

Cross-talk between CXCR4 and IGF-1R signal transduction pathways in a metastatic breast cancer cell line

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Abstract

Breast cancer metastasis has been known to be influenced by various homeostatic factors. The insulin-like growth factor 1 tyrosine kinase receptor (IGF-1R) and more recently, the chemokine G-protein coupled receptors, CXCR4 and CCR7, have been implicated in metastasis and invasion by breast carcinoma. The molecular mechanisms underpinning the involvement of these receptors in those processes are still largely unclear and require much more investigation. The present study investigated the expression and function of IGF-1R, CXCR4 and CCR7, in metastatic MDA-MB-231 and non-metastatic MCF-7 cells. The data generated in this study demonstrate that both cell lines express IGF-1R, CXCR4 and CCR7. However, MDA-MB-231 cells exhibit a significantly lower level of IGF-1R expression and function and, despite expression of CXCR4 and CCR7 observed in both cells, only these receptors in MDA-MB-231 cells are functionally active. Furthermore, this study strongly indicates a unidirectional transactivation of CXCR4 by IGF-I/IGF-1R in MDA-MB-231 cells. This cross-talk appears to depend on constitutive formation of the complex containing IGF-1R, CXCR4 and the G-protein subunits, $G_i\alpha$ and $G\beta$. This complex allows IGF-I to transactivate CXCR4 and G-protein signal transduction which partially mediates migrational response of the cells. Surface expression of IGF-1R and CXCR4 is coregulated by IGF-I in MDA-MB-231 cells. Co-internalisation of IGF-1R and CXCR4 is observed in response to IGF-I and this results in a reduction of CXCR4 activity. IGF-I-mediated degradation of IGF-1R also requires signalling of GPCRs, potentially of CXCR4. Finally, despite the existence of a constitutive complex of IGF-1R and CXCR4 in MCF-7 cells, the defect in CXCR4

signalling results in no transactivation of CXCR4 by IGF-I as well as no coregulation of IGF-1R and CXCR4 surface expression in this cell line.

In summary, the studies conducted in this thesis have demonstrated a novel cross-talk between a tyrosine kinase growth factor receptor, IGF-1R, and a G-protein coupled chemokine receptor, CXCR4. This interaction between these receptors may have a major impact on the IGF-1R- and CXCR4-mediated biological responses which have been shown in a wide range of cellular systems. Of particular importance, the lack of this cross-talk in the non-metastatic MCF-7 cells suggests its potentially important role in the metastatic and invasive ability of breast cancer cells. Together, these findings provide further understanding of the contribution of growth factors and chemokines to metastatic and invasive behaviour in breast carcinoma.

Statement of Originality

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Publications arising from this work

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Holland J, Kochetkova M, **Akekawatchai C**, Mara Dottore, Angel Lopez and McColl SR. Differential functional activation of chemokine receptor CXCR4 is mediated by G proteins in breast cancer cells. *Cancer Res*. 2006 Apr 15;66(8):4117-24.

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Abbreviations

Ab	antibody
BCA	bicinchoninic acid
bp	base pair
BSA	bovine serum albumin
°C	degrees celcius
[Ca ²⁺] _i	intracellular free calcium
CCL	CC chemokine ligand
CCR	CC chemokine receptor
cDNA	complementary deoxyribonucleic acid
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxynucleic triphosphate
dNTPs	deoxynucleoside triphosphates
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
FACS	fluorescence-activated cell-sorting
FAK	focal adhesion kinase
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
g	gram
GDP	guanosine diphosphate
GPCR	G-protein coupled receptor
G-protein	GTP-binding protein
Grb	growth factor receptor-bound protein
GRKs	G-protein coupled receptor kinases
GTP	guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
HER2	human epidermal growth factor receptor 2
HRP	horseradish peroxidase
Ig	immunoglobulin
IGF-I or -II	insulin-like growth factor I or II
IGF-1R	insulin-like growth factor-1 receptor
IGF-2R	insulin-like growth factor-2 receptor
IGFBP	insulin-like growth factor binding protein
IR	insulin receptor
IRS	insulin receptor substrate
JAK	Janus-family tyrosine kinase
kDa	kiloDalton
KIRA	kinase receptor activation assay
l	litre
m	metre
M	molar
mA	milliampare

MAPK	mitogen-activated protein kinase
MFI	mean fluorescence intensity
mg	milligram
ml	millilitre
mM	millimolar
mRNA	messenger RNA
μ	micron
μ g	microgram
μ l	microlitre
n	nano
NGF	nerve growth factor
nm	nanometre
nM	nanomolar
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PFA	paraformaldehyde
PI3K	phosphatidylinositol 3-kinase
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethylsulfonylfluoride
PTX	pertussis toxin
Raf	Ras activated factor
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RTK	receptor tyrosine kinase
RT-PCR	reverse-transcriptase PCR
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Shc	src homology/collagen proteins
SOS	son of sevenless
S1P	sphingosine 1-phosphate
S1P ₁	sphingosine 1-phosphate receptor 1
S1PRs	sphingosine 1-phosphate receptors
STAT	signal transducer and activator of transcription
TAE	tris acetate EDTA
TBS	tris buffered saline
TEMED	N,N,N',N'-tetramethyl ethylenediamine
Tween-20	polyoxyethylene sorbitan monolaurate
V	Volt
v/v	volume per unit volume
w/v	weight per unit volume

CHAPTER 1

General Introduction

CHAPTER 1

General Introduction

1.1 Cancer Cell Biology

Cancer comprises a large group of diseases which share an important characteristic of uncontrolled growth. In most tissues and organs, homeostasis is maintained by a balance between cell proliferation and cell death. Occasionally, cells lose the ability to respond to the normal growth control mechanisms and clones of cells arise which can expand to a considerable size, producing tumours or neoplasms. A tumour which remains in its original location is not able to grow indefinitely and does not invade surrounding tissue is termed benign, whereas a tumour which grows extensively and becomes invasive is known as a malignant tumour or cancer. In addition, most malignant tumours are able to exhibit metastasis, a process of new tumour formation and growth in distant organs, which is known as the cause of 90% of human deaths from cancer (Sporn 1996).

A number of previous studies indicate that cancer results from a series of molecular events that cause an imbalance of normal cell growth regulation. Various human genes which normally function in cell proliferation and death have been associated with different forms of cancer. Generally, these genes are classified into two main groups. The first group comprises oncogenes, abnormal forms of proto-oncogenes or cellular oncogenes which encode different proteins that drive cell division and inhibit cell death. Conversely, the second group comprises tumour suppressor genes which encode proteins that prevent cell

division and cause cell death. It is apparent that alterations of these genes by different genetic mechanisms such as mutation, insertion and translocation can interrupt the normal regulation of cellular forms of these genes causing transformation of normal cells to tumour cells and eventually to malignant cells (Ponder 2001).

Accumulating molecular studies have established the theory of somatic genetic changes, suggesting that tumour and cancer development, termed tumourigenesis and carcinogenesis respectively, are multistep processes and require a series of genetic changes. These stepwise genetic alterations drive the progressive transformation of normal cells to precancerous cells and subsequently to highly invasive malignant cells (Fearon and Vogelstein 1990; Gatenby and Vincent 2003; Ponder 2001). The alteration of gene activity allows tumour cells to develop characteristics that overcome anti-cancer mechanisms in hosts. In most types of human cancer, at least six essential changes in cell physiology are required in order to complete the progression to malignancy. These include becoming self-sufficient in terms of growth signals, resistance to anti-growth signals, evasion of programmed cell death or apoptosis, unlimited replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg 2000). In summary, there are many cancer-associated proteins, mostly products of oncogenes and tumour suppressor genes, that promote malignant characteristics, and there are homeostatic mechanisms which may enhance or prevent the progression of malignancy (Hanahan and Weinberg 2000).

