMP antibody solution (rabbit polyclonal antibody to cAMP) which was then incubated for 3 h at RT on a horizontal orbital microplate shaker at 500 rpm. Each well was aspirated and washed with 200 µL wash buffer for four times and incubated with 200 µL of substrate solution for 30 min at RT on the bench-top protected from light. Following this incubation 50 µL of stop solution was added to each well and the optical density was determined within 30 min using a
microplate reader set to 450 nm. The data was presented as a fold-induction of cAMP synthesis was relative to untreated cells.

2.4.7. **Proliferation Assay**

To measure the growth rates of various cell lines, breast cancer cells were seeded at $1 \times 10^3$ and $3 \times 10^3$ cells/well in a 96-well plate. After 24 h, the cells were washed twice in PBS and 150 µL XTT working solution (100 µL DMEM, 50 µL XTT solution and 100 µL PMS) were added. After a further 4 h incubation period, the absorbance of the samples was measured using an ELISA reader (Multiskan EX, Labsystems, MA) at a test wavelength of 492 nm and a reference wavelength of 690 nm.

2.5 **In vivo techniques**

2.5.1. **Mouse strains and conditions**

Six to eight week female CB-17 severely compromised immunodeficient (CB-17 SCID) mice were purchased from the Animal Resource Centre (Perth, WA, Australia). SCID mice were housed in pathogen free conditions in the immunocompromised barrier rodent facility at the Adelaide University Animal House (Adelaide, SA, Australia). Mice were kept under standard temperature and light conditions, and fed food and water *ad libitum*. All procedures performed on mice were in accordance with the strict guidelines of the NH&MRC and approved by the Adelaide University Animal Ethics Committee.
2.5.2. *Intravenous injections of tumour cells*

To directly assay lung metastases, female SCID mice received tail vein injections of $6 \times 10^5$ cells, suspended in a total volume of 200μl of PBS using 29G needles. The *in vivo* assay was performed using five groups with six mice per group. Group 1 mice were given an injection of control cells consisting of a 1:1 (3 × 10^5 each) ratio of MDA-MB-231^{RFP} and control siRNA^{GFP} cell line. Group 2 mice were given an injection of cells consisting of a 1:1 ratio of CXCR4^{+/GFP} and control siRNA^{RFP} cell line. Group 3 mice were given an injection of cells consisting of a 1:1 ratio of CCR7^{+/RFP} and control siRNA^{GFP}. Group 4 mice were given an injection of cells consisting of a 1:1 ratio of CXCR4^{+/GFP} and CCR7^{+/RFP}. For clarification, refer to Table 5.1.

The mice were monitored weekly for changes in their body weight, behavior and infection. In accordance with the institutional guidelines, mice were sacrificed by CO₂ inhalation at week 3 and 8 or in the event of major compromise in their quality of life. Mice were perfused with PBS and whole lungs were harvested and placed in a 6-well tissue culture tray in PBS with no further tissue preparation.

2.5.3. *Dual-colour fluorescent imaging*

At autopsy, the whole lungs from mice were examined first under normal light and then the same field was examined under blue light (wavelength 490 nm) to activate GFP fluorescence and then under green light (wavelength 550 nm) to activate RFP fluorescence. Light and fluorescence microscopy were carried out using a Leica stereo fluorescence microscope model MZ16FA equipped with a UV lamp power supply. Images were processed by Metamorph Version 3.0 software (Universal Imaging Corp., Downingtown, PA).
2.6 Statistical tests

All statistical tests were performed using GraphPad InStat software (San Diego, CA). Comparisons between data sets were made with the two-tailed, unpaired Student’s t-test and p values less than 0.05 were considered statistically significant.
Table 2.1. Primary antibodies used for Flow cytometry and Western blot analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Conc/Dilution</th>
<th>Source</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-hu CXCR4</td>
<td>Mu IgG2B</td>
<td>50 μg/mL</td>
<td>R &amp; D</td>
<td>FACS</td>
</tr>
<tr>
<td>α-hu CCR7</td>
<td>Mu IgG2A</td>
<td>50 μg/mL</td>
<td>R &amp; D</td>
<td>FACS</td>
</tr>
<tr>
<td>α-hu CXCR4</td>
<td>Mu IgG</td>
<td>1:1000</td>
<td>Chemoicon</td>
<td>Western and IP</td>
</tr>
<tr>
<td>α-hu CCR7</td>
<td>Mu IgG</td>
<td>1:500</td>
<td>Epitomics</td>
<td>Western and IP</td>
</tr>
<tr>
<td>α-hu Gαi</td>
<td>Rab IgG</td>
<td>1:500</td>
<td>Santa Cruz</td>
<td>Western and IP</td>
</tr>
<tr>
<td>α-hu Gαq</td>
<td>Rab IgG</td>
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<td></td>
<td>Western</td>
</tr>
<tr>
<td>α-hu Gβ(1-4)</td>
<td>Rab IgG</td>
<td>1:500</td>
<td>Santa Cruz</td>
<td>Western and IP</td>
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<tr>
<td>α-mu β-actin</td>
<td>Rab IgG</td>
<td>1:5000</td>
<td>Sigma</td>
<td>Western</td>
</tr>
</tbody>
</table>

hu; human, mu; mouse, rab; rabbit, IP; immunoprecipitation, FACS; Flow cytometry

* The anti-hu Gαq antibody was kindly provided by Michael Crouch (TGR Biosciences, Adelaide, Australia)
Table 2.2. Secondary antibodies used for Flow cytometry and Western blot analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Source</th>
<th>Application</th>
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<tbody>
<tr>
<td>Strep- FITC</td>
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<td>Rockland</td>
<td>FACS</td>
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<tr>
<td>Strep- PE</td>
<td>1:150</td>
<td>Rockland</td>
<td>FACS</td>
</tr>
<tr>
<td>α-muIgG HRP</td>
<td>1:10 000</td>
<td>Rockland</td>
<td>Western</td>
</tr>
<tr>
<td>α-rabbit IgG HRP</td>
<td>1:10 000</td>
<td>Rockland</td>
<td>Western</td>
</tr>
</tbody>
</table>

Strep; streptavidin, FITC; fluorescein isothiocyanate, PE; phycoerythrin, HRP; horseradish peroxidase
Table 2.3. Oligonucleotides for PCR amplification

<table>
<thead>
<tr>
<th>Oligo Name</th>
<th>Oligonucleotide Sequence 5' → 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR4 F</td>
<td>atgaaggaacccctgtttcgt</td>
</tr>
<tr>
<td>CXCR4 R</td>
<td>ttggaaatccactgtgcacagtgttctc</td>
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<tr>
<td>CCR7 F</td>
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</tr>
<tr>
<td>Gαi R</td>
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<tr>
<td>Gβ (4) R</td>
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</tr>
<tr>
<td>Gβ (4) R</td>
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<tr>
<td>Cycolphilin F</td>
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</tr>
<tr>
<td>Cycolphilin R</td>
<td>cttagcatgggagga</td>
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F; forward primer, R; reverse primer
Table 2.4. Summary of cell line culture conditions

<table>
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<th>Cell line</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>Complete RPMI</td>
</tr>
<tr>
<td>BT-549</td>
<td>Complete RPMI</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Complete DMEM</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>Complete DMEM</td>
</tr>
<tr>
<td>MDA-MB-134</td>
<td>Complete DMEM</td>
</tr>
<tr>
<td>MCF10A</td>
<td>Complete F12: DMEM + supplements</td>
</tr>
<tr>
<td>Jurakt T</td>
<td>Complete RPMI</td>
</tr>
<tr>
<td>HEK 293T</td>
<td>Complete RPMI</td>
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Table 2.5. Target sequences for siRNA knockdown

<table>
<thead>
<tr>
<th>Oligo Name</th>
<th>Oligonucleotide Sequence 5' → 3'</th>
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<tbody>
<tr>
<td>CXCR4</td>
<td>GGTGGTCTATGTTGGCGTCTG</td>
</tr>
<tr>
<td>CCR7</td>
<td>GGCCATCAAGGTGATCATCGC</td>
</tr>
<tr>
<td>Renilla</td>
<td>AAACAUGCAGAAAAUGCUGTTTTUUGUACGUCUUUUACGAC</td>
</tr>
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</table>
CHAPTER 3

RESULTS: *In vitro* expression and functional characterization of CXCR4 and CCR7 chemokine receptors in breast cancer cell lines
3.1 Introduction

Since the time of Paget (40), investigators have been challenged with the concept of metastasis. This process consists of a complex series of sequential, interrelated steps in which tumour cells from a primary tumour disseminate to specific sites in distant organs. Various oncogenes, tumour suppressor genes, growth factors and adhesion molecules have been shown to play both a direct and indirect role during this non-random homing process, however the precise mechanisms of their involvement still remain to be elucidated (34, 189). More recently the expression of chemokines and their receptors has been documented in a vast number of metastatic human cancers, raising the possibility that these molecules play an important role in the metastatic pathway (100, 104, 106). In this regard, the chemokine and receptor pairs CXCL12/CXCR4 and CCL19/CCR7 have received a great deal of attention due to their potential involvement in metastatic breast cancer (3, 4, 181, 190). Moreover a link between the level of CXCR4 and CCR7 expression with the incidence of metastasis has been clearly demonstrated. Although there have been conflicting reports showing that the expression of CXCR4 is initiated at a very early point in the transition from a normal to transformed phenotype in breast epithelium (191).

In spite of these previous studies, the relationship between chemokine receptor expression on cancer cells and its potential role in cancer metastatic progression is not clear and requires considerable clarification. Therefore in this chapter, a comprehensive investigation into the expression and function of CXCR4 and CCR7 throughout a panel of breast cancer cell lines was initiated. In order to increase the relevance of this study a number of cell lines ranging from non-transformed immortalized breast epithelial cells to highly aggressive breast cancer cell lines proven to be metastatic in nude mice, were used. The main objective of these
experiments was to explore the potential relationship between chemokine receptor expression and/or function and the invasive potential of transformed breast cancer cell lines.

3.2 Results

3.2.1 Evidence for the metastatic potential of breast cancer cell lines in vivo

The well-established human non-metastatic cell lines MDA-MB-453 (192), MDA-MB-134 (193) and MCF-10A (194) as well as the metastatic breast cancer cell lines MDA-MB-231 (195) and BT-549 (196) were selected as a panel of cells representing the progression of breast cancer. Originally the cell lines were derived from patients with different types of metastatic breast disease (Table 3.1) and display varying degrees of invasive potentials based on their ability to form metastatic lesions in immunosuppressed mice (89). They have all been formerly characterised to meet the recognised criteria of a bona fide continuous cell line, which include altered cytomorphology, increased growth, a tendency toward anchorage-independent growth, changes in ploidy, tumourigenicity in nude mice and an infinite lifespan (197). There has however been substantial evidence not only for intra-laboratory cell line heterogeneity within established cell lines, but also a drift away from the phenotype of the originating tumour. Thus, the tumourigenicity of the cell lines was verified in vivo by measuring their ability to form pulmonary surface metastases.

Breast cancer cell lines were infected with a retrovirus expressing enhanced GFP under the control of a constitutively active cytomegalovirus promoter and the neomycin resistance gene. Stably infected lines were selected with G418 for two weeks and the resulting cell populations remained uniformly fluorescent over several passages. The growth rates of each cell line, as measured by a proliferation assay, were not affected by these modifications (data not shown).
Chapter 3

Cells were injected intravenously into the tail vein of severely compromised immunodeficient mice (SCID). The mice were monitored over an 8-week period and at the end of this time-point the lungs were excised and whole organs were examined under the fluorescence microscope. The extent of metastatic burden correlated with the level of fluorescence intensity, with the previously described metastatic cells, MDA-MB-231 and BT-549 displaying the highest level of fluorescence (Fig. 3.1). MDA-MB-453, MDA-MB-134 and MCF10A cells were not capable of disseminating to the lungs of SCID mice and forming visible metastatic lesions, despite the fact that they were originally derived from effusions of invasive breast carcinomas. The results consistently ranked the 5 cell lines from the most to the least invasive as follows: MDA-MB-231 greater than BT-549 greater than MDA-MB-453, MDA-MB-134 and MCF10A.

3.2.2 Analysis of chemokine receptor expression in human breast cancer cells

3.2.2.1 Gene expression profiles of CXCR4 and CCR7

To determine the relative chemokine receptor expression in breast cancer cells, the messenger RNA (mRNA) levels of CXCR4 and CCR7 were examined in a selection of human epithelial mammary tumour cell lines by RT-PCR. The expression of the CXCR4 transcript was strong and evenly distributed across the panel of breast cancer cell lines [Fig. 3.2 (A)]. RNA isolated from the murine B300 pre-B cell line expressing human CXCR4 was included as a positive control and the housekeeping gene, GAPDH, was used in the analysis as a loading control to enable the direct comparison of RNA between the cell lines. PCR revealed more variation in CCR7 mRNA expression across the cells [Fig 3.2 (B)] with the metastatic cells MDA-MB-231 expressing mRNA levels similar to that by the non-metastatic cells MDA-MB-134 and MCF10A. The malignant but non-invasive cell line MDA-MB-453, as well as the metastatic...
BT-549 cell line expressed relatively low levels of CCR7 mRNA. However, overall chemokine receptor gene expression was prominent in both non-malignant and invasive breast cancer cell lines with RNA expression unrelated to the metastatic phenotypes of the cells.

### 3.2.2.2. Protein levels of CXCR4 and CCR7

The profiles of chemokine receptor protein expression in breast cancer cells were examined by Western blot analysis. Whole cell lysates were prepared from breast cancer cells, which were then subjected to SDS-PAGE and analysed for the presence of CXCR4 using a rabbit-polyclonal anti-human-CXCR4 antibody or a rat-polyclonal anti-human-CCR7 antibody. In accordance with mRNA transcript levels, total CXCR4 protein was expressed uniformly throughout the panel of cancer cells [Fig 3.3 (A), top panel]. Equal loading for each sample was verified by examining β-actin levels [Fig 3.3 (A), lower panel]. Lysates from the Jurkat T-cell line were included as a positive control as these cells have been shown by previous studies to exhibit high levels of CXCR4 (198, 199). Also the MDA-MB-231 cell line, in which CXCR4 expression was knocked down by siRNA was used to demonstrate antibody specificity. The analysis of CCR7 protein expression displayed a significant level of heterogeneity across the panel of breast cancer cell lines [Fig 3.3(B), top panel]. The metastatic cell line MDA-MB-231 as well as the non-metastatic cell line MDA-MB-453 expressed consistently higher levels of CCR7 with equal amounts of protein loaded shown for each sample [Fig 3.3 (B), lower panel]. The remaining non-metastatic cell lines expressed similar levels of CCR7 protein while the metastatic BT-549 cells expressed relatively low levels of CCR7.
In summary, the expression of CXCR4 and CCR7 as observed at the mRNA level could be detected in untransformed through to highly invasive breast cancer cells. Unlike what has been reported previously by others, the expression of chemokine receptors was not restricted to expression in only highly aggressive cancer cells. These results indicate that chemokine receptor expression does not correlate with the invasive characteristics of the cell lines used in this particular study.

3.2.2.3. Cell surface expression of CXCR4 and CCR7

Next the cell surface expression levels of chemokine receptors on breast cancer cells were evaluated using flow cytometric analysis. The cells were stained with either a monoclonal anti-human-CXCR4, a monoclonal anti-human CCR7 or with isotype-matched negative control antibodies. The percentages of cells staining positive for CXCR4 or CCR7 as well as the relative mean fluorescence intensities (MFI) were determined for each of the cell lines. The data revealed that a high proportion of cells, ranging from 95-98%, were positive for CXCR4 expression (Fig 3.4). A lower proportion of cells were positive for CCR7 with expression on the MDA-MB-453 cells as low as 40% positive for CCR7 and only as high as 78% for MDA-MB-231 cells (Fig 3.5). The MFI values obtained for each of the cell lines, with respect to CXCR4 expression, were similar indicating that the level of CXCR4, i.e. surface receptor density, was also uniform across the panel of cell lines [Fig 3.6 (A)]. By comparison the MFIs for CCR7 expression was considerably and consistently lower [Fig 3.6 (B)], again with no correlation observed between receptor expression and the phenotypes of the cell lines.

In summary our data demonstrate that the expression of CXCR4 and CCR7 is present in both non-metastatic and invasive breast cancer cells, shown at the mRNA, protein and cell surface
expression level. Since the expression of chemokine receptor was not correlated to the invasive phenotypes of the cell lines the focus of the study was to then examine the function of CXCR4 and CCR7 expression on breast cancer cells using agonist-dependent cellular assays.

3.2.3. *The examination of chemokine function in breast cancer cell lines*

3.2.3.1. *Chemotactic responses elicited by chemokines*

To examine in more detail the involvement of chemokines and their receptors in the metastatic pathway of cancer, the functional role of CXCR4 and CCR7 was investigated in the established mammary tumour cell lines. Since it has been suggested that tumour cells use chemokine-mediated migration to home to specific sites on distant organs (200), the ability of chemokines CXCL12, the ligand for CXCR4, as well as CCL19 and CCL21, the ligands for CCR7 to induce chemotaxis of breast cancer cells was examined. The transmigration of cancer cells treated with chemokines in a 96-well modified Boyden chamber assay was recorded and the migration index for each of the cell lines was determined.