1.2 Breast Cancer

1.2.1 Overview

Breast cancer is the most common neoplasm in women and a leading cause of cancer-related deaths worldwide. According to estimations in 2002, there were 1,151,298 new cases of breast cancer diagnosed, 410,712 deaths caused by breast cancer, and more than 4.4 million women living with breast cancer worldwide (Veronesi *et al.* 2005). Despite early diagnosis and significantly improved current treatments, the mortality rate is still very high, with approximately one in four diagnosed breast cancer patients dying from the disease. Most deaths from breast cancer result from invasive and metastatic carcinomas, which appear to be unresponsive to conventional therapeutic approaches. Although primary therapies such as surgical removal of the breast tumour are often effective in early stages of breast cancer, relapses of cancer in both local and secondary sites may occur because of a failure to remove individual tumour cells during surgery, or the presence of undetectable micrometastases at the time of diagnosis (Saphner *et al.* 1996). Therefore, it is apparent that invasion and metastasis are a critical stage of breast cancer pathogenesis.

Similar to other types of cancer, clinical features of breast carcinoma are very complicated and variable which leads to different treatments and care for patients, depending on the particular stage of disease. The development of breast cancer therapy began a long time ago and has led to current therapeutic strategies including surgery, radiotherapy and chemotherapy, all of which have limitations in effectiveness due to the complexity of the disease. Furthermore, these therapies also lack specificity on malignant cells and have inherent toxicity associated with them (Veronesi *et al.* 2005). Recently, increasing knowledge of the molecular pathogenesis of breast carcinoma has led to the development

of molecular-targeted therapeutic strategies. For instance, hormone-and endocrine-based therapies have significantly decreased the mortality rates of breast cancer in the past decade (Veronesi *et al.* 2005). Many new therapeutic targets are being identified. However, these rely on an increased understanding of molecular mechanisms underlying breast cancer progression.

1.2.2 Breast Cancer and Metastasis

1.2.2.1 Clinical Features of Breast Cancer

Breast carcinoma is a complex and heterogenous disease, with a variety of pathological and clinical appearances (Polyak 2001). Histological studies have indicated that, in spite of the heterogeneity of breast carcinoma, the progression of breast cancer disease is a sequential process, starting from progressive changes of normal tissues to hyperplasia, with or without atypical hyperplasia, carcinoma *in situ*, invasive, and eventually metastatic carcinoma (Shackney and Silverman 2003). Currently, even though the precursor of breast carcinoma is still uncertain, the stage of carcinoma *in situ* has been clinically identified as a preneoplastic lesion (Polyak 2001; Veronesi *et al.* 2005), which exhibits all malignant phenotypes except the ability to invade and metastasise, whereas stroma invasion and metastasis to regional lymph nodes and distant organs are the hallmark of fully developed disease.

1.2.2.2 Breast Tumourigenesis and Metastasis

Breast tumourigenesis is caused by both inherited and environmental factors (Polyak 2001). Approximately 10% of all breast cancer cases are caused by inherited mutations of

tumour susceptible genes such as *BRCA1* and 2 (Marcus *et al.* 1996). The majority of breast cancers in women, contributing to 90% of all cases, are non-inherited or sporadic breast cancers, which results mostly from environmental agents that influence the process of somatic genetic alteration. The molecular basis of the sporadic disease is less defined. However, several studies have shown that polymorphisms in several genes encoding hormonal receptors such as estrogen and progesterone receptor genes, proto-oncogene, *HRAS*, and DNA repair genes, *XRCC1* and *XRCC3*, promote breast cancer progression (Weber and Nathanson 2000).

Breast carcinomas arise from mammary epithelial cells and evidence from numerous recent studies supports the hypothesis that mammary tumours are derived from mutated mammary stem cells, located in the terminal end buds of branched ducts of mammary glands, from where most tumours originate (Dontu *et al.* 2003; Li *et al.* 1998; Russo and Russo 1999). First, stem cells have many characteristics of cancer cells including unlimited self-renewal and the capacity for sustained proliferation. Second, only subpopulations of tumour cells, are able to complete the process of tumour progression and metastasis, and not all patients develop metastatic cancers (Al-Hajj *et al.* 2003; Veronesi *et al.* 2003). Third, epidemiologic observations that radiation exposure in prepubertal girls increased the incidence of breast carcinomas later in life, suggests that damaged cells, which have genetic changes, were present and sustained in subsequent years (Little and Boice 1999). Finally, a recent study has proposed a model in which transformation of stem cells or early progenitor cells results in carcinogenesis. Transformation of mammary stem cells or progenitor cells appears to generate the phenotypic heterogeneity, found in human and rodent breast cancers (Dontu *et al.* 2003).

The concept of stem cells in breast cancer progression still requires more clarification and this may affect the direction of the development of breast cancer therapy.

1.2.2.3 Tumour Metastasis and The Hypothesis of “Seed” and “Soil”

Tumour metastasis is a complex process and consists of sequential and interrelated steps (Chambers *et al.* 2002; Fidler 2002). As depicted in figure 1.1, the process includes the initial transformation and growth at primary sites, the formation of a vascular network from surrounding tissue, a process called angiogenesis, shedding of tumour cells into the blood circulation, known as intravasation, survival in the circulation, arrest in the target organs, penetration into surrounding tissue, termed extravasation, and cell proliferation and secondary tumour growth at the sites of metastasis.

It has been suggested that metastasis is an inherently inefficient process, based on the evidence that only a few cells from primary tumours can complete the entire metastatic process (Weiss 1996). Radioactive labelling of tumour cells demonstrated that, within 24 hours after entry to blood circulation, less than 0.1% of tumour cells are still viable and less than 0.01% of the cells in the circulation survive to form metastases at the target sites (Fidler 1970). Several studies using *in vivo* video microscopy and quantitative cell-fate analyses that monitor the loss of cells over time during metastasis has led to the suggestion that the process from intravasation of tumour cells into the circulation to extravasation of the cells into the secondary organs is relatively efficient. In contrast, the subsequent steps of the metastatic process appear to be inefficient, with only a small numbers of extravasated cells surviving to form metastases at metastatic sites (Cameron *et al.* 2000; Luzzi *et al.* 1998). It has been suggested that the ability of these cells to

successfully metastasise is dependent on both intrinsic properties of the tumour cells and normal host response in all steps of the metastatic process (Fidler 2002).

Metastasis has also been recognised as a non-random but highly organ-selective process. In breast cancer, secondary tumours are usually found in the lung, liver, bone marrow and lymph nodes but rarely in other organs. The first investigation of factors regulating certain patterns of cancer metastasis, known as the hypothesis of “seed” and “soil”, dates back to 1889. Paget suggested that the metastasis occurred when tumour cells, referred to as the “seed”, are compatible with a particular organ microenvironment, equated to the “soil”. A review of this aspect shows that accumulating data mostly supports Paget’s hypothesis and provides a current definition of “seed” and “soil” theory (Fidler 2002; Fidler 2003). It has been suggested that primary tumours are inherently heterogenous and contain subpopulations of cells with a variety of biological characteristics and only cancer cells with particular phenotypes including metastatic and invasive properties succeed in the formation of secondary tumours at the metastatic sites. The outcome of metastasis also appears to be dependent on multiple interactions between tumour cells and host homeostatic mechanisms, especially at the level of the microenvironment in target organs, where the initiation of secondary tumour growth preferentially occurs (Cameron *et al.* 2000; Luzzi *et al.* 1998). In addition, three of the “fertile soil” concepts have recently been proposed to explain the preference of metastatic sites. Tumour cells randomly distribute through out the body and will selectively proliferate at the sites producing appropriate growth factors, which support survival and proliferation of tumour cells, adhesion molecules, which specifically trap the circulating cells, and chemoattractants, which promote tumour cells homing to particular sites (Liotta 2001; Liotta and Kohn 2001; Moore 2001; Murphy 2001).

1.2.3 Established Molecular Mechanisms for Breast Cancer Progression

Advances in molecular studies on mechanisms of cancer progression have led to the identification of a number of molecules that play important roles in tumourigenesis and metastasis of breast cancer. These include components of the endocrine and immune systems, oncogene products and proteases (Polyak 2001; Rogers *et al.* 2002). The well established hormonal nuclear receptors, estrogen receptor α and β isoforms (ER α and ER β), and their involvement in breast cancer progression was discovered more than three decades ago. It was demonstrated that ER β expression predominated in normal breast tissue, being detected in 22% of samples tested, whereas ER α was expressed in most tumours either alone or in combination with ER β . The coexpression of the two isoforms appears to be associated with poor prognosis of breast cancer (Speirs *et al.* 1999). An increase in ER β expression was also observed in chemical-transformed human breast epithelial cells, implicating it in the process of carcinogenesis (Hu *et al.* 1998). Numerous studies revealing the function of estrogen and estrogen receptors in breast cancer progression have led to the development of the first molecular targeted breast cancer drug, tamoxifen, an antagonist of both estrogen receptors (Veronesi *et al.* 2005). This drug has been successful in treating approximately 60% of breast cancer with ER α -positive lesions. However, many of these patients eventually develop recurrent cancers (Speirs and Kerin 2000).