The results from these experiments show that the highly-invasive cell lines, MDA-MB-231 and BT-549, were capable of migrating towards CXCL12 in a dose-dependent manner (Fig 3.7). The non-invasive cells were unresponsive, in spite of the fact that all cell lines migrated towards serum used as a positive control (data not shown). The data are expressed as mean ± SE of migration index from at least three separate experiments each performed in triplicate. Similarly, only the metastatic cell lines exhibited a chemotactic response to either CCL19 or CCL21 (Fig 3.8) shown only for MDA-MB-231 and BT-549 cells. The non-invasive cell line MDA-MB-453, chosen as a representative of the non-metastatic cell panel, was not capable of
migrating in response to either CCL19 or CCL21 stimulation. Interestingly in the cells that were responsive, CCL19 induced a more potent effect and was thus predominantly used in subsequently experiments.

3.2.3.2. Chemokine mediated actin-polymerization

In addition to measuring chemokine directed migration we examined the ability of chemokines to promote intracellular actin polymerization. Actin polymerisation represents one of the earliest events in the rearrangement in the cytoskeleton following chemokine receptor ligation that is a requirement for pseudopodia formation to assist the movement of cells (201). The changes in the amount of intracellular polymerization of F-actin induced by chemokines were quantified by flow cytometry. The data was normalised to the basal level of F-actin in untreated cells and presented as a fold-change. After 15 mins of exposure to CXCL12 (100 ng/mL), optimised conditions based on previous chemotaxis assays, the level of F-actin was significantly increased in the metastatic MDA-MB-231 and BT-549 cells [Fig 3.9 (A)] compared to the resting levels in untreated cells. In contrast the basal levels of F-actin in the non-metastatic cell lines were unchanged, with CXCL12 having no effect on these cells. Furthermore, fluorescence microscopy confirmed CXCL12-induced actin polymerization in the metastatic cells and not in the non-metastatic cells [Fig 3.9 (B)]. In addition, a time-course study examining the ability of CCL19 to induce actin polymerisation was performed. As with CXCL12, exposure to CCL19 generated significant changes in the level of F-actin in the metastatic cell lines, MDA-MB-231 and BT-549 (Fig 3.10).
3.2.3.3. Investigation of CXCL12 binding affinity to CXCR4

There are several points at which a defect may account for the unresponsiveness of non-metastatic cells to CXCL12. For instance, the ligand may not bind to the receptor or the receptor may not transduce the message to the downstream signalling machinery. The characteristics of CXCL12 binding to CXCR4 were compared between the cell lines. Radioligand binding experiments using $[^{125}\text{I}]$ CXCL12, ranging from 10 pM to 10 nM in the presence and absence of 100-fold excess of unlabelled CXCL12, were performed in metastatic MDA-MB-231 and BT-549 cells and the non-metastatic MDA-MB-453 and MCF10A cells. The specific binding was determined by subtracting cpm obtained in the presence of excess unlabelled CXCL12 from total cpm. Scatchard transformation of saturation binding curves revealed similar receptor numbers and binding affinities for the metastatic and non-metastatic (MDA-MB-453) cells, with $K_D$ being in the nanomolar range (Fig 3.11).

3.3 Summary

The aim of the experiments described in this chapter was to test the hypothesis that CXCR4 and CCR7 expression and function correlated with the metastatic phenotype of breast cancer cell lines. For this purpose a number of breast cancer cell lines were chosen. The reported behaviour of the cell lines in vivo with respect to their metastatic phenotypes, was confirmed in SCID mice and the cancer cell lines were then tested for expression of CXCR4 and CCR7 and functional responses to their ligands in vitro. An initial screen for the presence of CXCR4 and CCR7 expression revealed that both metastatic and non-metastatic cell lines exhibited chemokine receptor expression. CXCR4 was expressed more abundantly throughout the cell lines than CCR7 with expression uniform and there were no clear distinctions between the
metastatic and non-metastatic cells. CCR7 on the other hand, while still present in all cell lines was expressed at different levels among the cell lines. However, the level of expression was not related to the metastatic characteristics of the cells. Therefore, expression of CXCR4 and CCR7 was not correlated to the invasive characteristics of the cell lines. On the contrary, experiments aimed at investigating the functional responses to ligation of CXCR4 and CCR7 revealed an important difference between metastatic and non-metastatic cells. The data revealed that incubation with CXCL12, CCL19 or CCL21 resulted in the modulation of actin polymerisation and migration in the metastatic cells lines while the non-metastatic cells remained unresponsive to the chemokines. The results indicate that functional chemokine receptor expression is restricted to those cells with a metastatic phenotype. Expression of unresponsive receptors is not unique to breast cancer cells. Fedyk et al. (202) reported no response in human bone marrow B lineage cells to CXCL12. Mitra et al. showed that the HepG2 cell lines expressed high levels of CXCR4 but incubation with CXCL12 failed to induce calcium mobilisation, phosphorylation or CXCR4 internalisation (203).

To try to identify the mechanisms behind the lack of responsiveness in the non-metastatic cells, radioligand binding experiments using $[^{125}\text{I}]$-CXCL12 were conducted. However similar receptor numbers and binding affinities were found for the metastatic and non-metastatic cell thereby ruling out differences in binding characteristics as an explanation for the non-responsiveness of the non-metastatic cells. Therefore, in the non-metastatic cells which express CXCR4, CXCL12 is in fact capable of binding to the receptor. However, this binding does not result in the activation of chemotaxis or actin polymerisation. The lack of response in these cells is unlikely to be due to a mutation in the receptor since the non-responsive cells chemokine receptors can be activated non-specifically with serum. A more likely explanation is the existence of an aberration in signalling downstream of the receptor.
A range of signalling events downstream of chemokine receptors were therefore examined. The results of those experiments are presented in the following chapter.
Table 3.1.  *Details on the origin and pathology of selected breast cancer cell lines.* The range of metastatic and non-metastatic breast cancer cell lines used in this study was chosen based on their ability to form metastatic lesions *in vivo* as reported by the listed references.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Origin</th>
<th>Age</th>
<th>Pathology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>Pleural effusion</td>
<td>51</td>
<td>Adenocarcinoma</td>
<td>(194)</td>
</tr>
<tr>
<td>BT-549</td>
<td>Pleural effusion</td>
<td>72</td>
<td>Ductal carcinoma</td>
<td>(195)</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>Pericardiac effusion</td>
<td>48</td>
<td>Metastatic carcinoma</td>
<td>(191)</td>
</tr>
<tr>
<td>MDA-MB-134</td>
<td>Pleural effusion</td>
<td>47</td>
<td>Ductal carcinoma</td>
<td>(192)</td>
</tr>
<tr>
<td>MCF10A</td>
<td>Pleural effusion</td>
<td>36</td>
<td>Fibrocystic Disease</td>
<td>(193)</td>
</tr>
</tbody>
</table>
Fig 3.1. Evaluation of the metastatic potential of GFP-labelled human breast cancer cell lines in vivo. Each of the breast cancer cell lines was transduced with the pLNCX retroviral vector expressing enhanced GFP and the neomycin resistance gene. Cell lines stably expressing GFP were selected with G418 and 200 μL of $1 \times 10^5$ cells/mL were injected into the tail vein of SCID mice ($n = 3$). The mice were monitored for 8 weeks, the lungs were excised and the level of metastatic burden on the surface of whole lungs was qualitatively assessed by fluorescence microscopy. The data obtained from images presented are representative of 3 mice.

Magnification: × 1
Fig 3.2. *PCR analysis of chemokine receptor mRNA levels in breast cancer cell lines.* RNA was extracted from breast cancer cell lines, DNase-treated and reverse-transcribed. Full-length oligonucleotide primers were used to amplify the complete gene product of (A) CXCR4 (size ~ 1000bp) or (B) CCR7 (size ~ 1100bp), the GAPDH housekeeping gene control was included as a loading control and the B300 cell line transfected with human CXCR4 was used a positive control. The PCR-amplified products were resolved on a 2.0% agarose gel and detected by ethidium bromide staining. The data shown are representative of 2 independent experiments with similar results.
(A) CXCR4 and GAPDH expressions in different cell lines.

(B) CCR7 and GAPDH expressions in different cell lines.
Fig 3.3. Western blot analysis of total protein levels of chemokine receptors in breast cancer cells. Whole cell lysates were prepared from 1 × 10^6 breast cancer cells using a modified Triton-X lysis buffer. Protein samples (50μg) were resolved by SDS-PAGE under denaturing conditions. The membranes were incubated with either rabbit anti-human-CXCR4 (A), rat-anti-human CCR7 primary or mouse anti-human β-actin antibody (lower panels) for 1 h at 4°C, followed by extensive washes in TBS wash buffer. The bound primary antibodies were detected using HRP-conjugated anti-rabbit IgG and visualized using the ECL system. The presented immunoblots are representative of at least 3 separate experiments performed with similar results.
(A) CXCR4
MDA-MB-231  MDA-MB-453  MDA-MB-134  MDA-MB-468  MCF10A  CXCR4 siRNA

CXCR4  ACTIN

47kDa
42kDa

(B) CCR7
Jurkat-T  MDA-MB-231  BT-549  MDA-MB-453  MDA-MB-134  MDA-MB-468  MCF10A

CCR7  ACTIN

48kDa
42kDa
Fig 3.4. *Evaluation of CXCR4 cell surface expression on human breast cancer cell lines.* 1 x 10⁴ cells breast cancer cells fixed in 3.7% paraformaldehyde were incubated with monoclonal anti-CXCR4 fluorescein isothiocyanate (FITC) antibody (shaded histograms) or with an isotype-matched-labelled control (open histograms). The cells were then analysed on a BD FACScan using CellQuest 3.1 software with n=3. Representative histogram plots are shown for each cell line, the Jurkat-T cell line was included as a positive control and MDA-MB-231/CXCR4 siRNA knockdown cell line as a control for antibody specificity shown as the bold open histogram. The percentages of positive CXCR4 staining are shown in parentheses.
Fig 3.5. **Evaluation of CCR7 cell surface expression on human breast cancer cell lines.** 1 x 10^4 cells breast cancer cells fixed in 3.7% paraformaldehyde were incubated with monoclonal anti-CCR7 phycoerythrin red (PE) (shaded histograms) antibody or with an isotype-matched-labelled control (open histograms). The cells were then analysed on a BD FACScan using CellQuest 3.1 software with n=3. Representative histogram plots are shown for each cell line including Jurkat-T cell line as a positive control and MDA-MB-231/CCR7siRNA knockdown cell line as a control for antibody specificity shown as the bold open histogram. The percentages of CCR7-positive cells are shown in parentheses.
Fig 3.6. *Comparison of the relative cell surface expression of CXCR4 and CCR7 chemokine receptors on the panel of breast cancer cell lines.* Data shown represent the relative mean fluorescent intensity (MFI) of CXCR4 (A) and CCR7 (B) on breast cancer cells with the mean ± SE determined over three independent experiments.
Fig 3.7. *Chemotactic response of breast cancer cell lines to CXCL12.*
Breast cancer cells were exposed to the indicated concentrations of CXCL12 in a 96-well modified Boyden chamber. The ratio of cells migrating in each well towards CXCL12 relative to media-only controls is shown as the migration index. All panels are expressed as mean ± SE of migration index from at least three separate experiments each performed in triplicate.
Fig 3.8.  *Chemotactic response of breast cancer cell lines to CCL19 and CCL21*. Breast cancer cells were exposed to the indicated concentrations of either CCL19 or CCL21 in a 96-well modified Boyden chamber. The ratio of cells migrating in each well towards each chemokine relative to media-only controls is shown as the migration index. All panels are expressed as a mean ± SE of migration index from at least three separate experiments each performed in triplicate.
Fig 3.9. *Actin polymerisation induced by CXCL12 in breast cancer cell lines.* Breast cancer cells were incubated with CXCL12 (100 ng/ml) or with PBS control. At the indicated time points, the cells were fixed, permeabilised, stained with FITC-labelled phalloidin and analysed by flow cytometry (A). The data show the MFI and presented as a mean ± SE at all time points relative to the mean fluorescence of the samples treated with PBS (n = 3). (B) Fluorescence microscopy analysis of polymermerised F-actin in MDA-MB-231 and MDA-MB-453 cancer cell lines after exposure to CXCL12. Cells were grown on glass slides, fixed and stained with FITC-phalloidin for F-actin (red) and counterstained with DAPI to visualise cell nuclei (blue).
Fig 3.10. *Time course of CCL19-induced actin polymerisation in metastatic and non-metastatic breast cancer cell lines.* Each of the breast cancer cell lines was stimulated with CCL19 (750 ng/ml) or with PBS control. At the indicated time points, the cells were fixed, permeabilised, stained with FITC-labelled phallacidin and analysed by flow cytometry. The data show the MFI and represent the mean ± SE at all time points relative to the mean fluorescence of the samples treated with PBS (n = 3).
![Graph showing intracellular F-actin (% control) over time (mins) for various cell lines: MDA-MB-231, BT-549, MDA-MB-453, MDA-MB-134, MDA-MB-468, and MCF10A.](image-url)
Fig 3.11. Examination of CXCL12 binding to CXCR4 on the breast cancer cell lines. CXCL12 (2 μg) was labelled with $^{125}$I and incubated at different concentrations with each of the cell lines ($4 \times 10^6$ cells/mL) in the presence and absence of 100-fold excess of unlabelled CXCL12. Specific binding was determined using a γ-counter by subtracting the counts per minute obtained in the presence of excess unlabelled CXCL12 from the total counts per minute. Presented are Scatchard transformations of $^{125}$I-CXCL12 bound to breast cancer cell lines. These data are from one experiment representative of three independent experiments performed with similar results.
(A) MDA-MB-231

- $B_{\text{max}} = 1.21 \times 10^{-10}$
- $K_d = 4.49 \times 10^{-9}$

(B) BT-549

- $B_{\text{max}} = 1.05 \times 10^{-10}$
- $K_d = 4.98 \times 10^{-9}$

(C) MDA-MB-453

- $B_{\text{max}} = 8.58 \times 10^{-11}$
- $K_d = 6.64 \times 10^{-9}$

(D) MCF10A

- $B_{\text{max}} = 3.08 \times 10^{-11}$
- $K_d = 4.83 \times 10^{-9}$
CHAPTER 4

RESULTS: Investigation into the molecular mechanisms and signal transduction events mediated by chemokine receptors
4.1 Introduction

Chemokine receptors are members of the seven transmembrane heterotrimeric G-protein coupled receptor family (GPCR) (204). The G-protein family represents the largest and most diverse group of proteins with at least 800 potential GPCRs identified in the human genome (205, 206). They play critical roles in many physiological and pathological processes, including haematopoiesis, angiogenesis, inflammation, metabolic disease and viral infection (207). As a result, GPCRs have become the most important molecular targets for pharmaceutical companies with approximately 40% of all currently marketed drugs directed against as many as 30 types of GPCR (208).

GPCRs are associated with a heterotrimeric G-protein complex composed of an $\alpha$-subunit a $\beta$- and a $\gamma$-subunit, with the latter forming a covalently linked $\beta\gamma$-dimer. Upon GPCR activation various conformational changes occur leading firstly to the exchange of GDP to GTP on the $\alpha$-subunit which then allows dissociation of the GTP-bound $\alpha$-subunit from the $\beta\gamma$-subunit complex (209). The subunits of G-proteins show a wide range of heterogeneity. To date, 16 genes encoding $G\alpha$, 5 genes encoding $G\beta$ and 12 genes encoding $G\gamma$ have been identified in mammals (210). Although not all interactions are favoured, the multiplicity allows formation of many heterotrimeric combinations, which confers both diversity and specificity on G-protein signalling and overall function (211). In addition the expression and function of G-proteins may be redundant in a number of cells and tissues, which adds to the complexity of this system.

On the basis of amino acid similarities of the $\alpha$-subunit, G-proteins are further categorized into four major classes $G_s$, $G_{\alpha_i}$, $G_{q_1}$ and $G_{12/13}$ (212). Stimulation of the $G_s$ subfamily
activates adenyl cyclase whereas stimulation of the $G_i$ subfamily leads to the inhibition of adenyl cyclases. Activation through the $G_q$ subfamily stimulates phospholipase C (PLC) and the $G_{12/13}$ subfamily is implicated in the regulation of the function of small GTP binding proteins (213). Once the GTP-bound $\alpha$-subunit has dissociated from the $\beta\gamma$-subunit both interact with and regulate the function of a diverse array of effector molecules and pathways. As described for leukocytes, the major effector molecules which have been reported to be regulated by $\beta\gamma$-subunits include PLC$\beta$, phosphoinositidol-3-kinase (PI3-K) as well as its downstream modulator protein kinase B (PKB or Akt), mitogen activated protein kinase (MAPK), small GTPases such as RhoA, Rac and Cdc42 and members of the protein kinase C (PKC) family (214). Signalling via these kinases has been implicated in cell proliferation, survival, adhesion and motility (139, 215).