The family of growth factor receptor tyrosine kinases (RTKs), as well as their downstream signalling molecules, have also been implicated in breast cancer development. Indeed, these have become targets for treatment of breast carcinoma, particularly the estrogen receptor-negative cancers, which appear to be highly-aggressive and are unresponsive to

conventional anti-estrogen therapy (Andrechek and Muller 2000; Hynes 2000; Nahta *et al.* 2003; Zwick *et al.* 2001). Numerous studies have implicated HER2/ErbB2, a member of the epidermal growth factor receptor (EGFR) family and known as one of the most important oncoproteins in breast tumourigenesis and metastasis (Zwick *et al.* 2001). Overexpression of HER2/ErbB2 gene is found in human breast carcinomas, with a frequency of 25-30%, and also correlated with a more aggressive phenotype of breast cancer (Slamon *et al.* 1987; Slamon *et al.* 1989; Yu and Hung 2000). The first RTK-specific anti-oncogene drug, Herceptin, which inhibits the function of HER2/ErbB2, has been developed, and is used currently for HER2/ErbB2-positive breast cancer, with up to a 15% response rate (Cobleigh *et al.* 1999).

The role of several other types of RTKs in breast cancer development has also been increasingly investigated. For example, EGFR/ErbB1 is also expressed in 14-91% of breast cancer. Its expression is a modest prognostic indicator and inversely correlated with estrogen receptor status (Klijn *et al.* 1992; Nahta *et al.* 2003). Increased levels of EGFR/ErbB1 expression are associated with a late stage of the disease progression, and a high rate of tumour proliferation and metastasis (Zwick *et al.* 2001). In addition, the vascular endothelial growth factor receptors (VEGFRs), expressed on endothelial cells, appear to be key regulators of the formation of new vascular networks in tumours, termed angiogenesis (Zwick *et al.* 2001). Furthermore, the insulin-like growth factor receptors (IGF receptors) have generated much interest due to their ability to inhibit programmed cell death, which is thought to function aberrantly during tumourigenesis, and to their established roles in oncogenic transformation and metastasis (Hynes 2000). In addition, the roles of several signalling molecules have been implicated in breast cancer as important components of growth factor receptor signal transduction pathways, including

Src family kinases, phosphatidylinositol-3 kinases (PI3Ks), and cytoplasmic Shc-Grb2-Ras pathways (Andrechek and Muller 2000).

A wide range of molecules such as oncogene products, proteases and immune mediators generated from host cells have also attracted much attention. Aberrant functions of oncogene products such as Bcl-2, p53, and cyclin D1 have been shown to influence tumour growth, whereas overexpression of some proteases such as Cathepsin D, MMP9 and uPA, appears to increase the potential of tumour metastasis. These components are being identified as promising prognostic indicators in breast cancer (Rogers *et al.* 2002). Immune mediators, particularly chemokines, have been implicated in transformation, growth, angiogenesis, invasion and metastasis of several types of cancer (Tanaka *et al.* 2005). Recent evidence, both *in vitro* and *in vivo*, supports the concept that certain chemokines act as tissue-specific attractant molecules for tumour cells, promoting the metastatic process of breast cancer (Moore 2001; Muller *et al.* 2001). The identification and investigation of new factors involving the progression of breast cancer are increasingly being reported.

1.3 Molecular Basis of Breast Cancer Metastasis

1.3.1 Overview

Studies on the molecular biology of cancer metastasis have emphasised the importance of the interaction between malignant and host tissues. Accumulating data support the roles of various types of host cells, such as immune cells, endothelial cells, fibroblast cells, and platelets, and their paracrine cytokines in the establishment of tumour microenvironments (Nicolson 1993). At the tumour-host interface, several types of host factors such as growth

factors, adhesion molecules, cytokines and chemoattractants support tumour cell proliferation, survival, and migration that promote growth and invasion of tumours (Liotta and Kohn 2001; Singer *et al.* 2000). A complete summary of all the factors involved in cancer metastasis is beyond the scope of this thesis. Rather, this review focuses on the established roles of growth factors, in particular insulin-like growth factors, and chemokines in breast cancer progression, emphasising on the process of invasion and metastasis and on the molecular mechanisms by which these molecules influence those processes.

1.3.2 Growth Factors and Breast Cancer

1.3.2.1 The IGF system; IGFs and IGF receptors

The IGF system is composed of the ligands (IGF-I and IGF-II) and their respective receptors (IGF-1R, IGF-2R, insulin receptor (IR) and IGF-1R/IR hybrids), at least six IGF-binding proteins (IGFBP 1-6) and IGFBP proteases (Figure 1.2). These components form a complex network that regulates various types of signal transduction pathways, resulting in cellular responses including cell proliferation, survival, and migration. IGFs exert their biological effects through multiple cognate receptors, IGF-1R, IGF-2R, IR as well as hybrid IGF-1R/IR, with different affinities (Denley *et al.* 2005). However, it is apparent that the biological effect of IGF-I is mediated mainly through IGF-1R. Circulating IGFs are found to form complexes of high affinity with IGFBPs. Cleavage of IGFBPs by specific proteases or binding of such proteins to extracellular matrix (ECM) is thought to release IGFs in the circulation, thereby regulating the actions of IGFs (Denley *et al.* 2004; Sachdev and Yee 2001).

IGFs are small, single-chain polypeptide ligands with a molecular weight of approximately 7-8 kDa that are highly homologous to proinsulin. IGFs play, paracrine and endocrine roles in normal growth and development (Sachdev and Yee 2001). IGF-II is required for embryogenesis whereas IGF-I is more important in postnatal growth and development. IGF-II is predominately expressed during prenatal development and heterozygous IGF-II gene knockout mice are born with a 60% smaller size compared with their wild-type littermates (DeChiara *et al.* 1990). A homozygous deletion of IGF-I gene in mice results in a 60% decrease of birth weight compared with their wild-type mice. However, the majority of the mice die shortly after birth because of hypodevelopment of the lung and diaphragm (Liu *et al.* 1993; Powell-Braxton *et al.* 1993). In addition, IGF-I is also well-known as a mediator of growth hormone (GH) function, promoting growth after birth (Moschos and Mantzoros 2002).

There are two high affinity transmembrane receptors for IGFs, IGF-1R and IGF-2R (Figure 1.2)(Foulstone *et al.* 2005). IGF-1R can bind to IGF-I, IGF-II and insulin, but preferentially binds to IGF-I. IGF-2R binds to IGF-II with high affinity but binds to IGF-I with low affinity and does not bind to insulin (Denley *et al.* 2005). IGF-1R is a heterotetramer tyrosine kinase receptor sharing at least 60% homology with the insulin receptor (Fujita-Yamaguchi *et al.* 1986; Steele-Perkins *et al.* 1988; Ullrich *et al.* 1986), containing two 130-135 kDa α subunits and two 90-95 kDa β subunits that are linked by disulfide bonds (Figure 1.3). The extracellular α subunits have structural binding domains whereas the β subunits contain a single transmembrane domain and an intracellular segment, with tyrosine kinase activity and multiple binding sites for signalling substrates (Adams *et al.* 2000). IGF-1R is expressed in all cell types with the exception of hepatocytes and T lymphocytes (Sachdev and Yee 2001). Mice with null IGF-1R gene

mutations are 45% of the size of their littermates at birth and generally die after birth due to respiratory failure (Liu *et al.* 1993).

IGF-2R is a 300-kDa monomeric protein with a large extracellular domain and a small intracellular domain. In contrast to IGF-1R, IGF-2R does not have intrinsic tyrosine kinase activity and does not activate intracellular signalling molecules (Morgan *et al.* 1987; Scott and Firth 2004). Despite the unclear biological function of IGF-2R, the receptor is thought to be an inhibitor of IGF-II action and have anti-proliferative and anti-apoptotic activities. Binding of IGF-II to IGF-2R results in receptor internalisation and degradation, thereby reducing the half-life of IGF-II and decreasing the interaction between IGF-II and IGF-1R (Moschos and Mantzoros 2002). This is supported by studies on IGF-2R knock out mice, showing fatal overgrowth and perinatal lethality due to major cardiac abnormalities (Wang *et al.* 1994).

1.3.2.2 Signal Transduction Pathways Downstream of IGF Receptor

IGFs exert various biological effects through their multiple corresponding receptors. However, most of the information on IGF-triggered signal transduction described in this section has come from studies of the IGF-1R activation system. The ligand binding-induced signal transduction of IGF-1R is summarised in figure 1.4. Upon binding of the ligand IGF-I to IGF-1R, the intrinsic tyrosine kinase activity is activated, resulting in autophosphorylation on tyrosine residues thereby creating binding sites for other signalling substrates. The best-studied substrate, known as an adapter protein connecting the receptor to other effector molecules, is the insulin receptor substrate 1 (IRS-1), which binds to the receptor through a phosphotyrosine-binding (PTB) domain (Craparo *et al.* 1995; Surmacz 2000). IRS-1 contains about 20 potential tyrosine phosphorylation sites

and acts as a “docking” protein for other downstream signalling molecules containing src-homology (SH-2) domains, such as the p85 subunit of phosphatidylinositol 3 kinase (PI3K) and growth factor receptor-bound-protein 2 (Grb2) (Delahaye *et al.* 1998; Giorgetti *et al.* 1993; Sun *et al.* 1993). However, IGF-1R has also been shown to directly activate various intracellular molecules including src-homology 2 (Shc) (Giorgetti *et al.* 1994), the p85 subunit of PI3K enzymes (Lamothe *et al.* 1995), growth factor receptor-bound protein 10 (Grb10) (Morrione *et al.* 1996), focal adhesion kinase (FAK) (Baron *et al.* 1998), as well as receptor for the activated C kinase 1 (RACK1) (Hermanto *et al.* 2002).

The best-characterised signalling systems downstream of IRS-1 are PI3K and mitogen-activated protein kinase (MAPK). PI3K activation has been recognised as one of the most important signals for cell survival. The stimulation of PI3K subsequently activates the ser/threonine kinase Akt (protein kinase B (PKB)), which phosphorylates and inactivates pro-apoptotic protein BAD, attenuating its ability to induce apoptosis (Peruzzi *et al.* 1999; Shepherd *et al.* 1998). The activation of PI3K/Akt pathway is also known to be involved in the regulation of cell metabolism, induction of cell proliferation and cell growth in several growth factor receptor systems (Lawlor and Alessi 2001; Mitsiades *et al.* 2004). An association between IRS-1 and the Grb2/Sos complex results in the activation of the classical Ras/Raf/MAPK pathway, which leads to cell growth and differentiation (Goalstone and Draznin 1998; Skolnik *et al.* 1993). The activation of downstream signalling pathways by other direct substrates of IGF-1R has also been studied. For instance, Shc, which is now known as another major substrate for IGF-1R, is involved in the pathway leading to cell proliferation. Phosphorylation of Shc promotes an association with Grb2/SOS which activates the Ras/MAPK pathway (Giorgetti *et al.* 1994; Goalstone

and Draznin 1998). The FAK and RACK1 adapter proteins, which directly bind to both IGF-1R and IRS-1, can link IGF-1R to integrin-mediated signalling, which modulates actin polymerisation, and cell adhesion (Baron *et al.* 1998; Casamassima and Rozengurt 1998; Hermanto *et al.* 2002; Lebrun *et al.* 1998). In summary, signal transduction downstream of IGF-1R has been well studied, particularly the IRS-1-induced pathways involving the activation of the two main signalling systems PI3K and MAPK. While there is abundant information on IGF-1R-induced signalling pathways which lead to the cellular responses of mitogenesis, anti-apoptosis, and metabolisms, there is significantly less information available on the pathways leading to cell adhesion and migration (Brodt *et al.* 2000; Foulstone *et al.* 2005; Surmacz 2000).

Upon the activation of RTKs by the ligands, there is a rapid decrease in the cell surface number of receptors and an eventual decrease in the cellular receptor contents, called “downregulation”. This process is divided into two distinct steps. The first is internalisation of the surface receptors and the second is degradation of the internalised receptors (Lipkowitz 2003). Similar to other types of RTKs, it is suggested that the activated IGF-1R undergoes the process of downregulation to switch-off the signals and consequently attenuates or terminates cellular reactions (Carelli *et al.* 2006; Girnita *et al.* 2005; Lin *et al.* 1998). Currently, an understanding of the downregulation process of IGF-1R is still very poor, whereas there has been more information available for other RTKs such as EGFR and PDGFR (Bache *et al.* 2004; Lipkowitz 2003). The ligand-occupied RTKs are generally endocytosed via clathrin-coated pits, which mainly involves the activity of the best-characterised adapter protein, the E3 ubiquitin ligase (named c-Cbl). Following the activation of receptors, the ubiquitin ligase activity of c-Cbl is activated and then allows this protein to recruit an ubiquitin-loaded E2 enzyme which covalently tags

the receptors with ubiquitin, termed “ubiquitination”. It is suggested that ubiquitination in the RTK system is possibly essential for the internalisation of the receptors, as observed in other receptors such as hormone receptors (Govers *et al.* 1999; Strous *et al.* 1996). After internalisation, it has been suggested that the RTK complex in endocytic vesicles traffics intracellularly to lysosomes and/or proteasomes where it is eventually degraded by lysosomal and proteasomal mechanisms. This is supported by experiments in which the specific inhibitors of both the lysosome and proteasome can block the degradation of RTK complex (Ettenberg *et al.* 2001; Levkowitz *et al.* 1998).

1.3.2.3 Expression of IGF Components in Breast Cancer

IGFs are well-known as important contributors of normal growth, development and survival. IGF-I, as an effector molecule of growth hormone action (GH), appears to be an endocrine factor promoting growth and development after birth and also plays a role as a local mediator involved in mammary gland development (Gross and Yee 2003; Ruan and Kleinberg 1999; Sachdev and Yee 2001; Walden *et al.* 1998). Accumulating evidence has revealed an association of the expression of IGF components with breast cancer. Several studies indicate that high circulating levels of IGF-I and low levels of IGFBP-3 are correlated with an increase of breast cancer risk (Furstenberger and Senn 2002; Krajcik *et al.* 2002; Toropainen *et al.* 1995). The local production of IGFs in stroma compartment also provides a stimulatory effect on breast cancer cells (Singer *et al.* 2000). As demonstrated by *in situ* hybridisation, IGF-I is mainly found in stromal cells but very rarely in normal and malignant epithelial cells, whereas IGF-II is also expressed in the stroma but is occasionally found in malignant breast epithelial cells (Paik 1992; Yee *et al.* 1989). Several studies have shown significant overexpression of IGF-1R in cancer cells

compared with normal and benign breast tissues (Cullen *et al.* 1990; Papa *et al.* 1993; Peyrat and Bonneterre 1992). Taken together, these data suggest that, within breast tissue, tumour cells with the overexpression of IGF-1R are exposed to IGF ligands available as endocrine and paracrine factors, leading to the activation of various biological responses, which promote the development of malignancy.

1.3.2.4 Role of the IGF system in Transformation, Growth and Survival

A number of studies indicate a role for IGFs and IGF-1R in breast cancer transformation, growth and survival (Baserga *et al.* 2003; Gross and Yee 2003; Surmacz *et al.* 1998). Experiments in a mouse fibroblast cell line derived from IGF-1R knock out mice (R⁻) have demonstrated that different tumourigenic agents were not able to induce a transformed phenotype in these cells (Baserga 1995). In addition, inhibition of IGF-1R expression by anti-sense-IGF-1R RNA, or its function by anti-IGF-1R or dominant-negative mutants, resulted in growth inhibition and reduced transforming potential in various cell types including breast cancer cells (Baserga 1995; Surmacz 2000). The tumourigenicity of IGF-1R is suggested to be related to hyperactivation of IGF-1R signal transduction, which can be caused by an overexpression of ligands, receptors, signalling molecules such as IRS, and constitutive activation of the downstream signalling, PI3K/Akt pathways (Mauro and Surmacz 2004).