The results of the previous chapter clearly demonstrate a difference between chemokine receptor function in metastatic and non-metastatic breast cancer cells. The aim of the experiments in the present chapter was to compare the intracellular signaling events activated by chemokines in metastatic and non-metastatic cells in order to potentially identify the underlying molecular basis that may account for the differential responsiveness of chemokine receptors in the two cancer cell types.
4.2 Results

4.2.1 Analysis of G-protein subunit expression in human breast cancer cells

4.2.1.1 Gene expression profiles of G-protein subunits

To investigate the role of G-proteins involved in the regulation of chemokine receptor signaling, a preliminary screen for a range of G-protein subunits was conducted in breast cancer cells. These initial experiments were conducted on MDA-MB-231 cells and MDA-MB-453 cells as representative of metastatic and non-metastatic cells respectively. To begin with, Goi mRNA was amplified from RNA isolated from breast cancer cells using reverse-transcription PCR. The results shown in Figure 4.1 (A) illustrate that the full-length Goi gene product was expressed uniformly across the panel of cell lines. Next, Gβ subunit mRNA was examined and compared between the metastatic cell line MDA-MB-231 and the non-metastatic cell line MDA-MB-453. Gβ1-3 subunit transcripts were amplified from cDNA prepared from both cell types. However, mRNA for the Gβ4 subunit was not detected in the non-metastatic MDA-MB-453 cell line [Fig 4.1 (B)]. This observation was unique to the MDA-MB-453 cells as Gβ4 expression was observed in the other non-metastatic cell lines (data not shown). RNA levels were compared with cyclophilin expression as a loading control (Fig. 4.1, lower panels).

4.2.1.2 Protein expression of G-protein subunits

Western blot analysis was performed to confirm the presence of G-protein subunits at the protein level. Whole cell lysates were prepared from untreated breast cancer cell lines, subjected to SDS-PAGE and analysed for the expression of G-protein subunits using antibodies specific for Goi, Gαq and Gβ. Antibodies used for Gq detection were included in
these experiments due to several lines of evidence implicating Gq in chemokine receptor signal transduction (216), while for economical reasons, a pan Gβ(1-4) antibody was used to screen for Gβ expression. These experiments revealed that both Gα and Gβ subunits were expressed throughout the panel of breast cancer cell lines (Fig 4.2). The expression of Gαi as well as Gαq was notably and consistently lower in the non-metastatic cells while Gβ expression was uniform across the cell lines. As a loading control the western blots were reprobed with β-actin antibodies (bottom panel).

4.2.2 Assessment of functional Gα-subunit expression

4.2.2.1 Inhibition of cAMP production in breast cancer cells

Previous studies conducted initially in leukocytes show that the majority of chemokine receptors are coupled to the Gαi-subunit of the heterotrimeric G protein complex (103, 112). Signalling through chemokine receptors via the Gαi-subunit pathway was originally identified as the ability of Gαi to inhibit adenyl cyclase-mediated cAMP production (217). The ability of chemokines to inhibit forskolin-induced cAMP production was therefore examined in the two representative breast cancer cell lines.

The breast cancer cell lines were first incubated with forskolin for 30 min to induce cAMP production, after which chemokine ligands CXCL12, CCL19 or phosphatidic acid (PA as a positive control) were added. After 10 min of incubation the cells were harvested and cAMP levels were assayed for using a cAMP ELISA. The results shown in Figure 4.3, reveal that both chemokine ligands CXCL12 and CCL19 were capable of significantly suppressing forskolin-induced cAMP accumulation in the highly-invasive MDA-MB-231 and BT-549 cells. However, this effect was not observed in the non-invasive cells, MDA-MB-453 and
MCF10A. Similar results were obtained in breast cancer cells treated with the non-chemokine agonist, phosphatidic acid. Overall, the results of these experiments show that in metastatic cells chemokines are capable of inhibiting forskolin-induced cAMP production. The data also indicate that the chemokine receptors expressed in invasive breast cancer cells are coupled to functionally activate Gαi-subunit while in the non-metastatic cells, Gαi signalling cannot be activated, suggesting that coupling to Gαi in these cells is blocked. In order to investigate this phenomenon, further assays were performed to explore G-protein function in breast cancer cells.

4.2.2.2 Intracellular calcium mobilization induced by chemokines

Intracellular calcium is critical for a range of cellular processes (218). Calcium mobilization can be activated through the Gαi-coupled receptors that possess the ability to stimulate PLC-β to produce the intracellular messengers inositol trisphosphate (IP₃) and diacylglycerol (DAG) which has also been shown to be potentiated through the Gαq pathway (219). IP₃ triggers the release of calcium from intracellular stores and DAG recruits protein kinase C (PKC) to the membrane where it is activated (212). In addition, signal transduction through the Gαi pathway, but not the Gq pathway, can be inhibited by pertussis toxin (PTX) which uncouples GPCRs from Gαo (220). To investigate whether the Gαi/q-mediated pathway is activated in breast epithelial cells and to verify the role of functional chemokine receptor expression in invasive breast cancer cells, a series of experiments were performed to determine the ability of chemokines to induce calcium mobilisation.

The cells were loaded with the intracellular calcium probe, Fura-2AM before the addition of ligand and the changes in the concentration of intracellular calcium were monitored.
Metastatic cell lines incubated with CXCL12 produced a significant transient increase in intracellular calcium while the non-metastatic cells remained unresponsive (Fig 4.4). The results clearly demonstrate differential effects of CXCL12 in the metastatic MDA-MB-231 and non-metastatic MDA-MB-453 cells, presented in Figure 4.4 (A). A comparison of CXCL12-induced calcium mobilisation across the entire panel of breast cancer lines, expressed as a fold-induction is shown in Figure 4.4 (B). These data indicate that only the metastatic cell lines responded to CXCL12. In keeping with the results of previous findings (221), the response to CXCL12 in metastatic cells proved to be PTX-sensitive thereby implicating Goi in the signal transduction cascade [Fig 4.4 (C)].

In order to assess the function of CCR7 expression on breast cancer cells the ability of CCL19 to induce calcium mobilisation was investigated. Incubation with this chemokine resulted in a transient increase in intracellular calcium in the metastatic cell lines MDA-MB-231 and BT-549 (Fig 4.5). The result of a representative real time plot is shown in Figure 4.5 (A) while a summary of the ability of CCL19 to induce calcium mobilisation, presented as a fold-induction, is compared across the entire panel of cell lines in Figure 4.5 (B). CCL19 was capable of inducing a 1.5-fold increase in intracellular calcium concentrations in the metastatic cell lines which was similar to stimulation with CXCL12 [compare Fig 4.5 (B) with Fig 4.4 (B)]. Calcium mobilisation mediated by CCL19 was also completely blocked by the addition of PTX, confirming that calcium mobilisation in breast cancer cells is Goi dependent [Fig 4.5 (C)].

As observed with respect to inhibition of cAMP, the non-chemokine agonist PA, induced a similar calcium mobilisation response to that observed with CXCL12 and CCL19. More specifically, stimulation with PA significantly enhanced the level of intracellular calcium in
the metastatic cells while the non-metastatic cell lines did not respond (Fig 4.6). This is shown in a representative experiment in Figure 4.6 (A) for MDA-MB-231 and MDA-MB-453 cells with the data summarized for the responses of all cell lines used in the study in Figure 4.6 (B). The induction of intracellular calcium mobilisation mediated by PA was also shown to involve the Gαi pathway, since treatment with PTX abolished the response to PA in MDA-MB-231 cells [Fig 4.6 (C)].

In summary, this set of experiments aimed at investigating the functional role of the Gα-subunit, revealed a clear distinction between the metastatic and non-metastatic cell lines. Addition of chemokines resulted in the activation of Gαi pathways only in the metastatic cell lines as measured by cAMP and calcium mobilisation assays. The data implies that the expression of chemokine receptors in metastatic breast cancer cells are coupled to functional Gαi which was not observed in the non-metastatic cells.

4.2.3 Investigation of functional Gβγ expression

4.2.3.1. Assessment of chemokine-induced activation of signalling cascades downstream of the Gβγ-subunit.

The Gβγ-subunit complex has been shown to mediate numerous kinase cascades downstream of GPCRs described in leukocytes. As an example, studies have demonstrated that CXCL12 is capable of inducing activation of the PI-3K, MAPK and ERK1/2 signaling cascades in lymphocytes (214, 222). As a result, the range of molecules regulated by Gβγ include many small GTPases such as Rho and Ras, as well as PI-3K and molecules in the MAPK pathway (213). The activation of these pathways plays an important role in physiological processes such as chemotaxis, cell proliferation and survival (223). In the present study an investigation
of chemokine signaling in breast cancer cells was performed to determine if similar pathways described for leukocytes are activated in cancer cells and to determine whether these pathways are differentially activated in metastatic and non-metastatic cells.

A dose response to chemokines was initially performed to determine an optimal concentration to achieve phospho-activation in breast cancer cells. The results from a recent study have shown that the PI-3K/AKT pathway is constitutively active in MDA-MB-231 cells under culture conditions with serum (224). Therefore, the experiments were conducted in serum-free conditions to avoid activation of non-specific signalling pathways induced by serum components. Breast cancer lysates were prepared from the invasive MDA-MB-231 cells, shown previously to express functionally-active chemokine receptors (Fig 3.7-3.12). The cells were treated with varying concentrations of chemokine ligand for 15 min, lysed and then examined in a multi-plex phosphorylation assay to detect activated states of ERK1/2, IκBα, JNK, p38MAPK, GSK-3α/β and Akt. The data revealed incubation with 5-500 ng/mL of CXCL12 was capable of activating multiple kinase pathways (Fig 4.7). At the higher concentrations (100 and 500 ng/mL), CXCL12 treatment resulted in a 6-fold induction of phospho-p38MAPK and a 5-fold induction of phospho-ERK1/2 [Fig 4.7 (A)]. Alternatively, treatment with low to moderate concentrations of CCL19 (5-500 ng/mL) produced a 5-fold induction of p38MAPK and a 4-fold induction of ERK1/2 [Fig 4.7 (B)]. In these cells the phosphorylation of Akt was not significant which may have been related to the high basal levels. Incubation with a high dose of CCL19 (750 ng/mL) induced very high levels of kinase activation. Concentrations of 100 ng/mL CXCL12 and 500 ng/mL CCL19 were chosen for further experimentation because they reproducibly stimulated moderate to high levels of phosphorylation in the metastatic cells. From these experiments stimulation with 100 ng/mL of CXCL12 proved to be efficient in activating various signalling pathways downstream of
Gβγ while incubation with 500 ng/mL of CCL19 was optimal. As a result these conditions were used in further time course experiments performed in breast cancer cells.

4.2.3.2. Time course analysis of chemokine-induced phospho-activation

The cellular signal transduction pathways initiated by chemokines have been well characterized in leukocytes. However at this point of time there is little if no data available on the characterization of the kinetics associated with signal transduction in cell types other than leukocytes despite the fact that chemokine receptors are expressed in most tissues and organs. Therefore, a time course analysis of the signal pathways initiated by chemokines was conducted in adherent breast epithelial cells. Lysates were prepared from two representative metastatic and non-metastatic cell lines that were serum starved for 2 h. The cells were treated with CXCL12 or CCL19 for 1, 30 and 60 min using ligand concentrations optimized from the previous experiments (Fig 4.7). In the metastatic cells MDA-MB-231 or BT-549, CXCL12 exposure induced a rapid but sustained 2- to 5-fold activation of ERK1/2, p38MAPK, JNK, GSK-3α/β and IκBα which returned to basal levels over an hour of stimulation and again Akt activation could not be detected (Fig 4.8). At 30 min in MDA-MB-231 cells, activation of the kinase pathways was consistently at its highest while in the BT-549 cells this was achieved earlier. In contrast, CXCL12 did not result in significant activation of signaling pathways in the non-metastatic cells MDA-MB-453 and MCF10A at 1, 30 or 60 min of chemokine incubation.

To further substantiate the evidence supporting the activation of chemokine-mediated Gβγ-dependent pathways in metastatic breast cancer cells, the signaling pathways downstream of CCR7 were examined. Similarly, in metastatic cells the level of phosphorylation was initiated early, within 1 min, and could be sustained for up to 30 min and then returned to basal
conditions at 1 h (Fig 4.9). ERK1/2, GSK-3α/β and JNK activation reached peak levels in the metastatic cell lines after 30 min of CCL19 incubation, while a 2-6-fold increase of p38MAPK, IκBα and Akt could be detected as early as 1 min and continued for 30 min. CCL19 failed to induce detectable activation of the measured pathways downstream of Gβγ in the non-metastatic cells confirming that observed previously in CXCL12 stimulated cells.

In summary, phosphorylation induced by the chemokines was noted only in the metastatic cells which was detected within 1 min, sustained for 30 min and had returned to basal levels after 1 h of stimulation. Although various kinase pathways were activated in both the MDA-MB-231 and BT-549 cells, the kinetics involved in the phosphorylation of signaling pathways induced by chemokines were very different in these cells with responses occurring more rapidly in the BT-549 cells.

4.2.4 Molecular examination of the association between chemokine receptors and G-proteins

4.2.4.1 CXCR4 and G-protein subunit association

The interaction between a chemokine receptor with the subunits of the heterotrimeric G-protein complex is an essential step in triggering multiple signalling pathways upon ligand binding (225). The inability of CXCR4 or CCR7 to bind to either Ga- or Gβγ-subunits in the non-metastatic breast cancer cells may potentially be responsible for the lack of chemokine-induced signal transduction in these cell types. Therefore, an investigation into the mechanisms coupling chemokine receptors to their G-proteins was performed.
CXCR4/G-protein coupling was first studied in MDA-MB-231 and compared to the non-metastatic cell line MDA-MB-453 which had been treated or untreated with CXCL12 for 15 seconds. Protein complexes were precipitated from whole cell lysates with a polyclonal anti-Gαi antibody. The immunocomplexes were then analyzed for the association with CXCR4 by Western blot. The results shown in Figure 4.10 (A) demonstrate a constitutive association between CXCR4 and Gαi in resting breast cancer cells (compare lane 2 & 4). This association was reduced in the metastatic MDA-MB-231 cells upon stimulation by CXCL12 signifying efficient dissociation of Gαi from the receptor following ligand binding (compare lanes 2 & 3), a necessary step for signal transduction. However, CXCL12 failed to stimulate dissociation of CXCR4 and Gαi in the non-metastatic cells (compare lanes 4 & 5). Next, the interaction between CXCR4 and the Gβγ-subunit was examined. The breast cancer cell lysates were precipitated with a pan polyclonal anti-Gβ antibody and the immunocomplexes were examined for the presence of CXCR4. As observed with Gαi a constitutive association was observed between Gβγ and CXCR4 in both MDA-MB-231 and MDA-MB-453 cells [Fig 4.10 (B), compare lanes 2 & 4]. Again, in keeping with the data obtained for Gαi, the dissociation of Gβγ from the receptor after ligand binding was only observed in the metastatic cell lines [Fig 4.10 (B), compare lanes 2-3 with lanes 4-5].

The interaction between the α and the βγ subunit in breast cancer cells was also investigated. Whole cell lysates prepared from resting cells and precipitated with Gαi antibodies. The resulting immunocomplexes were analyzed for the presence of Gβγ using a pan polyclonal Gβ antibody. The data presented in Figure 4.10 (C) show that in MDA-MB-231 cells, Gαi and Gβγ are present as a complex, this association is reduced following CXCL12 stimulation (compare lanes 2 & 3). Surprisingly this association was not detected in the non-metastatic
cells (compare lanes 4 & 5). This indicates that the heterotrimeric G-protein complex is present in the metastatic MDA-MB-231 cells but absent in the non-metastatic MDA-MB-453 cells.

4.2.4.2  CCR7 and G-protein subunit interaction

While the previous findings offer a novel insight into chemokine-mediated G-protein coupling mechanisms for breast cancer cells with non-invasive and invasive phenotypes, the data shown for only CXCR4. To extend and strengthen these findings, the interaction between CCR7 and G-protein subunits was investigated in breast cancer cells. The protein complexes were precipitated from untreated or CCL19-treated whole cell lysates with a polyclonal anti-Gαi antibody and the immunocomplexes were analyzed for the presence of CCR7 or Gβ by Western blot [Fig 4.11 (A) & (B)]. Overall, the results of these experiments were qualitatively similar to that observed for CXCR4. Constitutive association of Gαi from CCR7 was observed in both cell lines [Fig 4.11 (A), compare lanes 2 & 4]. Stimulation with CCL19 resulted in the efficient uncoupling of Gαi from CCR7 in MDA-MB-231 cells only (compare lanes 2 & 3). In the non-metastatic MDA-MB-453 cells in which CCR7 and Gαi were shown to associate constitutively, uncoupling of this interaction after CCL19 stimulation was not observed (compare lanes 4 & 5). In addition, the Gβγ-subunit of the heterotrimeric G-protein complex was shown to constitutively interact with CCR7 in both metastatic and non-metastatic cell lines [Fig 4.11 (B)]. However, only in the metastatic cell line, MDA-MB-231 was this association was reduced following CCL19 stimulation (lanes 2 & 3).

The formation of heterotrimeric G-protein complex was also assessed in resting and activated breast cancer cells. Whole cell lysates were precipitated with Gαi antibodies and the
immunocomplexes were evaluated for their interaction with Gβγ. The data shown for the metastatic cells, MDA-MB-231, demonstrate that Gαi and Gβγ are associated in resting and this association is reduced upon exposure to CCL19 [Fig 4.11 (C)]. The formation of the heterotrimeric G-protein complex could not be detected in the non-metastatic cells, as was seen previously in MDA-MB-453 cells (lanes 5 & 6).

4.2.4.3  Gα- or Gβγ-subunit association

To further investigate G-protein coupling mechanisms in cancer cells, the association between Gαi and Gβ throughout the entire panel of breast epithelial cell lines under resting conditions was performed. Whole cell lysates prepared from unstimulated breast cancer cells were precipitated with Gαi antibodies and the resulting immunocomplexes were analysed for their association with Gβγ. Under these conditions, Gαi and Gβγ formed a heterotrimeric complex in the metastatic but not in the non-metastatic cell types (Fig 4.12, upper panel). The differences in Gαi and Gβ binding observed throughout the panel of breast cancer cells lines were not due to the absence of Gβ protein since all cells expressed Gβ subunit (lower panel).

4.3  Summary

The role of chemokines in the control of leukocyte traffic, angiogenesis, cell proliferation, and their involvement in the pathogenesis of infectious, inflammatory and malignant disease, has made these small molecules an attractive target for therapeutics (226). The involvement of chemokines in mediating directional cell migration has been well established and studied for many years, but more recently it has become evident that chemokines are able to couple to
distinct signalling pathways that are involved in not only chemotaxis, but also cell growth and transcriptional activation in a range of cell types, including epithelial cells. The signalling pathways involved in regulating these processes are a part of a very complex and integrated system of which very little is understood, particularly in non-leukocytes. Because the data presented in Chapter 3 indicated differential functional responses to the chemokines CXCL12 and CCL19, the molecular events resulting from G-protein activation throughout the panel of human breast cancer cells was investigated.

A rigorous examination of the signaling pathways downstream of CXCR4 and CCR7 chemokine receptors, was able to identify a key difference between the metastatic and non-metastatic breast cancer cells at the level of G-protein subunit coupling. As chemokine receptors are generally, although not exclusively, coupled to the Gi subclass of G proteins (114), the investigation commenced by examining the ability of chemokines to inhibit forskolin-mediated cAMP production and intracellular calcium mobilization, signaling events regulated at the Gi subunit level. The data revealed significant transient increases in intracellular calcium concentrations after chemokine stimulation as well as the ability to suppress forskolin induced cAMP accumulations in the metastatic cell lines only. This effect was also noted for metastatic breast cancer cells treated with phosphatidic acid, a non-chemokine GPCR ligand, pointing to a common disruption of Gi-protein signaling. Furthermore chemokine-mediated calcium mobilization in the responsive cell lines was inhibited by pre-treatment with pertussis toxin, an inhibitor of Goi. At the same time, stimulation with CXCL12 or CCL19 was not capable of eliciting these responses in the non-metastatic cells. Altogether this set of data show that Gi-dependent signaling could only be activated in the metastatic breast cancer cell lines.
Since heterotrimeric G-proteins not only consist of the α-subunit but also the βγ-subunit, the ability of chemokine to regulate the function of a number of kinases downstream of the Gβγ-subunit complex was investigated. CXCL12 and CCL19 modulated the MAPK and the PI-3K pathways, only in the metastatic cell lines. Interestingly the kinetics of chemokine-induced phosphorylation of the measured kinases was slightly different in cells stimulated with CXCL12 compared with cells stimulated with CCL19 however activation was rapid and sustained to up to half an hour in both cases.

Given the distinct differences in the activation of signalling pathways demonstrated by the two cell types the coupling of G-protein subunits to the chemokine receptors was investigated. Co-immunoprecipitation experiments revealed that uncoupling of the α-subunit and Gβγ-subunit from either receptor, a necessary step for signal transduction, was not occurring the non-metastatic breast cancer cells. On the other hand, uncoupling of the α-subunit and Gβγ-subunit from the receptors was observed in the metastatic breast cancer cells, which also demonstrate cellular responsiveness (calcium mobilization, actin polymerization, chemotaxis and cAMP inhibition) to chemokine stimulation. The results of previous studies examining GPCR activation indicate a requirement for the association of the G-protein heterotrimer with the GPCR in the resting state for signal transduction to occur (209). Importantly, the interaction of the α-subunit and the Gβγ-subunit in the non-metastatic cells was completely absent even in resting conditions. The data implies there is a deficiency in the non-metastatic cells in the expression and function of the critical machinery required for signal transduction.

In summary, the results presented in this chapter show a clear distinction between metastatic and non-metastatic breast cancer cells, at the level of biological function, receptor signalling...
and G-protein coupling. At this stage, further studies are required to uncover the complete mechanisms responsible for the lack of functional responses observed in the non-metastatic cells. This will be the subject of future investigations and are beyond the scope of the present study.
Fig 4.1. *RT-PCR analysis of G-protein subunit expression in human breast cancer cell lines.* Total cellular RNA from breast cancer cells was isolated and 1 µg of RNA template was used for the first strand synthesis. Full-length oligonucleotide primers were used to amplify the gene product of (A) Goi (size ~ 1000 bp) while gene specific primers were used to amplify fragments of (B) Gβ_{(1,4)} subunits (size ~ 300 bp). The housekeeping gene, cyclophilin, was included as a RNA loading control and the PCR-amplified products were analysed by 2.0% agarose gel electrophoresis followed by ethidium bromide staining. The data are representative of 2 independent experiments.
Fig 4.2. Western blot analysis of G-protein subunit expression in human breast cancer cell lines. Equal amounts of protein (50 µg) from whole cell lysates were prepared from $1 \times 10^5$ cells breast cancer cells and subjected to SDS-PAGE under denaturing conditions. Proteins were transferred onto PVDF membrane and the Western blots were incubated with specific polyclonal anti-human antibodies against G\(\alpha\)i subunit (first panel), G\(\alpha\)q subunit (second panel) or G\(\beta\)1,4 (third panel) and the bands were visualised by ECL detection. The same blots were stripped and re-probed with \(\beta\)-actin (shown only for the G\(\alpha\)i western blot) to assess for equal protein loading with a representative blot shown (bottom panel). Lines indicate the position and size of the G-protein subunits and \(\beta\)-actin. Shown are representative blots each from one experiment with similar results obtained in 3 others.
Go, Ga
GΓ
{ 
Gαi

Gαq

Gβ
{ 
Gβ(1-4)

Actin

Jurkat-T | MDA-MB-231 | BT-549 | MDA-MB-453 | MDA-MB-134 | MCF10A

41kDa

36kDa

36kDa

40kDa
Fig 4.3. Chemokine-mediated inhibition of cAMP production as a measure of functional Gai-subunit activation in breast cancer cell lines. Cells were pre-treated with 5 μM forskolin (FSK) for 30 mins followed by the addition of either CXCL12 (100 ng/mL), CCL19 (750 ng/mL) or phosphatidic acid (PA; 100 ng/mL). Cells were harvested 10 mins later and cAMP levels were assayed from cell lysates. Values are the mean ± SE from triplicate measurements of a representative experiment performed twice.
The effect of CXCL12 on intracellular calcium mobilisation in breast cancer cells as a measure of functional Gα/γ-subunit expression. Breast cancer cells (1 × 10^6 cells/mL) were labelled with Fura-2AM and treated with CXCL12 (300 ng/mL) as indicated by the arrow. Fluctuations in the level of intracellular calcium were monitored over 100 s and recorded as [Ca^{2+}]_i (nM). (A) Real-time traces are shown for the metastatic cell line MDA-MB-231 and the non-metastatic cell line MDA-MB-453 following addition of CXCL12. (B) Comparison of the changes in calcium mobilisation in response to CXCL12 across the panel of human breast cancer cell lines, summarised as a fold-increase in [Ca^{2+}]_i (nM) relative to cells treated with PBS control. Data are shown as the mean ± SE determined in three independent experiments for all cell lines. (C) Inhibition of CXCL12-induced calcium mobilisation in the MDA-MB-231 metastatic cell line by PTX. MDA-MB-231 breast cancer cells were incubated overnight with PTX (100 ng/mL) and the response to CXCL12 (300 ng/mL) was measured as described above. The data shown are representative results from a typical experiment repeated at least three times.
(A)

\[ [\text{Ca}^{2+}] \text{(nM)} \]

\[ \text{MDA-MB-231} \quad \text{MDA-MB-453} \]

\[ \text{CXCL12} \quad 300\text{ng/mL} \]

\[ \text{Time (secs)} \]

\[ 0 \quad 20 \quad 40 \quad 60 \quad 80 \quad 100 \]

(B)

Fold Increase in [Ca\(^{2+}\)](nM)

MDA-MB-231  BT-549  MDA-MB-453  MDA-MB-134  MDA-MB-468  MCF10A

(C)

\[ [\text{Ca}^{2+}] \text{(nM)} \]

\[ \text{ CXCL12 } \quad \text{CXCL12 + PTX} \]

\[ \text{CXCL12} \quad (300\text{ng/mL}) \]

\[ \text{Time (secs)} \]

\[ 20 \quad 40 \quad 60 \quad 80 \quad 100 \]
Fig 4.5. The effect of CCL19 on intracellular calcium mobilisation in breast cancer cells as a measure of functional Gαq-subunit expression. Breast cancer cells (1 × 10⁶ cells/mL) were labelled with Fura-2AM and treated with CCL19 (750 ng/mL) as indicated by the arrow. Fluctuations in the level of intracellular calcium were monitored over 100 s and recorded as [Ca²⁺]ᵢ (nM). (A) Real-time traces are shown for the metastatic cell line MDA-MB-231 and the non-metastatic cell line MDA-MB-453 following addition of CCL19. (B) Comparison of the changes in calcium mobilisation in response to CCL19 across the panel of human breast cancer cell lines, summarised as a fold-increase in [Ca²⁺]ᵢ (nM) relative to cells treated with PBS control. Data are shown as the mean ± SE determined in three independent experiments for all cell lines. (C) Inhibition of CCL19-induced calcium mobilisation in the MDA-MB-231 metastatic cell line by PTX. MDA-MB-231 breast cancer cells were incubated overnight with PTX (100 ng/mL) and the response to CCL19 (750 ng/mL) was measured as described above. The data shown are representative results from a typical experiment repeated at least three times.
(A) Changes in [Ca^{2+}] for MDA-MB-231 and MDA-MB-453 cells with CCL19 (750 ng/mL) treatment.

(B) Comparison of fold increase in [Ca^{2+}] for various breast cancer cell lines.

(C) Effect of CCL19 (750 ng/mL) and CCL19 + PTX on [Ca^{2+}] levels over time.
Fig 4.6. *The effect of PA on intracellular calcium mobilisation in breast cancer cells as a measure of functional Gq/11 subunit expression.* Breast cancer cells (1 x 10^6 cells/mL) were labelled with Fura-2AM and treated with PA (300 ng/mL) as indicated by the arrow. Fluctuations in the level of intracellular calcium were monitored over 100 s and recorded as [Ca^{2+}]_i (nM). (A) Real-time traces are shown for the metastatic cell line MDA-MB-231 and the non-metastatic cell line MDA-MB-453 following addition of PA. (B) Comparison of the changes in calcium mobilisation in response to PA across the panel of human breast cancer cell lines, summarised as a fold-increase in [Ca^{2+}]_i (nM) relative to cells treated with PBS control. Data are shown as the mean ± SE determined in three independent experiments for all cell lines. (C) Inhibition of PA-induced calcium mobilisation in the MDA-MB-231 metastatic cell line by PTX. MDA-MB-231 breast cancer cells were incubated overnight with PTX (100 ng/mL) and the response to PA (300 ng/mL) was measured as described above. The data shown are representative results from a typical experiment repeated at least three times.
(A) [Ca^{2+}](nM) vs Time (secs)

(B) Fold increase in [Ca^{2+}](nM) for different cell lines.

(C) [Ca^{2+}](nM) vs Time (secs) for PA and PA + PTX treatment.
Fig 4.7. *Dose-dependent activation of chemokine-mediated signalling pathways downstream of the Gβy subunit in metastatic breast cancer cells.* Cells were serum-starved for 2 hrs and then treated with various concentrations of (A) CXCL12 or (B) CCL19 as indicated. Cells were harvested 15 mins later and analysed in a multi-plex phosphoprotein assay to simultaneously measure the activated states of ERK1/2, IκBα, JNK, p38MAPK, GSK-3α/β and Akt. The values were expressed as fold-activation relative to the amount of phosphoprotein present in untreated cells. The data represent the mean ± SE from triplicate determinations of two independent experiments.
Fig 4.8. Time course evaluation of CXCL12-induced Gβγ-dependent signalling pathways activated in breast cancer cell lines. The phosphorylation levels of selected kinases were assayed in lysates prepared from breast cancer cells treated with CXCL12 (100 ng/mL) at 1 or 30 mins intervals. Phosphorylation of signalling molecules in CXCL12-treated cells was compared with the basal levels in unstimulated cells in a multi-plex phosphorylation assay and represented as fold-activation. The data are presented as the mean ± SE of duplicate determinations and are representative of at least three independent experiments.
MDA-MB-231
BT-549
MDA-MB-453
MCF10A

pERK1/2
Fold Activation
CXCL12 (100ng/ml)

p38MAPK
Fold Activation
CXCL12 (100ng/ml)

pAkt
Fold Activation
CXCL12 (100ng/ml)

pGSK-3α/β
Fold Activation
CXCL12 (100ng/ml)

pJNK
Fold Activation
CXCL12 (100ng/ml)

pIκBα
Fold Activation
CXCL12 (100ng/ml)
Fig 4.9. *Time course evaluation of CCL19-induced Gβγ-dependent signalling pathways activated in breast cancer cell lines.* The phosphorylation levels of selected kinases were assayed in lysates prepared from breast cancer cells treated with CCL19 (500 ng/mL) at 1 or 30 mins intervals. Phosphorylation of signalling molecules in CCL19-treated cells was compared with the basal levels in unstimulated cells in a multi-plex phosphorylation assay and represented as fold-activation. The data are presented as the mean ± SE of duplicate determinations and are representative of at least three independent experiments.
Fig 4.10.  *Comparison of CXCR4/G-protein subunit coupling mechanisms in metastatic and non-metastatic breast cancer cells.* Cells were incubated with CXCL12 (100 ng/mL) or PBS for 15 seconds and then lysed in a modified Triton-X buffer. Equal amounts of lysate were immunoprecipitated with anti-Goαi antibody (A) or anti-Gβ (B) and subjected to SDS-PAGE. Immunocomplexes were analysed by Western blot for the presence of CXCR4. Immunocomplexes pulled down with Goαi were also analysed for the presence of Gβ (C). MDA-MB-231 whole cell lysate was included as a positive control (lane 1) and immunocomplexes precipitated with β-actin as a negative control to demonstrate antibody specificity (lane 6). *WB,* Western blot, *IP,* immunoprecipitation.
(A)

![Blot Diagram](image)

CXCL12: [Abbreviations]

WB: CXCR4

MDA-MB-231 MDA-MB-453

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47kDa

IP: anti-Gαᵢ

(B)

![Blot Diagram](image)

CXCL12: [Abbreviations]

WB: CXCR4

MDA-MB-231 MDA-MB-453

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47kDa

IP: anti-Gβ

(C)

![Blot Diagram](image)

CXCL12: [Abbreviations]

WB: Gβ(1-4)

MDA-MB-231 MDA-MB-453

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36kDa

IP: anti-Gαᵢ
Fig 4.11. Comparison of CCR7/G-protein subunit coupling mechanisms in metastatic and non-metastatic breast cancer cells. Cells were incubated with CCL19 (500 ng/mL) or PBS for 15 seconds and then lysed in a modified Triton-X buffer. Equal amounts of lysate were immunoprecipitated with anti-\(G_{\alpha}i\) antibody (A) or anti-\(G_{\beta}\) (B) and subjected to SDS-PAGE. Immunocomplexes were analysed by Western blot for the presence of CCR7. Immunocomplexes pulled down with \(G_{\alpha}i\) were also analysed for the presence of \(G_{\beta}\) (C). Immunocomplexes were precipitated with \(\beta\)-actin as a negative control to demonstrate antibody specificity (lane 1) and MDA-MB-231 whole cell lysate was included as a positive control (lane 2). \(WB\), Western blot, \(IP\), immunoprecipitation.
(A) MDA-MB-231  MDA-MB-453
CCL19: - - + - + -
WB:CCR7  - - - - - -
1 2 3 4 5 6
48kDa
IP: anti-Gαi

(B) MDA-MB-231  MDA-MB-453
CCL19: - - + - + -
WB:CCR7  - - - - - -
1 2 3 4 5 6
48kDa
IP: anti-Gβ

(C) MDA-MB-231  MDA-MB-453
CCL19: - - + - + -
WB:Gβ(1-4)  - - - - - -
1 2 3 4 5 6
36kDa
IP: anti-Gαi
Fig 4.12. *Analysis of the association of Gi with Gβ throughout the entire panel of breast cancer cell lines.* Whole cell lysates were prepared from unstimulated cells, immunoprecipitated with anti-Gβ antibodies and subjected to SDS-PAGE followed by protein transfer. The immunoblots were analysed for the presence of Gαi (upper panel) or Gβ(1-4) (upper panel) subunits. Whole cell lysates from Jurkat-T cell line was included as a positive control (lane1) and immunoprecipitations with anti-β actin antibodies were used as a negative control (lane 6).
WB: Gα₁

WB: Gβ(1-4)

IP: anti-Gβ
CHAPTER 5

RESULTS: The development and optimization of an experimental mouse model system to study human breast cancer metastasis
5.1 Introduction

In the last two chapters evidence for the expression and functional involvement of chemokine receptors in breast cancer metastasis in vitro was established. However, the ultimate proof of a role for chemokines in metastasis is considered to come from studies conducted in vivo. As a result, the aim of this part of the research was to develop and optimize the conditions for a human breast cancer model to investigate the function of chemokines and their receptors during the process of metastasis in vivo. The development of animal models has been one of the most important advances in understanding human cancers including breast cancer and to date there are several good models available to study the metastatic aspect of breast cancer, each with their own advantages and limitations.