IGF-I is a potent mitogen and the activation of IGF-1R by IGF-I promotes proliferation in particular types of breast cancer cells. Numerous studies have suggested the existence of considerable cross-talk between the IGF and ER systems and this is of particular importance to breast cancer because ER status is related to its prognosis and treatment (Gross and Yee 2003; Sachdev and Yee 2001). ER⁺ breast cancer cell lines, such as MCF-

7 and T47D, show a proliferative response to low concentrations of IGF-I and IGF-II whereas ER⁻ cells lines such as MDA-MB-231, MDA-MB-435 and MDA-MB-468, are minimally responsive or do not proliferate in response to IGFs (Bartucci *et al.* 2001; Karey and Sirbasku 1988; Sachdev and Yee 2001). ER⁺ breast cancer cells also show synergistic proliferative response to IGF-I and estrogen (Stewart *et al.* 1992). It is possible that estrogen induces the upregulation of components in the IGF-1R signalling system, particularly IGF-1R and IRS-1/2 (Lee *et al.* 1999). Conversely, IGF-I has been reported to transactivate the ER system based on the evidence that treating MCF-7 cells with an antagonist of ER, tamoxifen, decreases IGF-I-induced proliferation and the stimulation of IGF-1R causes the activation of estrogen-responsive genes in breast cancer cells (Lee *et al.* 1997; Sachdev and Yee 2001). The cross-talk between the two activation systems appears to play an important role in promoting breast tumour growth.

IGF-1R also has anti-apoptotic activity which has been shown to protect breast cancer cells from internal and external apoptotic inducers (Baserga *et al.* 2003; Gross and Yee 2003). Several studies suggested that inhibition of IGF-1R function by dominant-negative IGF-1R mutants abolishes the protective effects of IGF ligands (Kulik *et al.* 1997). IGF-1R activation by IGF-I ligands elicits anti-apoptotic effects through the pathway of IRS/PI3K/Akt (Gross and Yee 2003). It has been thought that cancer cells exploit the IGF-1R activation pathways to protect themselves from programmed cell death, an important mechanism by which chemotherapeutic agents and radiation kill cancer cells.

1.3.2.5 Role of the IGF system in Metastasis and Invasion

The involvement of the IGF system in metastasis and invasion of various types of cancers including breast cancer has become more evident in the last 10 years (Furukawa *et al.*

2005; Gross and Yee 2003; Kornprat *et al.* 2006; Long *et al.* 1995; Surmacz 2000). A study in premenopausal women suggests that high IGF-I and low IGFBP-3 plasma levels is associated with a risk of breast cancer recurrence (Decensi *et al.* 2003). While some studies identify IGF-1R as a positive regulator of the invasion and metastasis of cancer (Brodt *et al.* 2000; Dunn *et al.* 1998; Long *et al.* 1995; Long *et al.* 1998; Sachdev *et al.* 2004), several studies have demonstrated that different types of advanced cancers have low levels of IGF-1R expression (Nakamura *et al.* 2004; Sarfstein *et al.* 2006; Schnarr *et al.* 2000). It has also been suggested that the levels of IGF-1R expression in breast cancer may decrease over the course of disease (Surmacz 2000). For instance, downregulation of IGF-1R and its signalling molecule IRS-1 has been reported in advanced human breast cancer (Schnarr *et al.* 2000). In breast cancer cells, ER⁺ breast cancer cell lines with less aggressive phenotypes show high levels of IGF-1R expression (Guvakova and Surmacz 1997; Lee *et al.* 1999) whereas the low levels of IGF-1R and lack of an IGF-I-induced proliferative response is found in the more aggressive ER⁻ breast cancer cell lines (Bartucci *et al.* 2001; Sepp-Lorenzino *et al.* 1994). In addition, experimentation in non-metastatic MCF-7 cells has also demonstrated that a reduction of IGF-1R expression in those cells by stable transfection of anti-sense constructs for IGF-1R results in more metastatic phenotypes (Pennisi *et al.* 2002).

Despite the reduction of IGF-1R expression in metastatic breast cancer, several studies have suggested that the receptor is required for the metastatic potential of breast cancer cells. For instance, *in vitro* studies have also demonstrated that IGF-I elicits a chemotactic response from both non-metastatic MCF-7 and metastatic MDA-MB-231 human breast cancer cells and a neutralising anti-IGF-1R inhibits IGF-I-induced chemotaxis in those cells (Doerr and Jones 1996). The inhibition of IGF-1R function by dominant-negative

IGF-1R mutants suppresses adhesion, invasion and metastasis of breast cancer cells both *in vitro* and in a mouse model (Dunn *et al.* 1998; Sachdev *et al.* 2004). Several recent studies have suggested that IGF system components may play an important role in promoting some of the cellular phenotypes associated with invasive and metastatic potential of breast cancer cells such as angiogenic ability, cell-cell adhesion and cell migration (Bae *et al.* 1998; Gross and Yee 2003; Lee *et al.* 2000; van Golen 2003).

In summary, extensive research of breast cancer progression mediated by the IGF system indicates that IGF components are key regulators of malignant phenotypes controlling transformation, proliferation, survival (Gross and Yee 2003; Sachdev and Yee 2001; Surmacz *et al.* 1998) as well as invasion and metastasis of breast cancer cells (Gross and Yee 2003; Surmacz 2000). While it is clear that IGF-I and IGF-1R mediate transformation and survival, thus promoting tumour growth in the early steps of tumour development, their function in the later stage, invasion and metastasis, is not well understood.

1.3.3 Chemokines and Breast Cancer

1.3.3.1 Chemokines and Chemokine Receptors

The chemokines, a superfamily of low molecular weight chemotactic cytokines, are well-known as mediators of leukocyte trafficking and other biological activities such as development, angiogenesis and haematopoiesis. Chemokines are approximately 8-14 kDa in weight and generally comprise 70-80 amino acids in length with few exceptions. Chemokines contain at least four conserved cysteines at certain positions, which form the disulfide bonds essential for their distinctive structure, one between the first and the third and one between the second and the fourth cysteine. Most chemokines have two important

regions, an exposed loop in the backbone between the second and the third cysteine, believed to be required for binding to the receptors, and a variable region at the NH₂-terminus prior the first cysteine, thought to be critical for receptor specificity and chemokine-triggered signalling effects (Baggiolini *et al.* 1997; Clark-Lewis *et al.* 1995; Olson and Ley 2002).

Basically, chemokines are classified into four main groups, the CC, CXC, CX₃C and C, based on the number and spacing of the conserved cysteines (Figure 1.5A) (Allen *et al.* 2007; Horuk 2001; Rajagopalan and Rajarathnam 2006). The two main groups of chemokines, the CXC or α chemokines and the CC or β chemokines contain a majority of the known chemokines. In the CXC chemokines, an amino acid is positioned between the first and second cysteines whereas, in CC chemokines, these cysteines are adjacent. Minor chemokine families have also been described, the C or γ chemokines, which has lost the first and the third cysteines, and the CX₃C or δ chemokines, which contains three amino acids between the first two cysteines (Baggiolini *et al.* 1997; Gale and McColl 1999; Mellado *et al.* 2001; Olson and Ley 2002).

Chemokine receptors are members of the seven transmembrane G protein-coupled receptor (GPCR) family (Figure 1.5B) (Allen *et al.* 2007; Horuk 2001; Rajagopalan and Rajarathnam 2006). Most chemokine receptors are composed of approximately 350 amino acids and have a molecular weight of around 40 kDa. Functionally, these receptors can be considered to consist of two main parts, three extracellular loops with an NH₂-terminus which act as a binding site for chemokine ligands and three intracellular loops with a COOH-terminus which coordinately transduce intracellular signals. Most of the receptors contain a conserved 10-amino acid sequence in the second intracellular loop, required for

heterotrimeric G-protein coupling, and a cysteine residue in each of the extracellular loops, forming disulfide bridges to maintain their three-dimensional structure. Chemokine receptors can be grouped into four major families according to the classification of their ligands. The receptors, CR, CCR, CXCR and CX₃CR, are counterparts of the ligands, C, CC, CXC and CX₃C respectively (Baggiolini *et al.* 1997; Horuk 2001; Mellado *et al.* 2001; Olson and Ley 2002). The known human chemokines in systematic names together with original names, and their respective receptors are summarised in Table 1.1.