The relevance of any particular animal model depends on how closely they are able to replicate the histology, physiological effects, biochemical pathways and metastatic patterns observed in the same human tumour type. In addition, mouse models that are able to evaluate specific molecular events that contribute to the development of metastases are especially important as they are the main determinants of the clinical course of the disease and patient survival and are the target of systemic therapy (227). The generation of clinically relevant mouse models however may require humanization since differences in the process of transformation and oncogenesis have been proposed between the human and mouse systems (88). Such models most commonly use immunodeficient animals, principally nude or SCID mice, which have been shown to be useful for the experimental analysis of human breast cancer metastasis (64, 81, 90, 228). In addition, models of orthotopic xenografts of human tumours, or tumour cell lines, in immunodeficient animals have been shown to partially reproduce the histology and metastatic pattern of most human tumours at an advanced stage. In breast cancer models, mammary tumours have proven to be one of the more difficult
tumours to transplant directly into anatomically appropriate tissue (the mammary fat pad) in experimental animals (66). However, other routes of injection can be used to assess the ability of human breast cancer cells to form metastatic lesions in the lungs (i.v. injection), the liver (intrasplenic), the brain (direct or intracarotid artery injection) and the bone marrow or bone (injection into the left ventricle of the heart). These different approaches have provided methods in which to accurately and reproducibly study human breast cancer growth and metastasis with the use of immunodeficient mice.

Based on the current literature some of the most promising models come from xenograft transplantations of human tumours in immunodeficient mice. As a result the MDA-MB-231 human metastatic breast cancer cell line, characterised in the previous chapter, was used to the role of CXCR4 and CCR7 during the metastatic process of cancer cells in SCID mice. The effect of modulating the expression of these chemokine receptors on this cell line on the metastatic potential was assessed utilizing gene-silencing techniques. To summarize, the aim of these experiments was to provide definitive proof for the essential role of CXCR4 and CCR7 expression during the process of breast cancer dissemination in vivo.
5.2 Results

5.2.1. *In vitro* characterization of siRNA-mediated down-regulation of chemokine receptor expression

5.2.1.1. Assessment of chemokine receptor knockdown

In recent studies the inhibition of the interaction between CXCR4 and CXCL12 using selective antagonists (229) or neutralising anti-CXCR4 antibodies (4) has been shown to block breast cancer metastasis *in vivo*. In the present examination, the expression of CXCR4 and CCR7 on breast cancer cells was specifically knocked-down by the use of short interfering RNAs (siRNAs). A retroviral-based delivery system was employed to introduce the siRNA sequences into breast cancer cells. The sequence designed to block CXCR4 expression targeted the nucleotides 470 to 490 of the CXCR4 open reading frame (230). The sequence directed against CXCR4 expression targeted bases 332 to 351 of the CCR7 open reading frame. A sequence from the *Renilla* luciferase gene was used as a negative control (230). Individual clones with reduced chemokine receptor expression determined by Flow cytometric analysis were selected in media containing puromycin and expanded.

The results from these experiments revealed that CXCR4 siRNAs were capable of reducing the overall cell surface expression of CXCR4 on metastatic breast cancer cells by up to 45% of that detected on the parental cell line (Fig 5.1). A number of clones were selected from the original polyclonal cell population each displaying different degrees of receptor knockdown activity [Fig 5.1 (A)]. From these clones high [45%, clone 7 (i)], moderate [30%, clone 19 (ii)] and low (18%, clone 3 (iii)) levels of CXCR4 inhibition was achieved. In addition, CXCR4-specific knockdown was confirmed, with the cell surface expression of CCR7 on CXCR4 siRNA cells similar to that observed in wild-type MDA-MB-231 cells [Compare Fig

- 98 -
Clones displaying greater than 30% reduction of chemokine receptor expression were further analysed in functional assays.

The effect of siRNAs targeting CCR7 mRNA in breast cancer cells was also examined by Flow cytometry. Overall, the results for CCR7 siRNA-mediated knockdown were similar to those obtained for CXCR4, with the selected clones displaying a range of reduced receptor expression levels. Shown in Figure 5.2 (A) are representative clones that exhibited variations in receptor knockdown efficiency; clone 5 presented a 65% decrease in receptor expression (i), clone 28 a 40% reduction (ii) while clone 3 resulted in a 20% inhibition (iii). Furthermore the level of CXCR4 expression was not altered on these cells after infection with CCR7 siRNAs as the amount of CXCR4 expression was comparable to levels previously observed in wild-type MDA-MB-231 cells [Compare Fig 5.2 (B) to Fig 3.1]. CCR7 siRNA-expressing clones characterised and demonstrated to exhibit more than a 30% reduction of receptor expression were selected and assayed for their functional responsiveness to chemokines.

Overall, the data indicated that the specific siRNA sequences chosen to target chemokine receptor expression knockdown resulted in significantly reduced expression. Although there was variation in the level of receptor knockdown across the selected clones, maximal knockdown of 45% for CXCR4 and 65% for CCR7 expression was achieved. Importantly, silencing of chemokine receptor expression appeared to be specific, with the endogenous levels of the non-targeted chemokine receptor unaffected. Furthermore, the morphology of cells infected with retroviruses containing siRNA sequences was compared with uninfected wild-type cells and revealed no apparent differences (data not shown).
5.2.1.2. Functional evaluation of siRNA-induced knockdown on chemokine receptors

To investigate if the effect of partial chemokine receptor knockdown on breast cancer cells led to an inhibition of function, the ability of cells to mobilise intracellular calcium in response to chemokines was examined. These experiments were conducted on clones that displayed greater than 30% receptor knockdown. The results from these experiments revealed that clone 19 (presenting a 30% reduction of CXCR4 expression) and clone 7 (presenting a 45% reduction of CXCR4 expression), were not able to mobilise intracellular calcium in response to CXCL12 [Fig 5.3 (A)]. Breast cancer cells that were infected with a control siRNA and treated with CXCL12 demonstrated a transient increase in intracellular calcium.

In order to substantiate the previous findings, the effect of migration in response to chemokines in the knockdown cells was examined. Cells were exposed to the indicated concentrations of CXCL12 and the migration index was recorded for the two clones and control cell lines (wild-type MDA-MB-231 cells and MDA-MB-231 cells infected with a negative control siRNA virus). The data revealed that CXCL12 induced migration in the MDA-MB-231 cell line infected with a control siRNA and in the parental cell line [Fig 5.3 (B)] whereas the chemotactic response to CXCL12 in the CXCR4 cells was significantly reduced. This effect was more pronounced at a higher concentration of CXCL12 with clone 7 displaying the greatest reduction in cellular migration.

The same approach as described above was employed to investigate the effect on receptor function in the CCR7 siRNA-mediated knockdown cells. Similarly, the data demonstrated that CCL19 induced a rapid increase in intracellular calcium [Fig 5.4 (A)] and a chemotactic response [Fig 5.4 (B)] in the control cell lines. However, the response in CCR7 knockdown cells was inhibited.
In summary, the characterisation of receptor activity in clones expressing significant levels of CXCR4 or CCR7 knockdown resulted in a significant inhibition of their functional activity. This was confirmed by calcium mobilisation and chemotaxis assays which are activated through independent chemokine receptor-mediated pathways. The role of CXCR4 and CCR7 during metastasis was further examined \textit{in vivo} using these cell lines.

\textbf{5.2.2. Characterization of knockdown cells for \textit{in vivo} experimentation}

\textbf{5.2.2.1. Development of a dual fluorescence system}

To examine the effect of chemokine receptor knockdown on breast cancer cells during the metastatic process \textit{in vivo}, a reproducible detection system to assay the formation of metastases was developed. While studies of a similar nature have been undertaken previously in metastatic lung, breast, colon, prostate and breast cancers \cite{231}, the majority of conventional methods use laborious histological and molecular biological techniques to examine micrometastases. However, tumour cells stably expressing fluorescent proteins \textit{in vivo} have proven to be a powerful new tool for cancer research allowing the visualisation in real-time of important aspects of cancer in living animals, including tumour cell mobility, invasion, metastasis and angiogenesis \cite{232}. Furthermore, fluorescent proteins of many different colours have now been characterised and can be used to ‘colour-code’ cancer cells of a specific genotype or phenotype \cite{233,234}. In this study a dual-colour fluorescence system was adopted. This allows for examination of metastatic properties of two different cancer subpopulations within the same microenvironment. Furthermore, such an approach permits an internal reference within the same animal, thus reducing the total number of mice used and the potential experimental variation that may exist between animals.
To achieve this, the previously characterised knockdown cells displaying maximal receptor knockdown were pooled to generate a ‘mixed’ cell population. The resulting cell line was then infected with viruses encoding GFP or RFP, and stable cell lines were produced. Shown in Figure 5.5 is a representative image of pooled CXCR4 knockdown clones expressing GFP and pooled CCR7 knockdown clones expressing RFP. These cell lines were visualised by fluorescent microscopy using the appropriate fluorescent filters. An overlay of red and green fluorescence is also presented to demonstrate that the cells express approximately uniform levels of fluorescence. Importantly, the use of the fluorescent proteins as well infection with siRNA sequences did not change the overall morphology of MDA-MB-231 cells.

5.2.2.2. Functional characterization of pooled knockdown clones

The mixed cell lines generated above were assessed for their functional responsiveness to chemokines determined by calcium mobilization. The main objective of these experiments was to confirm that following infection with retroviruses encoding fluorescent proteins that the newly-derived polyclonal knockdown population continued to exhibit a loss of function due to reduced chemokine expression. The data indicate that the pooled CXCR4 knockdown cells response to CXCL12 was inhibited to a similar degree as that observed for the individual clones [compare Fig 5.6 (A) to Fig 5.3]. The control siRNA cells were included in these experiments, to provide an indication of a normal response to CXCL12. Importantly the effect of receptor knockdown on function appeared to retain its specificity since CXCR4 knockdown cells responded to CCL19 indicating no significant effect on CCR7 function. Similar results were obtained in the CCR7 knockdown cells with the mixed cell line unable to mobilize calcium in response to CCL19 reproducing the pattern observed for the individual clones [Fig 5.6 (B)].
Prior to performing *in vivo* experimentation with the CXCR4 or CCR7 knockdown cell lines, an assessment of their relative proliferative capacity was conducted to eliminate the potential involvement of variations in inherent growth rates of the cell lines and any differences in metastatic potential observed *in vivo*. The breast cancer cells were seeded in 96-well plates at two different cell densities, $3 \times 10^3$ and $1 \times 10^3$ per well. The cells were then given sufficient time to re-attach, survive and grow. Following 24 h in culture, XTT solution was added to the cells for 3 hours and the absorbance, which correlates to cell density of each of the cell lines, was evaluated by a colorimetric assay. The data presented in Figure 5.7 show no significant differences in the growth rate of cells infected with any of the siRNA expressing viruses and the un-infected parental cell line. This effect was observed at both low and moderate cell densities.

5.2.3. *The effect of chemokine receptor expression on breast cancer cell metastasis in vivo*

5.2.3.1. **Generation of mixed cell lines for use in a dual fluorescence system**

A total of four mixed cell lines were produced to allow investigation of the effect of CXCR4 and/or CCR7 knockdown on metastasis *in vivo* (Table 5.1). Each of the fluorescently-labelled single knockdown cell lines was combined with a fluorescently-labelled internal control cell line (wild-type$^{RFP}$, control siRNA$^{RFP}$ or control siRNA$^{GFP}$). This resulted in a cell mix consisting of RFP- and GFP-expressing cells.

5.2.3.2. **Optimization of in vivo metastasis detection using control mixed cell lines**

The metastatic properties of the control group *in vivo*, consisting of the parental MDA-MB-231 cell line (expressing RFP) and the control siRNA cell line (expressing GFP), were first
examined to establish optimal conditions for the model. A range of cell concentrations and different time points was examined (data not shown). Following the preliminary experiments a dose of $6 \times 10^5$ cells in 200 µL of PBS and time points of 3 and 8 weeks were selected for these experiments. At these time-points mice were sacrificed, the lungs were perfused in PBS and then excised from the pleural cavity. No additional tissue preparation was performed on the whole lungs which were then examined under a fluorescence microscope. The level of either red or green fluorescence was used as an index of metastatic burden.

The results shown at week 3 after tail vein injections of metastatic cells, revealed evident tumour cell colonisation of lungs of SCID mice [Fig 5.8 (A)]. At this early stage the presence of the malignant lesions were small in size and diffuse as indicated by green and red colonies originating from the control siRNA and wild-type cell line respectively (overlay image). After 8 weeks, large (1-3mm) metastatic lesions were visible, constituting approximately 50% of all lesions on the surface of the lung which were also evident under normal light [Fig 5.8 (B-D)]. Of the mice injected with the control group, 100% developed surface lung metastases originating from the wild-type and control siRNA cells in similar numbers indicating both cells lines formed metastases at comparable rates in vivo. Furthermore, although a mix of GFP- and RFP-expressing clones were injected into mice, the resulting lung tumour colonies presented as pure green or red lesions with little overlap or yellow fluorescence.

Overall the data demonstrate that detection using dual-colour fluorescence provides an easy and effective method to distinguish the metastatic potential of two cancer cell lines within the same animal. Retroviral infection as well as the incorporation of fluorescent markers did not affect the ability of tumour cells to metastasize in vivo with the control siRNA$^{GFP}$ and parental MDA-MB-231$^{RFP}$ cells producing similar numbers of metastatic lesions.
5.2.3.3. Examination of chemokine receptor knockdown in vivo

Having established that the method of dual-colour fluorescence was useful for studying the behaviour of breast cancer cells in vivo, the effect of modulating chemokine receptor expression on breast cancer cell metastasis was subsequently investigated. The consequence of CXCR4 knockdown on breast cancer cells in vivo was first examined using the mixed CXCR4^-GFP and control siRNA^RFP cell line. The results from this experiment show that within 3 weeks the control cells produced numerous metastatic lesions as seen by diffuse red fluorescence while the breast cancer cells in which CXCR4 was knocked-down generated very few metastatic lesions (shown by a slight green speckling effect) [Fig 5.9 (A)]. An overlay image of red (control breast cancer cells) and green (CXCR4 knockdown cells) at higher magnification illustrates a clear dominance of red fluorescence. This effect was more pronounced after the lungs were examined at week 8 [Fig 5.9 (B-D)]. The images revealed a predominance of red fluorescence on the surface of lungs of all mice belonging to this group.

The role of CCR7 in breast cancer metastasis was examined using the mixed CCR7^-RFP and control siRNA^GFP cell lines. Co-injection of these cell lines revealed after 3 weeks green fluorescence only, corresponding to control breast cancer cell growth [Fig 5.10 (A)]. The presence of red fluorescence was not detected at week 3 or 8 indicating CCR7 knockdown cells were not metastatic [Fig 5.10 (A) & (B)-(D) respectively]. This pattern was observed for all of the experimental animals in this group.

Having established that CXCR4 and CCR7 were independently essential for metastasis in vivo, the effect on metastasis of CCR7^-RFP cell line mixed with CXCR4^-GFP cells line was examined in vivo as described above. Three weeks following intravenous injection of breast
cancer cells in which CXCR4 and CCR7 were silenced, the metastatic ability of MDA-MB-231 cells was completely inhibited with no fluorescence visualised [Fig 5.11 (A)]. Furthermore, at week 8 the absence of metastatic growth remained in all of the animals [Fig 5.11 (B-D)]. The data implies that the specific knockdown of CXCR4 or CCR7 directly inhibited the metastatic ability of MDA-MB-231 cells in vivo.

Overall, the data from these experiments demonstrate that siRNAs targeting the expression of either CXCR4 or CCR7 had a significant impact on the ability of breast cancer cells to form metastatic lesions in vivo (summary in Table 5.2). The use of an internal control cell line allowed a direct comparison between ‘normal’ tumour behavior and the effect of siRNA-mediated knockdown, giving a clear indication of the specific effect of CXCR4 and CCR7 knockdown in vivo.

5.3 Summary

In the previous chapters, evidence for the involvement of chemokine receptor expression and biological function in metastatic breast cancer cells was established in vitro. Furthermore, an insight into the molecular basis for differential functional responses of metastatic and non-metastatic breast cancer cells in terms of receptor signalling and G-protein coupling was also studied. However, the link between chemokine receptor expression in breast cancer metastasis in vivo remained to be investigated. To establish this connection a suitable metastatic mouse model system in which to examine the effect of chemokine receptor expression was characterised. The expression of chemokines on breast cancer cells was silenced using a retroviral-based delivery of siRNAs targeting CXCR4 or CCR7. While partial knockdown was achieved, a complete or significant inhibition of chemokine receptor function resulted, as
assessed by calcium mobilisation and chemotaxis assays. Chemokine receptor knockdown in SCID mice following intravenous administration in breast cancer cells dramatically reduced in the metastatic ability of these cells to the lung. The data imply the knockdown of either CXCR4 or CCR7 or both receptors has a dramatic effect on the metastatic potential of cancer cells thus highlighting an important role for these chemokine receptors during the metastatic process.