Chemokines are found in a variety of tissues and produced by a wide range of cells including leukocytes, platelets, fibroblasts, endothelial, epithelial and malignant cells (Balkwill 1998). They can also be classified into two main groups, depending on their function and pattern of expression, inflammatory (inducible) and homeostatic (constitutive) chemokines. However, some chemokines share characteristics of both types and are called dual-function chemokines (See Figure 1.6)(Moser *et al.* 2004). Most of the known chemokines are inflammatory chemokines, whose respective receptors are expressed by particular subsets of leukocytes. The ligands are expressed in response to proinflammatory cytokines and their role is to recruit particular leukocytes to inflammatory sites. Examples of inflammatory chemokines, which are responsible for attracting various types of leukocytes, include CXCL1, 2, and 3 (GRO α , β , and γ), CXCL5 (ENA-78), CXCL6 (GCP-2) and CXCL7 (NAP-2) for myeloid-lineage cells, CXCL10 (IP-10) for Th1 cells, and CCL5 (RANTES) for Th1 and Th2 cells (Olson and Ley 2002). In contrast, homeostatic chemokines are constitutively expressed in certain types of cells and tissues, and are thought to play roles in embryonic development and in the maintenance of the haematopoietic and immune systems. For instance, CXCL12 (SDF-1) is required for bone marrow and follicular B-cell emigration, and thymocyte

homing. CCL19 (MIP-3 β) and CCL21 (SLC) are responsible for T and B cell homing to secondary lymphoid organs and the microanatomical formation of secondary lymphoid organs (Baggiolini 1998; Olson and Ley 2002).

Chemokines are well-known to have chemoattractant properties and therefore play a major role in directional cell migration, termed chemotaxis. The ligation of chemokines to their receptors leads to many cellular responses essential for cell migration including cytoskeleton rearrangement, cell polarisation, and integrin-dependent adhesion (Balkwill 1998; Mellado *et al.* 2001). The function of chemokines is primarily well established in leukocytes. However, it is apparent that chemokines also participate in biological activities of various types of other cells. It has become more evident that they contribute not only to homeostasis but also to many pathological conditions such as autoimmune, infectious and cancer diseases (Balkwill 1998; Gale and McColl 1999; Tanaka *et al.* 2005).

1.3.3.2 Signal Transduction Downstream of Chemokine Receptors

Despite advances in understanding of chemokine-triggered signal transduction leading to leukocyte chemotaxis, the signalling system in other cell types is less well documented. This section will therefore describe the signal transduction pathways downstream of the chemokine G-protein coupled-receptors based mainly on the knowledge of leukocyte systems. It has become clear that chemokines trigger cellular signal transduction through various known effectors including heterotrimeric G-proteins, JAK/STAT, and other tyrosine and serine/threonine kinases (Mellado *et al.* 2001; Thelen 2001) (Figure 1.5). These molecules coordinately activate downstream cascades that are required for chemokine-triggered biological responses.

The ligation of chemokine receptors to their cognate ligands results in conformational changes to the receptor, leading to the dissociation of heterotrimeric G-proteins into the $G\beta\gamma$ complex and GTP-bound $G_i\alpha$ subunit. So far, the role of $G_i\alpha$ in chemokine signal transduction is still unclear even though a great importance of $G_i\alpha$ activity to leukocyte chemotaxis has been shown by the fact that a majority of chemokine-induced cell responses including chemokines are inhibited by treatment with the exotoxin of *Bordetella pertussis*, pertussis toxin (PTX), which inactivates the G_i protein (Murphy 1994) (Thelen 2001). Some studies indicate that $G_i\alpha$ itself is not essential for cell migration but the activation of $G_i\alpha$ is required to release $G\beta\gamma$ complex from $G_i\alpha$ and the receptors (Arai *et al.* 1997; Neptune and Bourne 1997; Neptune *et al.* 1999). However, a direct activity of $G_i\alpha$ can not be ruled out. Recent evidence has suggested that $G_i\alpha$ directly activates one or more members of the Src tyrosine kinase family, which links chemokine receptors to a mediator of cytoskeleton adhesion, focal adhesion kinase (FAK), and possibly to other downstream effector enzymes, the class IA PI3K and MAPK systems (Ganju *et al.* 1998; Le *et al.* 2005; Ma *et al.* 2000; Thelen 2001; Wang *et al.* 2000).

The $G\beta\gamma$ heterodimer is known to play a major role in chemokine-mediated cellular responses. The $G\beta\gamma$ subunits trigger the activation of phosphoinositide specific phospholipase C (PLC), leading to inositol-1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG) production (Jiang *et al.* 1996). Through binding to its specific receptor in the endoplasmic reticulum (ER), IP_3 induces release of calcium stored in the ER into cytoplasm, resulting in an increase of intracellular free calcium ($[Ca^{2+}]_i$) (Kiselyov *et al.* 2003). The formation of DAG results in the activation of protein kinase C (PKC), which is also an important effector system that regulates many subsequent intracellular signal

transduction events. The pathway of PLC is not required for chemokine-induced chemotaxis as shown by the evidence that neutrophils isolated from mice lacking the PLC gene do not show any defect in migration mediated by chemokines (Li *et al.* 2000). For chemoattractant-mediated chemotaxis of neutrophils, $[Ca^{2+}]_i$ elevation is not essential for cell spreading, polarisation and pseudopod extension. However, it has been shown that $[Ca^{2+}]_i$ transient is required for the processes of attachment/detachment of the cells from the extracellular matrix and therefore regulates cell motility through biological tissues (Hendey and Maxfield 1993; Mandeville and Maxfield 1997). The PLC pathway, in particular the elevation of $[Ca^{2+}]_i$ levels, is commonly used to test the responsiveness of cells to chemokines. The $G\beta\gamma$ complex released from the receptors also activates the class IB PI3K γ enzyme, and its important downstream effector, the pleckstrin homology (PH) domain containing protein kinase B (PKB) or Akt. Chemokine-induced PI3K γ activity is crucial for leukocyte migration as shown by the reduction of chemotactic ability of PI3K γ -deficient leukocytes both *in vitro* and *in vivo* (Hirsch *et al.* 2000; Li *et al.* 2000; Sasaki *et al.* 2000). The activated serine/threonine PKB subsequently phosphorylates various signalling molecules, one of which is the Rho GTPases family, which is known to be associated with chemotaxis. The members of the Rho family such as Rac, Rho and Cdc42 appear to modulate the process of actin polymerisation and the cytoskeleton rearrangements required for cell motility. The subcellular localisation of PI3K γ and its downstream molecules, Rho GTPase, has now been thought to regulate cell polarity which is required for directional movement of leukocytes (Curnock *et al.* 2002; Procko and McColl 2005; Ward 2004). In contrast to PLC and PI3K, the MAPK activation system as a downstream of $G\beta\gamma$ complex is unclear. However, several recent studies have demonstrated that the activation of MAPK by chemoattractants is dependent on the

activity of PI3K γ (Bondeva *et al.* 1998; Sasaki *et al.* 2000). This leads to a proposed mechanism that PI3K γ , which is activated by G $\beta\gamma$ complex may, in turn, stimulate a src-like kinase, initiating a classical growth factor MAPK cascade through Shc, Grb2, Sos, Ras and Raf. Although the activation of MAPK by chemokines is well documented, their role in a chemotactic response remains poorly understood (Thelen 2001).