Overall, the model chosen in this study offered an easy and reliable way to directly study the role of these receptors in breast cancer metastasis \textit{in vivo}. An advantage of the system was the use of dual-colour fluorescence which provided a means to colour-code test and control tumour cell populations and to visually assess their metastatic potential within the same animal microenvironment. Detection of fluorescence provided a quick method for detection of both macro- and micro-metastases thus eliminating tedious and laborious detection methods described for other cancer models.
Fig 5.1. SiRNA-mediated down-regulation of CXCR4 expression in breast cancer cells. The MDA-MB-231 cell line was stably infected with the retroviral vector expressing a short hairpin RNA sequence targeting the CXCR4 mRNA open reading frame. Cells were selected with G418 (200 ng/mL) and the level of CXCR4 expression was examined by Flow cytometry analysis. The selected cells were stained with an anti-human CXCR4 antibody and the data was compared with cells infected with a virus encoding a control siRNA. The clones were also assessed for expression of CCR7. Shown are representative histogram plots of CXCR4 staining for (A) individual CXCR4 siRNA clones; clone 7 (i), clone (ii) and clone 3 (iii) as well as (B) CCR7 and isotype-matched control staining on CXCR4 siRNA cells (shown only for clone 7) to demonstrate receptor knockdown specificity. The percentages of reduced CXCR4 expression are shown in parentheses.
(A) (i) % Inhibition

Clone 7

(45%)

Counts

Control siRNA

(ii) Clone 19

(30%)

Counts

Control siRNA

(iii) Clone 3

(18%)

Counts

Control siRNA

CXCR4 Expression

(B)

Counts

Isotype Control

CXCR4/siRNA

CCR7 Expression
**Fig 5.2.**  *SiRNA-mediated down-regulation of CCR7 expression in breast cancer cells.* The MDA-MB-231 cell line was stably infected with the retroviral vector expressing a short hairpin RNA sequence targeting the CCR7 mRNA open reading frame. Cells were selected with G418 (200 ng/mL) and the level of CCR7 expression was examined by Flow cytometry analysis. The selected cells were stained with an anti-human CCR7 antibody and the data was compared with cells infected with a virus encoding a control siRNA. The clones were also assessed for expression of CXCR4. Shown are representative histogram plots of CCR7 staining for (A) individual siRNA clones; clone 5 (i), clone 28 (ii) and clone 17 (iii) as well as (B) CXCR4 and isotype-matched control staining on CCR7 siRNA cells (shown only for clone 28) to demonstrate receptor knockdown specificity. The percentages of reduced CCR7 expression are shown in parentheses.
(A) (i) Clone 5
Counts
Control
siRNA

(ii) Clone 28
Counts
Control
siRNA

(iii) Clone 17
Counts
Control
siRNA

CCR7 Expression

(B) Isotype Control
Counts
CCR7/siRNA

CXCR4 Expression
Fig 5.3.  *In vitro functional characterisation of MDA-MB-231 cells infected with CXCR4 siRNAs.* (A) CXCL12-mediated intracellular calcium mobilisation in CXCR4 knockdown cells. Cells in which CXCR4 was knocked-down were loaded with the intracellular calcium indicator Fura-2AM, treated with 300 ng/mL of CXCL12 (indicated by the arrow) and the changes in the level of intracellular calcium concentration were monitored. A typical experiment is shown for the indicated cells lines and clones with the data being representative of at least three experiments. (B) Chemotactic response of breast cancer cells infected with CXCR4 siRNAs. Breast cancer cells were exposed to the specified concentrations of CXCL12 in a 96-well modified Boyden chamber. The ratio of cells migrating in each well towards CXCL12 relative to media-only controls is shown as the migration index for the indicated cell lines. All panels are expressed as a mean ± SE of migration index from at least two separate experiments each performed in triplicate. Asterisks indicate statistical significance (Students unpaired T-test) at *p<0.05 or ** p<0.004.
Fig 5.4. *In vitro functional characterisation of MDA-MB-231 cells infected with CCR7 siRNAs.* (A) CCL19-mediated intracellular calcium mobilisation in CCR7 knockdown cells. Cells in which CCR7 was knocked-down were loaded with the intracellular calcium indicator Fura-2AM, treated with 750 ng/mL of CCL19 (indicated by the arrow) and the changes in the level of intracellular calcium concentration were monitored. A typical experiment is shown for the indicated cell lines and clones with the data being representative of at least three experiments. (B) Chemotactic response of breast cancer cells infected with CCR7 siRNA. The breast cancer cells were exposed to the specified concentrations of CCL19 in a 96-well modified Boyden chamber. The ratio of cells migrating in each well towards CCL19 relative to media-only controls is shown as the migration index for the indicated cell lines. All panels are expressed as a mean ± SE of migration index from at least two separate experiments each performed in triplicate. Asterisks indicate statistical significance (Students unpaired T-test) at *p<0.05, ** p<0.005 or ***p<0.0001.
(A) 

\[ \text{F} = \text{F} + \text{E} \text{C} \]

\( \text{EI} \)

\( \text{CCRT} / \text{siRNA Cl.28} \)

\( \text{CCRT} / \text{siRNA Cl.5} \)

\( 0 \text{xx} 50 \text{75} \text{500} \text{100} \) seconds

\( [\text{Ca}^{++}] \text{(nM)} \)

(B) 

\( \text{Wt MDA-MB-231} \)

\( \text{Control/siRNA} \)

\( \text{CCR7/siRNA Cl.28} \)

\( \text{CCR7/siRNA Cl.5} \)

\( 0 \text{xx} 10 \text{100} \) CCL19 (nM)

Migration index

** **
Fig 5.5. Dual colour fluorescent imaging of breast cancer cells stably expressing GFP and RFP proteins. Pooled MDA-MB-231 cells expressing CXCR4 or CCR7 siRNAs were transduced with a retroviral vector expressing elhXGFP and RFP respectively. Stably expressing GFP were selected with puromycin (200 ng/mL) while RFP-expressing cell lines were selected with G418 (200 ng/mL) and fluorescent cells were visualised by microscopy. Shown are representative images taken for the CXCR4\textsuperscript{+\rm GFP} and CCR7\textsuperscript{+\rm RFP} breast cancer cells using the appropriate filters.

Magnification: $\times 400$. 

(A) CXCR4 -/- GFP

(B) CCR7 -/- RFP

(C) Overlay GFP/RFP
Fig 5.6. *Confirmation of chemokine receptor responsiveness in knockdown cell mixes.* A. CXCL12-mediated intracellular calcium mobilisation in pooled CXCR4 knockdown cells. Clones were selected on the basis of displaying maximal CXCR4 reduction and pooled together to generate a mixed population. These cells were loaded with the intracellular calcium indicator Fura-2AM and were treated with 300 ng/mL of CXCL12 (indicated by the arrow). In addition, cells were exposed to CCL19 (750 ng/mL) to demonstrate receptor knockdown specificity in the CXCR4-knockdown mix cells. The changes in the level of intracellular calcium concentration were monitored over 100s and recorded as [Ca^{++}]i (nM). A typical experiment is shown for the indicated cells lines with the data representative of at least three experiments. B. CCL19-mediated intracellular calcium mobilisation in CCR7 knockdown pooled cells. As described above.
Fig 5.7.  *Proliferation rates of breast cancer cells infected with siRNAs targeting CXCR4 or CCR7.* The indicated pooled cell lines were seeded at $1 \times 10^3$ and $3 \times 10^3$ cells per well. After 24 h, the cells were washed twice in PBS and 150 μL XTT working solution (Section 2.4.7) were added. After a further 4 h incubation period, the absorbance of the samples was measured using an ELISA reader at a test wavelength of 492 nm and a reference wavelength of 690 nm. Data are presented as mean values for cell number ($n = 3$) ± SE of a representative experiment. ns, non-significant (Students T-test).
Absorbance (490nm)

- CCR7/siRNA
- CXCR4/siRNA
- siRNA Control
- MDA-MB-231

Absorbance values are shown for each condition, with error bars indicating standard deviation. The y-axis represents the number of cells, with tick marks at 1000 and 3000 cells.
Table 5.1. A summary of the cell line mixes generated for in vivo experimentation. The indicated cell lines were counted in equal cell numbers and mixed together to produce a single cell line. The resulting cell population contained a combination of GFP- and RFP-expressing cells which were injected into SCID mice and analysed for their metastatic potential.
<table>
<thead>
<tr>
<th>Mixed Cell Lines</th>
<th>Control siRNA&lt;sup&gt;RFP&lt;/sup&gt;</th>
<th>CCR7&lt;sup&gt;-/RFP&lt;/sup&gt;</th>
<th>Wild Type&lt;sup&gt;RFP&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control siRNA&lt;sup&gt;GFP&lt;/sup&gt;</td>
<td></td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>CXCR4&lt;sup&gt;-/GFP&lt;/sup&gt;</td>
<td>×</td>
<td>×</td>
<td></td>
</tr>
</tbody>
</table>
Fig 5.8. **Comparison of surface lung metastases at 3 and 8 weeks following intravenous injection of fluorescently-labelled control cell lines.**

Female SCID mice were injected into the tail-vein with the control cell line mix \( (6 \times 10^5 \text{ cells in } 200\mu\text{L}) \). The mix consisted of the parental breast cancer cell line MDA-MB-231\(^{\text{RFP}} \) and the control siRNA\(^{\text{GFP}} \) cell line. The animals were monitored over an 8-week period and whole lungs were excised at weeks 3 (A) and 8 (B-D), which were visualised using fluorescent microscopy to detect the extent of metastatic burden. Representative low and high magnification images of lungs excised after intravenous injections are shown for lungs viewed under bright field and fluorescent filters. The top right insert (white square) denotes images taken at a higher magnification and GFP-RFP overlay images are also presented (the bottom panel).
Normal light

RFP Filter

GFP Filter

Overlay

Week 3

(A)

Week 8

(B)
Fig 5.9. **Comparison of surface lung metastases at 3 and 8 weeks following intravenous injection of fluorescently-labelled CXCR4-knockdown cells.** Female SCID mice were injected into the tail-vein with the CXCR4-knockdown cell mix (6 x 10^5 cells in 200µL). The mix consisted of the control siRNA_{RFP} and CXCR4^{-/-}_GFP cells. The animals were monitored over an 8-week period and whole lungs were excised at weeks 3 (A) and 8 (B-D), which were visualised using fluorescent microscopy to detect the extent of metastatic burden. Representative low and high magnification images of lungs excised after intravenous injections are shown for lungs viewed under bright field and fluorescent filters. The top right insert (white square) denotes images taken at a higher magnification and GFP-RFP overlay images are also presented (the bottom panel).
Week 3
(A)

Normal light

RFP Filter

GFP Filter

Overlay

Week 8
(B)
Fig 5.10. Comparison of surface lung metastases at 3 and 8 weeks following intravenous injection of fluorescently-labelled CCR7 knockdown cells.

Female SCID mice were injected into the tail-vein with the CCR7-knockdown cell mix ($6 \times 10^5$ cells in 200µL). The mix consisted of the control siRNA$^{GFP}$ and CCR7$^{RFP}$ cells. The animals were monitored over an 8-week period and whole lungs were excised at weeks 3 (A) and 8 (B-D), which were visualised using fluorescent microscopy to detect for the extent of metastatic burden. Representative low and high magnification images of lungs excised after intravenous injections are shown for lungs viewed under bright field and fluorescent filters. The top right insert (white square) denotes images taken at a higher magnification and GFP-RFP overlay images are also presented (the bottom panel).
Normal light

RFP Filter

GFP Filter

Overlay

Week 8 (C)

Week 8 (D)
Fig 5.11.  *Comparison of surface lung metastases at 3 and 8 weeks following intravenous injection of fluorescently-labelled CXCR4 and CCR7 knockdown cells.* Female SCID mice were injected into the tail-vein with a mix of CXCR4 and CCR7 knockdown cells \((6 \times 10^5\) cells in 200µL). The mix consisted of CXCR4\(^{-/-}\)-GFP and CCR7\(^{-/-}\)-RFP cells. The animals were monitored over an 8-week period and whole lungs were excised at weeks 3 (A) and 8 (B-D), which were visualised using fluorescent microscopy to detect the extent of metastatic burden. Representative low and high magnification images of lungs excised after intravenous injections are shown for lungs viewed under bright field and fluorescent filters. The *white square* (top right) denotes images taken at a higher magnification and GFP-RFP overlay images are also presented (the bottom panel).
Normal light

RFP Filter

GFP Filter

Overlay

Week 3

(A)

Week 8

(B)
Table 5.2.  *A summary of the results obtained from siRNA-mediated chemokine receptor knockdown in vivo.* The table summarizes the data generated from the characterisation of chemokine receptor knockdown on breast cancer cells examined in a metastatic mouse model. The images shown are overlays of red and green fluorescent lesions of representative experiments.
<table>
<thead>
<tr>
<th>Cell Line Mixes</th>
<th>Control siRNA&lt;sup&gt;RFP&lt;/sup&gt;</th>
<th>CCR7&lt;sup&gt;-/&lt;/sup&gt;-&lt;sup&gt;RFP&lt;/sup&gt;</th>
<th>Wild Type&lt;sup&gt;RFP&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control siRNA&lt;sup&gt;GFP&lt;/sup&gt;</td>
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<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
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<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>
CHAPTER 6

DISCUSSION
6.1 Introduction

A considerable body of evidence highlights the importance of chemokines and their receptors during tumour progression and metastasis. Initially chemokines were noted for their ability to selectively regulate the recruitment and trafficking of leukocyte subsets during homeostasis and inflammation (115, 235). However, it is becoming increasingly clear that they are also responsible for controlling the function of a number of tumour-promoting processes including cancer cell growth, angiogenesis, host immune responses against malignant cells, and metastasis (106, 107, 236). In fact, up-regulation of specific chemokine/receptor pairs has been reported in many human cancers including breast, lung, prostate, colon, gastric and melanoma (2, 137). In particular, CXCR4 and CCR7 have been identified for their important role in breast and skin cancers (4). These chemokines have been implicated in facilitating tumour dissemination at each of the key steps of metastasis: adherence of tumour cells to endothelium; extravasation from blood vessels; metastatic colonisation; angiogenesis; proliferation and protection from the host response via activation of key survival pathways (100), although at this point in time their precise mechanism of action is still being investigated. Accordingly, the aim of the research presented in this thesis was to examine the role of CXCR4 and CCR7 expression on breast cancer cells by studying the molecular events mediated by chemokine receptor activation in these cells during the metastasis process.

To achieve this goal, a comprehensive assessment of CXCR4 and CCR7 expression and functional relationship with the pathology of breast cancer was performed. Initially the expression of CXCR4 and CCR7 was analysed in a range of non-metastatic and metastatic breast cancer cell lines. As there was no clear association between receptor expression and the phenotypes of the cancer cell lines, the function of chemokine receptors in terms of molecular and signalling events was subsequently assessed. Finally, the biological relevance
of CXCR4 and CCR7 expression \textit{in vivo} was investigated using a humanised metastatic mouse model of breast cancer which was developed and optimised.

The results generated from this research project provided several novel insights into the molecular mechanisms mediated by CXCR4 and CCR7 in breast epithelial cancer cells. In terms of their role during breast tumourgenesis, the data confirm that these chemokine receptors are important regulators of the metastatic process and for this reason may potentially be useful therapeutic targets to control the spread of tumour cells during breast cancer and potentially for other solid tumour malignancies.

6.2 The expression and functional role of chemokine receptors in breast cancer cells \textit{in vitro}

The expression of CXCR4 and CCR7 on tumour cells has recently received a great deal of attention due to the discovery of their direct involvement in promoting cancer and metastasis. Expression of these chemokine receptors has been reported on a number of cancer cell lines, tissues and primary tumours and the interaction with their associated ligands has been demonstrated to stimulate migration of cancer cells \textit{in vitro} and \textit{in vivo} (4). Of particular importance are retrospective studies in clinical samples, which use the expression of these chemokine receptors to correlate the outcome of cancer patients. In one study, the presence of CCR7 in non-small cell lung cancer (NSCLC) cells was directly associated with the development of lymph node metastasis (171). More specifically, CCR7 mRNA was expressed at higher levels in cancer tissue and not in the neighbouring normal lung tissues. Furthermore, the expression of CCR7 mRNA correlated with the stage of lymphatic invasion suggesting that CCR7 may participate in the emigration of cancer cells from peripheral tissue
to lymph nodes via lymphatics. As a result, the expression of CCR7 could be used as a potential diagnostic tool for predicting lymph node metastasis before surgery thus improving the overall disease treatment planning of NSCLC. It has also been reported that CCR7 expression specifically enhances the metastatic ability of B16 melanoma cell through a lymph-mediated but not a blood-borne pathway (167). Initially, CCR7 was shown to play a critical role in the migration of activated dendritic cells to regional lymph nodes via afferent lymphatic vessels. Subsequently, it has been proposed that neoplastic cells can also enter lymphatic vessels to enhance dissemination to the lymph nodes, which has been shown for B16 murine melanoma cells (167). Indeed, the formation of lymph node metastasis of CCR7 expressing B16 cells was blocked by neutralizing anti-CCL2\(^\_\)\(^\_\) antibody, confirming a role for CCR7 during metastasis.