Apart from the G-protein-dependent activation pathway, it has been proposed that the interaction of chemokines and chemokine receptors also induces receptor dimerisation or oligomerisation and tyrosine phosphorylation of the receptors, which initiates the activation of the JAK/STAT pathway (Mellado *et al.* 2001). The chemokine receptors, CCR5 and CXCR4, have been shown to activate different members of JAK/STAT family (Rodriguez-Frade *et al.* 1999; Rodriguez-Frade *et al.* 1999). CCL2 (MCP-1) induces dimerisation and tyrosine phosphorylation of CCR2 receptors and the activation of JAK2/STAT3. A JAK kinase inhibitor also inhibited CCL2-mediated calcium mobilisation and chemotaxis of a monocytic cell line (Mellado *et al.* 1998). The recognition of chemokine-triggered JAK/STAT activation pathways is relatively recent and these are suggested to be independent of G-protein-related signalling events (Mellado *et al.* 2001).

Chemokines elicit short transient signals and rapidly terminate receptor activity by the processes of receptor desensitisation, internalisation, and degradation and/or recycling. These processes have a great impact on the magnitude and duration of chemokine signals and therefore play an important regulatory role in chemokine-mediated cellular responses. A classical molecular mechanism for GPCR downregulation is the clathrin-mediated endocytosis pathway, which involves the activity of major effector molecules, G-protein-

coupled receptor kinases (GRKs) and arrestin-type proteins (Aragay *et al.* 1998; Bohm *et al.* 1997; Neel *et al.* 2005). Following the ligation of chemokine receptors, activated protein kinases, in particular GRKs, subsequently phosphorylate the ligand-occupied receptor at serine and threonine residues in the C-terminus domain, providing binding sites for β -arrestin proteins, which can act as both adaptor and transducer proteins (Aragay *et al.* 1998; Franci *et al.* 1996; Mueller *et al.* 1997; Shenoy and Lefkowitz 2005). The binding of β -arrestin prevents further coupling of G-proteins to the activated receptor, therefore attenuating subsequent chemokine signals. The β -arrestin proteins were originally identified as adaptor proteins connecting the receptor complex to clathrin-mediated internalisation pathways. The latter process removes the receptors from the cell surface into intracellular compartments, known as clathrin-coated vesicles. This is followed by the processes of degradation and/or recycling back to the cell surface (Bohm *et al.* 1997; Fan *et al.* 2003; Fernandis *et al.* 2002; Goodman *et al.* 1996). Additional activity of β -arrestin as a transducer has been recently discovered and it has been shown that the β -arrestin-bound receptors recruit the Src kinases, leading to the activation of MAPK pathways (Shenoy and Lefkowitz 2005). The critical roles of both GRKs and β -arrestins in chemokine receptor signalling and downregulation have been addressed in several chemokine receptor-signalling systems (Barlic *et al.* 1999; Huttenrauch *et al.* 2005; Vila-Coro *et al.* 1999; Yang *et al.* 1999).

A possible alternative mechanism for intracellular trafficking pathway of chemokine receptors is via lipid raft/caveolae-dependent internalisation and degradation (Neel *et al.* 2005). Lipid rafts are cholesterol-and sphingolipid-enriched microdomain of the plasma membrane and a caveolae is a subfamily of lipid rafts containing a specific set of proteins,

caveolae 1, -2 and -3. Some GPCRs are internalised within a caveosome, an endosomal compartment of phosphorylated calveolin proteins, and this possibly plays an important role in the recycling of GPCRs (de Weerd and Leeb-Lundberg 1997; Feron *et al.* 1997; Gagescu *et al.* 2000). Recent studies show that, in response to CCL2, CCR2 is internalised and downregulated dependent on the presence of caveolae in human astrocytes (Andjelkovic *et al.* 2002; Ge and Pachter 2004). CXCR4 and CCR5 have also been identified as lipid raft-associated proteins and lipid raft structure seems to be important for signalling and intracellular trafficking of these receptors (Hug *et al.* 2000; Manes *et al.* 1999; Nguyen and Taub 2002). At this point of time, chemokine-induced receptor internalisation via lipid raft/caveolae-dependent pathways is increasingly being investigated (Neel *et al.* 2005).

1.3.3.3 Role of Chemokines in Transformation, Survival, Growth and Angiogenesis

A number of chemokines and their receptors have been implicated in multiple steps of cancer progression, including transformation, survival, clonal expansion and growth (Arya *et al.* 2003; Tanaka *et al.* 2005; Vicari and Caux 2002). It has been suggested that the expression of particular chemokine receptors is associated with tumourigenesis. CXCR2, the receptor for multiple inflammatory chemokines such as CXCL1 and CXCL8 (IL-8) demonstrates a high degree of homology to the GPCR encoded by Kaposi's sarcoma-associated herpesvirus-8, called KSHV-GPCR. A point mutation of CXCR2 results in the constitutive signalling of the receptors and cellular transformation in a manner similar to that by KSHV-GPCR (Bais *et al.* 1998; Burger *et al.* 1999). The overexpression of the ligands for CXCR2 receptors, CXCL2 and CXCL3, in melanocytes also increases their tumourigenicity *in vitro* and in nude mice (Owen *et al.* 1997). A proliferative effect of

chemokines on tumour cells has also been demonstrated. Two ligands for CXCR2, CXCL1 and CXCL8 have been identified as autocrine growth factors of melanoma cells, which constitutively express CXCR2. Blocking the CXCL1 or CXCR2 function with specific antibodies appears to inhibit melanoma cell growth (Moser *et al.* 1993; Norgauer *et al.* 1996; Schadendorf *et al.* 1993). In addition, some chemokines may support tumour growth by providing anti-apoptotic signals as shown by the evidence that CXCL12 and CXCL9 (MIG) enhance the survival of tumour cells, expressing their counterpart receptors CXCR4 and CXCR3 respectively, in serum-free conditions (Kawada *et al.* 2004; Zhou *et al.* 2002).

Chemokines also affect the growth of tumours by regulating the formation of vascular networks from surrounding tissues. Particular types of CXC chemokines, containing an ELR (Glu-Leu-Arg) motif at the NH₂-terminus, appear to promote blood vessel growth in tumours. The ELR-positive chemokine CXCL8 elicits an angiogenic effect on prostate tumours. CXCL8 is found to be elevated in serum of prostate cancer patients compared with healthy subjects or patients with benign tumours, and also in tissue samples of prostate cancer but not in normal and benign prostatic hyperplasia (Ferrer *et al.* 1998; Veltri *et al.* 1999). In a human prostate cancer/scid mouse model using prostate cancer cells which constitutively produce CXCL8, the administration of a neutralising antibody to CXCL8 inhibits tumour growth and tumour-related angiogenesis (Moore *et al.* 1999). Other ELR-positive CXC chemokines such as CXCL1, CXCL2 and CXCL3 also exhibit angiogenic properties as shown by experiments in which the overexpression of these chemokines in non-tumorigenic mouse melanocytes caused the formation of highly vascular tumours in mice (Luan *et al.* 1997; Owen *et al.* 1997). In contrast, ELR-negative CXC chemokines such as CXCL4 (PF-4), CXCL9 (MIG) and CXCL10 (IP-10) have the

ability to inhibit angiogenesis, called angiostatic activity (Angiolillo *et al.* 1995; Arenberg *et al.* 1997; Strieter *et al.* 1995). In SCID mice, the intratumoural injection of CXCL10 attenuates the growth and neovascularisation of non-small cell lung cancer, whereas blocking the function of CXCL10 by the administration of a specific neutralising antibody enhances tumour growth and angiogenesis (Arenberg *et al.* 1996). However, the ELR-negative CXC chemokine CXCL12 appears to exhibit angiogenic ability both *in vitro* and *in vivo* (Gupta *et al.* 1998; Salcedo *et al.* 1999). Overall, it is believed that a balance between the activities of angiogenic and angiostatic factors, including certain CXC chemokines, is required for the formation of the neovascular network in tumours (Arenberg *et al.* 1997; Strieter *et al.* 1995).