CXCR4, on the other hand, appears very important for different reasons. Firstly, CXCR4 is easily the most common chemokine receptor expressed in tumour cells being implicated in over 25 different epithelial, mesenchymal and haemopoietic human cancers (2). However, not all cancer cells studied are CXCR4-positive. Some of the cell lines derived from ovarian cancer, acute myelogenous leukaemia (AML), anaplastic thyroid cancer and glioma are CXCR4-negative, as are primary cells from acute myeloid AML, erythroid AML and undifferentiated AML. Moreover, within primary tumours such as ovary and NSCLC, only a sub-population of cells express CXCR4. Secondly, CXCR4 activation on certain malignant cells plays a significant role in regulating metastasis to the lung, liver, bone marrow and brain (4, 237, 238). Since these organs are enriched with CXCL12 it was suggested that CXCR4-expressing tumours are attracted to these areas, in which the ‘seed and soil’ are compatible. Finally, CXCR4 expression has been linked to promoting the
production of other factors involved in the processes of malignancy. For example, CXCL12 stimulation of ovarian cancer cell lines and primary cells isolated from ascitic disease caused production of the pro-inflammatory cytokine TNF-α (42), which has been implicated in tumour/stromal communication in this disease.

The majority of these studies have focused on correlating the level of chemokine receptor expression with the metastatic disease state of the tumour. However, conflicting evidence shows that the expression of CXCR4 on cancer cells is not simply confined to cancer cells exhibiting metastatic abilities. One study shows that CXCR4 expression was initiated at a very early point in the transition from normal to a transformed phenotype in breast epithelium. This chemokine receptor was highly expressed in ductal carcinoma in situ (DCIS), a precursor of invasive ductal carcinomas (191). Importantly, high levels of CXCR4 were detected in 94% of studied cases of atypical ductal hyperplasia, potentially the first clonal pre-neoplastic expansion of ductal epithelial cells, representing an early stage in tumourigenic transformation (191). Therefore, these observations raise the possibility that expression of important chemokine receptors implicated in metastasis, such as CXCR4 and CCR7, are not restricted to the more malignant forms of breast cancer.

In view of these contrasting studies, the precise relationship of chemokine receptor expression in cancer cells and their potential role in metastatic tumour progression is still not clear and requires considerable clarification. To establish if the level of chemokine receptor expression relates to the disease stage of breast cancer, the analysis of CXCR4 and CCR7 expression in breast cancer cells was investigated in vitro. A panel of cell lines ranging from non-transformed immortalized breast epithelial cells to highly aggressive breast cancer cell lines was used in the study. Each of the cell lines was originally derived from patients with
different types of metastatic breast disease displaying various degrees of invasiveness based on their ability to form metastatic lesions in immunocompromised mice. The cell lines have been previously characterised in rigorous examinations and have been shown to meet the 'golden standards' of a continuous immortalized cell line resulting in their extensive use in the cancer field (90, 239, 240). There are however limitations involved in comparing cells that originate from different tumours as well as genetically different individuals, but the main purpose of the panel of selected cell lines was to model the major transitions of breast tumourgenesis. The cell lines chosen for the study satisfy this criterion.

The examination of CXCR4 and CCR7 expression in the breast cancer cell lines selected for this study revealed significant levels of expression throughout the different cell lines. CXCR4 and CCR7 expression was not restricted to the metastatic cell types with MCF10A cells, a non-transformed immortalized breast cancer cell line, exhibiting similar levels to that observed in highly invasive transformed breast cancer cell line, MDA-MB-231. These data challenge previous reports and strongly suggest that CXCR4 and CCR7 expression alone is not an indicator of aggressive breast cancer and therefore would not be useful as a prognostic marker of metastasis.

The assessment of chemokine receptor functionality in vitro is routinely performed by measuring the ability of chemokine ligands to induce calcium mobilisation, actin polymerisation and chemotaxis, which are early physiological changes associated with chemokine receptor activation (136, 241). The biological role of CXCR4 and CCR7 expression across the breast cancer cell lines was not the same with chemokine ligand treatment eliciting functional responses in the highly metastatic breast cancer cells but not in the non-metastatic cells. Radio-ligand binding assays revealed the presence of similar receptor
numbers as well as ligand binding affinities on the metastatic and the non-metastatic cells. Since these parameters were similar in both cell types, the observed differential signalling could not be accounted for and it was concluded that lack of functional receptor activity was probably attributed to a disruption in chemokine-mediated intracellular signalling.

The phenomenon of non-functional chemokine receptor expression is not common in the literature, although there have been several examples described. In a recent study, Trentin et al., reported differential function of CXCR4 and other chemokine receptors in non-Hodgkin lymphomas (242). Their findings revealed the expression of non-responsive CXCR4 in normal B cells (with respect to chemotaxis and calcium mobilization) and fully functional receptors in leukemic cells, similar to that observed for breast cancer cells examined in this study. In a different study, an early signalling defect in the CXCR4-expressing hepatocellular carcinoma cell line, HepG2, was identified (203). In those cells, CXCL12 was shown to bind to CXCR4 in Hep2G cells although the activation of downstream signalling events was not triggered. However, the transfection of exogenous CXCR4 into the Hep2G cell line was able to restore function upon CXCL12 treatment resulting in phosphorylation of p44/42, indicating the presence of intact signalling machinery in HepG2 cells transfected with CXCR4. Also a subpopulation of neuronal cells, cerebellar granule cells, have been reported to express non-functional CXCR4 while glial cells and cortical neurons were able to undergo chemotaxis and mobilise calcium in response to CXCL12 (124). Finally, differences in CXCR4 functionality have been reported in normal and malignant human hematopoietic cells (199). It was demonstrated that the activation of CXCR4 in human T cell lines (Jurkat and ATL-2) rapidly induced phosphorylation of mitogen-activated protein kinases (MAPK) (p44 ERK-1 and p42 ERK-2). The CXCR4-mediated signalling in normal haematopoietic cells, human megakaryoblasts, which highly express CXCR4 as a model, also led to the phosphorylation of
MAPK and the serine/threonine kinase AKT after CXCL12 treatment. However neither MAPK nor AKT was phosphorylated in normal human platelets after stimulation by CXCL12.

These findings, together with the observations from the present study strongly point to the existence of distinct mechanisms regulating the activation of CXCR4 and CCR7, which is likely to be cell type-dependent. In the case of cancer progression it is feasible to predict that cells that acquire functional chemokine receptors receive selective advantages to survive, migrate, colonise or proliferate at secondary tumour sites. At this point in time, the physiological function of CXCR4 or CCR7 expression in non-metastatic breast cancer cells is not known, nor is it known for normal breast epithelial cells. The determination of their role would be valuable but to achieve this, however further experiments will be required.

6.3 Molecular aspects of chemokine receptor signalling in breast cancer

To date there has been a great deal of interest in chemokine receptor signaling in cancer in terms of the specific pathways and effector molecules that regulate cell survival, proliferation, chemotaxis, migration and adhesion (100, 237, 243, 244). It is clear that a large number of downstream effector molecules are regulated by chemokines, which may account for the multiple effects of these receptors in the pathobiology of tumours. In particular the activation of chemokine receptors expressed in breast, prostate, melanoma and colon cancer, for example, has been shown to facilitate tumour dissemination at several key steps of metastasis including adherence of tumour cells to the endothelium, extravasation from blood vessels, metastatic colonization, angiogenesis, proliferation, and protection from host responses (100).
It is thought that these responses are activated through key survival pathways such as the MAP kinase, JAK/STAT, and PI-3K and Akt (224, 245, 246). However, at this stage the role of various effectors of chemokine receptors in primary and metastatic tumours has not been well established. Moreover, investigations into chemokine signalling have predominantly been conducted in leukocytes with little evidence presently available on signaling cascades in cell types other than leukocytes. The purpose of this part of the study consisted of two components. Firstly, chemokine-mediated signalling events in breast cancer cells were examined to address the paucity of existing knowledge. Secondly, as the previous findings presented in this study point to a difference in the metastatic and non-metastatic breast cancer cells with respect to chemokine receptor function, chemokine-mediated signalling events were compared in the two cell types.

Although at the present time there is relatively little known regarding the signalling pathways activated by chemokines in cancer cells, preliminary data show that CXCR4 and CCR7 are capable of activating several different intracellular events such as chemotaxis, invasion and adhesion, all of which are properties that correlate with metastatic behaviour of tumour cells (58, 136, 247, 248). These initial stages of cancer metastasis can be viewed as a tissue remodelling process involving various alterations in cell morphology such as cell polarization, redistribution of membrane receptors, the rearrangement of the actin cytoskeleton and the formation of filopodia and lamellipodia (extensions required for cell spreading and cell motility). Shown in this investigation, CXCR4 and CCR7 induced a transient increase in actin polymerisation and chemotaxis in metastatic breast cancer cell lines. However, the non-metastatic cell lines did not display significant changes in the cytoskeleton or migration after chemokine treatment under any of the conditions tested. The ligation of CXCL12 to CXCR4 has been proven to stimulate the PI-3K pathway that subsequently activates the protein kinase...
B or Akt. Activated Akt phosphorylates a wide variety of intracellular targets, functioning to inhibit apoptosis and prolong cell survival in many different types of cancer cells (245, 246). Beyond the role of promoting cell survival, the PI-3K pathway also has been implicated in effects of CXCR4 on proliferation of cells and migration (139, 249). This was further confirmed by the ability of CXCR4 and CCR7 ligands to induce chemotaxis in metastatic breast cancer cell lines used in this study, while the non-metastatic cells exhibited no ability to migrate. Clearly CXCR4 and CCR7 are involved in early migratory responses such as cytoskeleton remodelling and chemotaxis in the metastatic breast cancer cells while the non-invasive cell types show no changes during this stage. This implies that the expression of CXCR4 and CCR7 in non-metastatic cells are non-functional.

As actin polymerisation and chemotactic responses are not induced following the binding of CXCR4 and CCR7 ligands in non-metastatic breast cancer cells, it is possible that the signalling intermediates further upstream of these events are disrupted. Chemokine signalling pathways are mediated through GPCRs and generally use the Gi subclass of G proteins (114, 215) that results in the activation of the Gαi subunit which mediates the inhibition of adenylyl cyclase-mediated cAMP production and the mobilisation of intracellular calcium (212). Treatment with CXCL12 or CCL19 inhibited forskolin-induced adenylyl cyclase-mediated cAMP in metastatic breast cancer cell lines, however this was not the case for the non-metastatic cells. In addition chemokines could only induce Gαi-dependent intracellular calcium mobilization in the metastatic breast cancer cells. This induction of intracellular calcium mobilization was inhibited by pre-treatment with pertussis toxin, an inhibitor of Gαi. Therefore, functional Gαi signalling was only observed in the metastatic breast cancer cells after chemokine treatment, even after addition of a non-chemokine GPCR/phosphatidic acid, non-metastatic cells did not respond.
In cell types such as leukocytes the βγ subunit of the G protein complex is responsible for the activation of numerous kinase cascades downstream of GPCRs (248, 250). In metastatic breast cancer cells a rapid and sustained activation of ERK1/2, IκBα, JNK, Akt, p38MAPK and GSK-3α/β was observed in response to CXCR4 and CCR7 ligands, while little to no activation was detected in any of the non-metastatic representative cell lines. Because several of these effector molecules play a significant role during the migration of leukocyte cells (103, 251) these data imply that breast cancer cells use similar chemokine-mediated mechanisms to regulate processes such as migration, proliferation and survival and also metastasis. Moreover, the fact that Gβγ-dependent signalling is not activated in the non-metastatic cell types suggests that the block in functional signalling occurs further upstream of these signalling pathways. Overall, the analysis of the chemokine-mediated signalling events further downstream of the G-protein α and βγ subunits in breast cancer cells suggests that the blockade of CXCR4 and CCR7 function in non-metastatic cells occurs at the level of G protein activation.

6.3.1 Chemokine receptor and G-protein coupling mechanisms

Heterotrimeric G proteins act as molecular switches in signalling pathways by coupling the activation of heptahelical receptors at the cell surface to intracellular responses (252, 253). This role depends on the ability of the Ga subunit to cycle between a resting conformation primed for interaction with an activated receptor and a signalling conformation capable of modulating the activity of downstream effector proteins. In the resting state, the Ga subunit binds GDP and Gβγ. Receptors activate G proteins by catalysing GTP for GDP exchange on the Ga subunit, leading to a structural change in the Ga (GTP) and Gβγ subunits that allows
the activation of a variety of downstream effector proteins (Fig 1.5). The G protein returns to the resting conformation following GTP hydrolysis and subunit re-association.

The complexity of the molecular mechanisms whereby G protein-mediated signal transduction occurs is only beginning to be completely appreciated. G proteins play an important role in determining the specificity and temporal characteristics of the cellular responses to a diverse array of extracellular stimuli. For a long time, the study of GPCR signalling has been focused on classical second-messenger-generating systems. However it is becoming clear that these intracellular signalling molecules are not sufficient to explain their wide array of biological responses. It has been proposed that the ability of GPCRs to exert numerous physiological roles is a reflection of the receptor expression levels in the cell, the repertoire of signalling molecules expressed in each cellular system and the specificity of G-protein subunit coupling or a combination of all these factors (207, 213, 230, 254).

The results of previous reports have indicated that in the resting state, the heterotrimeric G proteins are constitutively associated with the cytoplasm surface of the GPCR as a trimer (209). In this study, chemokine receptor and G-protein coupling examined in resting and chemokine-activated metastatic and non-metastatic breast cancer cells demonstrated that CXCR4 and CCR7 formed complexes with the Gαi subunit constitutively in both cell types. However following chemokine stimulation, the dissociation of Gαi from the receptor occurred in only the metastatic cells. In parallel, the Gβ subunit associated with chemokine receptors in both non-invasive and metastatic breast cancer cells and dissociation of Gβ from the receptor upon ligand stimulation occurred only in the metastatic cells. Further investigations revealed that the formation of the heterotrimeric G-protein complex could only be detected in the invasive cell types that express functional chemokine receptors. The
differences in \( \Gamma \alpha \) and \( \Gamma \beta \) binding observed throughout the panel of breast cancer cells lines were not due to the absence of \( \Gamma \beta \) protein since all cells examined expressed \( \Gamma \beta \) subunits. Therefore, this novel finding indicates that in non-invasive cells with non-functional CXCR4 or CCR7, \( \Gamma \alpha \) and \( \Gamma \beta \gamma \) do not form the functional heterotrimeric structure which is critical for GDP to GTP transfer and thus activation of signalling pathways downstream of \( \Gamma \) proteins (255).

The major finding of the first part of this study is that highly metastatic breast cancer cell lines express functional chemokine receptors whereas non-metastatic cells do not. This points to the existence of potential regulatory mechanisms that may be switched on or off during the metastatic progression of breast cancer. Furthermore, the data indicate that the potential functional ‘on-switch’ for chemokine receptors expressed in breast cancer cells is controlled at the level of the receptor and G-protein subunit interactions with CXCR4 and/or CCR7. In summary, these data point to the existence of a novel mechanism for post-translational regulation of CXCR4 or CCR7 function, which may have potentially important implications for the acquisition of an invasive, metastatic phenotype by the cells of breast and possibly other tumours. Although at this point in time, no further insight into the precise molecular mechanisms mediated by G-proteins in cancer cells are available, the results from these studies may shed some light in the area. The results, however of recent studies suggest that various \( \alpha \), \( \beta \) and \( \gamma \) subunits form preferred G protein heterotrimers which in turn form complexes with GPCRs that are specific for these trimers (256). Therefore, lack of trimer formation in the non-invasive cells may be due to the expression of “incompatible” \( \alpha \) and \( \beta \gamma \) subunits in those cells. It is of note that the family of heterotrimeric G proteins consist of 27\( \alpha \), 5\( \beta \) and 14\( \gamma \) subunits, which leads to a very high number of possible \( \alpha \beta \gamma \) subunit combinations of varying affinity for a multitude of GPCRs (257). Alternatively G-protein heterotrimers may
potentially act in a tissue- and cell-specific fashion (230, 258). Another plausible explanation for the inability of Gα and Gβγ subunits to form stable complexes in selective cell lines may be the expression of one or more inhibitory molecules. Of relevance, Soriano et al., recently found that SOCS3 up-regulation by cytokines led to functional inactivation of CXCR4 via blockade of the Gαi pathway (259). Specifically, SOCS3 overexpression stimulated by cytokines impaired the response to CXCL12 as determined by cell migration in \textit{in vitro} and \textit{in vivo} experiments. It was proposed that this effect is mediated by SOCS3 binding to the CXCR4, thereby blocking JAK/STAT and Gαi pathways.

While the major focus of the present study was on the chemokine receptors CXCR4 and CCR7, the question of whether other GPCRs are similarly regulated in these cell lines is still open. Interestingly, in the present study as well as in others (260) an observation of a distinction between non-metastatic and metastatic cells in terms of their ability to respond to the lysophosphatidic acid (LPA) precursor, phosphatidic acid (PA). PA has been implicated in a host of important biological and pathological effects, including in cancer progression (261-263). This raises the possibility that other GPCRs, including other chemokine receptors, may be subject to the same control mechanism and warrants further investigation.

To gain a broader understanding of the functional roles chemokine receptor signaling pathways in human breast cancer cells, additional studies will be required to identify which G protein αβγ subunit combination interact with CXCR4 and CCR7. While these previous studies raise some insight into the observations presented in this study, determination of the precise molecular basis underlying CXCR4 or CCR7 blockade in the non-invasive cells should be considered, however these experiments were not feasible for this particular study due to time constraints.
Many aspects of G protein-mediated signalling remain to be elucidated. Nonetheless, many components of the system have been identified but new approaches are required to determine the exact composition of individual signalling units and to define their exact cellular localization. This will probably lead to the identification of even more proteins and non-protein factors that modulate G protein-mediated signalling. The use of in vivo models is necessary to test the significance of signalling mechanisms described in vitro under normal conditions as well as in disease states. The parallel application of genetic, genomic and of proteomic approaches will be required to continue to define how the G protein-mediated signalling system works on a molecular, cellular, and systemic level. Such an integrated view will provide the basis for a complete understanding of the physiological and pathophysiological role of G protein-mediated signalling and will allow the full exploitation of this multifaceted signalling system as a target for pharmacological interventions.

6.4 The role of chemokine receptors on breast cancer metastasis

in vivo

Metastasis is one of the more difficult tumour processes to study in vitro since the progression involves a sequence of events that is dependent on the properties of a tumour cell and its interactions with the tissue environment (227). Although the role of chemokine receptor function in mammary epithelial tumour cells has been assessed from cells maintained as monolayer in culture, there are several potential pitfalls in relying exclusively on in vitro studies. Over several passages the traits of tumour cells in culture can change and for the population of cells to consistently reflect the same metastatic phenotype at any given time is rare. In addition, using cell lines in vitro to model distinct steps and signalling events during the metastatic pathway can only take into account the ability of tumour cells to perform one
step in the progression of metastasis which may not represent the true nature of the multi-stage disease process (239). Finally, one of the biggest drawbacks of in vitro culture of established breast cancer cell lines is its limited use when it comes to studying cancer cell stromal interactions. Therefore, more relevant studies of metastasis are thought to come from those performed in vivo. Indeed, mouse models of human cancer have become a central part of many types of biomedical research as the laboratory mouse provides the most experimentally accessible mammalian model that shares organ structure, physiology and genes with the human system (67).

To date, there are a number of in vivo models available to study metastasis (64, 81, 227). Commonly used models include the transplantable xenogeneic or syngeneic systems, conditional or inducible transgenic mouse models and the chemically-induced mouse models (Section 1.3). It should also be pointed out that the entire complexity and heterogeneity of breast cancer cannot be represented in one model. For example, chemically-induced rodent models are excellent for investigating early events in the tumourigenic process while transplantable cancer models of metastasis are useful for studying later stages of the metastatic cascade. These models have proven to be valuable tools for the elucidation of the mechanisms of mammary tumourgenesis, each with their specific advantages and limitations. It is therefore important for investigators to critically assess theses factors in light of the hypotheses to be tested and to choose the appropriate model accordingly. Furthermore it is strongly believed that the most widely used animal models do have a limited role in cancer research as the biology of rodents and their tumours differ significantly from that of humans (67). In the field of cancer, there is a tendency to characterize the value of a mouse model in terms of the similarities shared between the animal and human cancer that is being
reproduced. The developmental differences in mouse tumours compared with humans tumours manifest in many ways, with size of the organism being the most obvious as humans are about 3,000 times larger than mice and are formed from a proportionally larger number of cells. Moreover, humans live on average 30-50 times longer than mice and given the continued, lifelong turnover of cells and more cell divisions, humans are predicted to experience vastly higher rates of cancer incidence (67). Overall, a smaller number of genetic changes, in comparison with human tumours are required for rodent cell transformation in vitro (264), and this is probably also true for murine tumours in vivo. This may contribute to the notable differences in tumour biology and pathology observed between the species.

A method, which attempts to eliminate these potential problems, is the widely used tumour xenograft models that involves the engraftment of human tumours in immunocompromised mice (265). This model represents a system with which to study certain complex pathobiological processes of human tumourgenesis, such as invasion and metastasis, that are not readily mimicked by the genetically engineered mouse models and that cannot be recapitulated by use of in vitro models. Xenograft transplantation of human tumours by orthotopic, intravenous, intrasplenic or intracardiac methods in nude mice have been shown to accurately reproduce, to a high degree, the histology and metastatic pattern of most human tumours at an advanced stage. For example, the histology of xenografts often closely reflects the variety of human adenocarcinomas (266). In human breast cancer sometimes the tumours can be difficult to transplant orthotopically into experimental animals with reported low success/take (7-20%) (267). Therefore, the use of experimental metastasis by intravenous injection of tumour cells directly into the systemic circulation, also known as the haematogenous route, has provided several advantages over the orthotopic method. The time...
course for this model is generally rapid, the biology of metastasis is reproducible and the number and type cells introduced into the system can be controlled (81). Since this model is based on cancer cells being injected into the bloodstream of mice for the purpose of producing pulmonary metastatic lesions, particular aspects of metastasis such as extravasation, migration, attachment, adhesion, survival or proliferation can be studied. Of note, it should be recognized that there are some advantages of xenogeneic models. For example, there is no intact immune system which means important immunological responses which affect tumour growth and metastasis are not considered. In addition, the reliance on the interaction between human and mouse molecules has not been fully elucidated so caution needs to be taken when analyzing the effect of human cancers in mouse model systems.

Several experimental approaches using specific chemokine antagonists, neutralising antibodies, knockout mice, and targeted gene disruptions have proven to be a useful tools for discerning the role of chemokines and their receptors in vivo (268) and these methods may potentially provide a basis for the development of future therapeutic agents. A knockout system to completely disrupt CXCR4 gene is embryologically lethal in mice, thus impeding the direct investigation of CXCR4-mediated metastasis. Alternatively the design of selective synthetic polypeptides against CXCR4, such as TN14003, has provided a means to investigate the role of CXCR4 during breast cancer metastasis (229). Moreover, an inducible system involving short interfering RNAs (siRNAs) was used to silence the expression of CXCR4 to determine the effect on migration of breast cancer cells in vitro (269). While neutralizing antibodies have been used to block CXCL12/CXCR4 interaction in vivo and were able to significantly impair metastasis of breast cancer cells to regional lymph nodes and lung in SCID mice (4). Studies involving the inhibition of CCR7 expression on cancer cells are yet to be performed at both the in vitro and in vivo level.
While these findings have identified a specific role of CXCR4 in breast cancer, the precise mechanisms involved as well as its role in vivo during the metastatic process have not been addressed. In order to provide a further insight into chemokine-mediated metastatic mechanisms, the expression of CXCR4 as well as CCR7 on mammary epithelial cell lines was modulated using an siRNA system to study the effect on the metastatic propensity of breast cancer cells in vivo. A retrovirus transgene-delivery system was selected based on the ability to deliver and stably express siRNAs within mammalian cells, both in vitro and in vivo (270) overcoming the problem of poor transfection efficiency demonstrated with plasmid-based systems (271). In this study the effect of siRNA on breast cancer cells resulted in an approximate 45-65% reduction of CXCR4 and CCR7 surface expression. More importantly, this partial knockdown resulted in a complete inhibition of function of both chemokine receptors in vitro. This effect of partial knockdown resulting in significant functional inhibition is not uncommon in the literature with the down-regulation of a proportion of CXCR4 having a significant effect on the growth of orthotopically transplanted breast cancer cells (238) as well as cancer cell invasion in vitro (269).

To facilitate the detection of CXCR4 or CCR7 siRNA infected breast cancer cells in vivo, tumour cells were colour coded with fluorescent markers, GFP or RFP. The first use of GFP to visualize cancer cells in vivo was performed by Chishima et al. in which stably transfected tumour cells with GFP were transplanted into several mouse models, including xenogeneic models with high metastatic capacity (272). The data revealed that in excised live tissue, with no additional preparation, metastases could be observed in any organ at the single-cell level. In addition, cells could be visualized in the process of intravasation and extravasation. With respect to the present study, adaptation of this method allowed for the visualization of colonised metastatic cells on the surface of the mouse organs, which is beyond the capabilities
of standard histological techniques. Furthermore the major advantage of this system was that micrometastases could be visualized in unfixed or unprocessed tissue reducing tedious histological procedures that are associated with conventional methods.

Cell growth and formation of metastatic lesions following i.v. delivery into SCID mice was examined at early and late stages of metastatic breast disease process using fluorescence microscopy. Down-regulation of chemokine receptor expression on breast cancer cells dramatically reduced the formation of metastases to the lung. From the experiments using either CXCR4 or CCR7 knockdown cells, it was clear that these receptors were independently required for metastatic spread of tumour cells. Mice in which the parental breast cancer cells were implanted developed large and frequent pulmonary metastatic lesions, which were detected at early and late stages of the disease process. Analysis of the lung colonies showed that the majority of the metastatic colonies were of clonal origin. Pure green and red metastatic lesions were found in approximately the same ratio on the lungs even although mixed implantations of GFP- and RFP-labelled clones were injected into the tail-vein of the same mouse. The resulting metastatic lesions on the lungs seem to be ultimately derived from single cells. Since it is thought that metastases are clonal and originate from rare cells in primary tumours that are heterogeneous in genotype and phenotype (273), it can be interpreted that this model offers a reliable system to study metastasis. This has also been reported in a study where GFP-labelled or RFP-labelled HT-1080 human fibrosarcoma cells were used to determine clonality (274). The resulting pure red or pure green colonies were scored as clonal, whereas mixed yellow colonies were scored as non-clonal. In a spontaneous metastasis model originating from tail vein injection in SCID mice, 95% of the resulting lung colonies were either pure green or pure red, indicating monoclonal origin of tumour cells, whereas only 5% were of mixed colour, indicating polyclonal origin (275). The clonality of
the experimental metastasis model depended on the number of input cells. Overall this simple fluorescence method of determining clonality of metastases allows for large-scale clonal analysis in numerous types of metastatic models and moves away from previously used complicated karyotype or molecular analyses.

These data indicating that the formation of metastases was inhibited in the cells lacking CXCR4 or CCR7 suggest that tumour cell colonisation, proliferation, survival or migration in vivo could be directly dependent on the expression of these receptors. Although from these experiments it is not clear at which stage the receptors play a role, it is obvious that their expression during metastasis is essential in this model. Several lines of evidence demonstrate that signalling via CXCR4 can modulate tumour cell expression of integrins, which can then facilitate adhesion of cancer cells (235, 276). For example in small cell lung cancer cells (SCLC), CXCL12 stimulation induced firm adhesion to marrow stromal cells via activation of α4β1 integrin and was able to induce SCLC cell invasion into the extracellular matrix (277). It has been hypothesized that during migration tumour cells use CCR7-dependent lymphatic migration mechanisms, which are reminiscent of the physiological migration mechanisms used by dendritic cells in the draining lymph nodes (107). As an example, cutaneous melanoma cells that express CCR7 functionally respond to CCL21/SLC in a manner that facilitates metastasis of these cells from the primary site to the sentinel lymph nodes. Therefore it is plausible that CXCR4 and CCR7 may act sequentially, at the same or different stages in the metastatic process with one essential for adhesion while the other essential for migration or proliferation. These possibilities could be addressed in future studies.
6.5 Concluding remarks and future studies

In this study, three novel observations with respect to the role of the chemokine receptors in cancer progression and metastasis were made. Firstly, CXCR4 and CCR7 were expressed throughout a panel of non-malignant and metastatic breast cancer cells. The expression of functional CXCR4 and CCR7 was however restricted to the invasive cell types with the chemokine receptor responsiveness reflecting the metastatic potential of the cell lines. Secondly, the acquisition of functional chemokine receptor expression on metastatic breast cancer epithelial cells involved the formation of G-protein-receptor trimeric complex, which was altered in the non-malignant cells. Thirdly, blocking the expression of CXCR4 and CCR7 with stable siRNAs on breast cancer cells in vivo significantly inhibited experimental pulmonary metastases formed from cancer cells injected intravenously. Collectively, the results point to a direct involvement of these chemokine receptors during the metastatic process of breast cancer.

While the findings of this study have provided an insight into chemokine-mediated mechanisms performed in breast cancer cells in vitro and in vivo there still are important questions that remain to be answered. For example, it will be valuable to gain a broader understanding of the functional role of chemokines and their receptors in different cancers other than breast. Also given the complex and multi-step process of metastasis, it is likely that chemokine receptors may function at several steps and so the use of specific in vivo models may assist in elucidating the precise stage at which chemokines and their receptors influence the metastatic pathway. Finally, it would be interesting to examine the effect of chemokine receptor expression on the growth of primary tumours using an orthotopic cancer model to verify the precise role of chemokines during metastasis.

The understanding of chemokine-mediated pathways and consequently their potential disruption may provide a useful method for the design of new strategies to treat breast cancer. It should be recognised that with the complex nature of tumourgenesis and the enormous
heterogeneity between breast cancer cells, it is not likely that any single inhibitor or functional modulator of chemokines or their receptors will be a 'cure' for breast cancer. It is more probable that, when used in conjunction with other therapeutic regimens, newly discovered chemokine- or chemokine-receptor-targeting agents will contribute significantly to the control of tumour cell invasion and metastasis. Such an approach may lead to many cancers becoming dormant and clinically manageable.
REFERENCES


- 135 -


180. Simonetti, O., Goteri, G., Lucarini, G., Filosa, A., Pieramici, T., Rubini, C., Biagini, G., and Offidani, A. Potential role of CCL27 and CCR10 expression in melanoma


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CORRIGENDUM

Corrected errors are highlighted in *bold-italic*.

**Chapter 1**
- **P. 22, 1st paragraph**
The sentence should read: Furthermore the expression of chemokine receptors has been reported in a variety of human tumours and have been identified as playing a critical role in several key steps in tumourgenesis and/or metastasis (Table 1.3) (104-107).
- **P. 35, 1st paragraph**
The sentence should read: As an example, PF-4-CTF, a derivative of PF-4, an anti-angiogenic chemokine, which retains the ability to block angiogenesis - a critical step in the conversion of micrometastases to macroscopic tumours, has proved to be a therapeutic agent of interest since the inhibitor significantly blocks the growth of established intracranial glioma in nude and syngeneic mouse models and improves survival (176).

**Chapter 2**
- **P. 53, last paragraph**
The sentence should read: To achieve GFP gene transduction, breast cancer cells were plated in a 60-mm tissue culture dish at ~40% confluence, and 24 h later the cell medium was removed before 5 ml of specific or control viral supernatants were added.
- **P. 54, Section 2.3.10, clarification:**
The RNAi Designer tool from Clontech was used to select 3 potentially efficient target sequences [http://bioinfo2.clontech.com/rnadesigner/sirnaSequenceDesign.do](http://bioinfo2.clontech.com/rnadesigner/sirnaSequenceDesign.do). The efficiency of the shRNA was tested by transient transfection of shRNA expression cassettes into HEK293 cells and one shRNA was selected for further use.

**Chapter 3**
- **Table 3.1,**
  Correction: MCF10A is made from fibrocystic breast tissue
  - Correct Figure 3.5 is shown in the Figures section of the corrigendum.
- **P. 76, last paragraph**
The sentence should read: The lack of response in these cells is unlikely to be due to a common mutation in the receptors since the probability of both CXCR4 and CCR7 simultaneously mutated in 3 different cell lines is extremely low.

**Chapter 4**
- Correct Figure 4.11 is shown in the Figures section of the corrigendum.

**Chapter 5**
- Amended figures 5.1 and 5.2 are shown in the Figures section of the corrigendum.
  - Figure legend for figure 5.1:
    The selected cells were incubated with an anti-human CXCR4 antibody and the data was compared with the wild type MDA-MB-231 cells and cells MDA-MB-231 infected with a control/siRNA.
  - Figure legend for figure 5.2:
    The selected cells were incubated with an anti-human CCR7 antibody and the data was compared with the wild type MDA-MB-231 cells and cells MDA-MB-231 infected with a control/siRNA.
  - Figure legend for figure 5.5:
    Three MDA-MB-231 clones expressing CXCR4 or CCR7 siRNAs were transduced with a retroviral vector expressing GFP or RFP. Stably expressing GFP or RFP cell lines were selected with G418 (750 μg/mL) and fluorescent cells were visualised by microscopy.
Fig. 4.11

(A) MDA-MB-231 MDA-MB-453

CCL19: - - - + - +
WB:CCR7 - - - - - - 48kDa

IP: anti-Gαi

(B) MDA-MB-231 MDA-MB-453

CCL19: - - - + - +
WB:CCR7 - - - - - - 48kDa

IP: anti-Gβ

(C) MDA-MB-231 MDA-MB-453

CCL19: - - - + - +
WB:Gβ(1-4) - - - - - 36kDa

IP: anti-Gαi
Fig. 3.4.

MMA-MB-231 (98%)

Fig. 5.1. (A)

(i) Clone 7

% Inhibition

(45%)

(ii) Clone 19

(30%)

(iii) Clone 3

(18%)

CXCR4 Expression
Fig. 5.2. (A)

(i) Clone 5

% Inhibition

(65%)

(ii) Clone 28

(40%)

(iii) Clone 17

(20%)

Counts

$10^0$ $10^1$ $10^2$ $10^3$

Control
siRNA

wt

Counts

$10^0$ $10^1$ $10^2$ $10^3$

Control
siRNA

wt

CCR7 Expression