1.3.3.4 Role of Chemokines in Metastasis and Invasion

Accumulating evidence has also implicated various chemokines in the late stages of cancer diseases, metastasis and invasion. For instance, expression of CXCL8 as well as its corresponding receptors, CXCR1 and CXCR2, increases the invasiveness of human melanoma cells and correlates with the metastatic potential of the cells in mice (Singh *et al.* 1994; Varney *et al.* 2003). Furthermore, overexpression of CXCL8 also results in an increase in invasion and metastasis both *in vitro* and in a mouse model of prostate cancer (Inoue *et al.* 2000). CCR7, the receptor for CCL19 and CCL21, has also been implicated in melanoma metastasis. The functional expression of CCR7 enhances the metastasis of B16 murine melanoma cells, and the metastasis is inhibited by a neutralising antibody to CCL21 (Wiley *et al.* 2001). Of particular relevance to breast cancer, certain chemokines promote the invasive and metastatic potential of breast cancer cells. A wide range of chemokines including CCL3 (MIP-1 α), CCL4 (MIP-1 β), CCL2 (MCP-1), CCL5

(RANTES), CXCL10, CXCL7 (NAP-2), CXCL1 and CXCL2 have been shown to induce the migration of human breast cancer cells (Youngs *et al.* 1997). The expression of CXCL8 has also been correlated with the metastatic potential of breast cancer cell lines (De Larco *et al.* 2001). At the present time, the involvement of certain chemokines in the metastasis of breast cancer is becoming increasingly well established (Moore 2001; Murphy 2001).

The first experimental evidence revealing a molecular mechanism for chemokine-mediated organ-specific metastasis of breast cancer was reported by Muller and colleagues (Muller *et al.* 2001). Amongst 17 different chemokine receptors, CXCR4 and CCR7 were markedly expressed in human breast cancer cell lines, malignant breast tumour and metastases, compared with normal mammary epithelial cells. The functionality of CXCR4 and CCR7 was demonstrated by *in vitro* experiments showing that the stimulation of human breast cancer cells with CXCL12 and CCL21 mediates actin polymerisation, chemotaxis and invasion. A panel of normal human organs was screened for their respective ligands, CXCL12 for CXCR4 and CCL21 for CCR7. It was found that CXCL12 was preferentially expressed at high levels in all target organs for breast cancer metastasis: the lung, liver, bone marrow, and lymph nodes and at low levels in all other organs. On the other hand, CCL21 was expressed selectively in lymph nodes. Protein extracts of the target organs exerted chemotactic activity on breast cancer cells, supporting the existence of chemotactic agents. In a metastatic model of human breast cancer, involving injection of MDA-MB-231 cells either orthotopically into the mammary fat pad or intravenously in immunodeficient SCID mice, blocking the interaction of CXCL12 and CXCR4 by the administration of a neutralising antibody to CXCR4 attenuated the metastasis of breast cancer to the regional lymph node and lung. This study indicates a

significant involvement of CXCR4/CXCL12 and potentially CCR7/CCL21 in the development of breast cancer metastasis and invasion. Several more recent studies, using different methods to block the activity of CXCR4 also support such a role. Downregulation of CXCR4 receptors by inducible small-interfering RNA (siRNA) inhibits the metastasis of breast cancer cells both *in vitro* and *in vivo* (Chen *et al.* 2003; Liang *et al.* 2005) and blocking CXCR4 activity using a specific antagonist delayed the growth of breast cancer cells in the lung (Smith *et al.* 2004). The impact of CXCR4/CXCL12 on metastasis has also been reported by several studies in other types of metastatic cancers, including prostate, melanoma, pancreatic and ovarian cancers (Balkwill 2004).

CXCR4 and its corresponding ligand CXCL12, are widely expressed in normal tissues and play a fundamental role in fetal development, mobilisation of haematopoietic stem cells and trafficking of naïve lymphocytes (Balkwill 2004; Kucia *et al.* 2004). Mice in which either CXCR4 or CXCL12 have been knocked out die perinatally due to a defect in the development of heart, brain, haematopoietic system, gastrointestinal tract, cerebellum and vasculature (Lazarini *et al.* 2003; Nagasawa *et al.* 1996; Tachibana *et al.* 1998; Zou *et al.* 1998). The aforementioned study by Muller and colleagues has clearly implicated the CXCR4/CXCL12 axis in organ-selective metastasis thereby providing further for the “seed” and “soil” hypothesis. These findings suggest that the CXCR4/CXCL12 axis are an important part of the local microenvironment at target organs that can arrest tumour cells and promote the initiation and growth of secondary tumours (Moore 2001; Murphy 2001). However, at this point of time, the role of CXCR4/CXCL12 in the process of breast cancer metastasis still requires much more investigation. A recent study has demonstrated that CXCR4 modulates growth of both primary and secondary breast tumours (Smith *et*

al. 2004) and based on previous data in various cellular systems, it is suggested that the CXCR4/CXCL12 axis potentially regulates multiple parameters that are associated with tumour metastasis including directed migration, adhesion, proliferation and survival of cells (Balkwill 2004; Kucia *et al.* 2004). Even though it has been shown in a number of *in vitro* experiments that CXCR4 and CXCL12 are able to mediate chemotaxis and cell adhesion of various cell types, including leukocytes and tumour cells, their effect on cell survival and proliferation is relatively less well documented (Kucia *et al.* 2004). Thus, the molecular mechanisms involved in CXCR4/CXCL12-mediated breast cancer metastasis still require further investigation.

1.3.4 Cross-talk between Signal Transduction Downstream of G-protein-coupled Receptors and Receptor Tyrosine Kinases

The families of G-protein-coupled receptor (GPCR) and receptor tyrosine kinase (RTK) are major groups of receptor proteins on the cell surface. They play important roles in mediating the signals from extracellular stimuli such as hormones, growth factors and cytokines and the function of both receptors is involved in a variety of physiological and pathological conditions (Krause and Van Etten 2005; Spiegel and Weinstein 2004). Accumulating studies in GPCR- and RTK-mediated signal transduction cascades have indicated that the two receptors share some downstream signalling pathways and may modulate each other to transduce the signals, a process termed “cross-talk” (Luttrell *et al.* 1999; Waters *et al.* 2004). Cross-talk between these receptors adds a significant complexity to their downstream signal transduction networks, which translate multiple signals from extracellular stimulus into cellular activity. To date, three forms of cross-talk between the GPCR and RTK systems have been demonstrated in different cellular

systems. First, RTKs can be transactivated by GPCRs. For instance, the epidermal growth factor receptor (EGFR) is phosphorylated in bronchial epithelial cells in response to CCL11, a ligand for the GPCR CCR3, leading to MAPK activation and CXCL8 (IL-8) production (Adachi *et al.* 2004). Second, GPCRs can be transactivated by RTKs. For example, IGF-I stimulates phosphorylation of CCR5 in MCF-7 cells. This appears to be indirect, requiring the production of CCL5, a ligand for CCR5 (Mira *et al.* 2001). Finally, bidirectional transactivation between the two receptor systems has also been observed. It has been demonstrated that the platelet-derived growth factor receptor (PDGFR) is phosphorylated by sphingosine 1-phosphate (S1P), a ligand for GPCR S1P receptors (S1PRs), leading to activation of downstream effectors including Shc, and the p85 regulatory subunit of class 1A PI3K (Tanimoto *et al.* 2004) and PDGF has been demonstrated to transactivate the S1P receptors (Waters *et al.* 2003). While cross-talk between GPCR and RTK activation pathways and their mechanisms have been studied and documented, this is a relatively new area of study in cell biology.

1.4 The research project

1.4.1 Significance and Rationale of the research

The literature to date has implicated IGF-1R and more recently CXCR4 and CCR7 in metastasis and invasion of breast cancer. The understanding of chemokine- and IGF-I-mediated signal transduction pathways leading to cell migration remains incomplete particularly when the interactions between the two signalling systems are considered. Therefore, this study was specifically aimed at investigating potential cross-talk between the signalling pathways induced by the chemokine receptors, CXCR4 and CCR7, and that

by IGF-1R in human breast cancer epithelial cells. A clearer understanding of these signal transduction pathways may lead to more effective therapeutic approaches for breast carcinoma.

1.4.2 The Central Hypothesis to be tested

That there is cross-talk between chemokine receptors, CXCR4 and CCR7, and IGF-1R signal transduction pathways in human breast cancer epithelial cells

This hypothesis was addressed by investigating the following aims

Aim 1 To characterise the expression and function of chemokine receptors, CXCR4 and CCR7, and IGF-1R in breast cancer cell lines

Aim 2 To investigate transactivation between chemokine receptors, CXCR4 and CCR7, and IGF-1R signal transduction pathways in breast cancer cells

Aim 3 To investigate coregulation of CXCR4 and IGF-1R expression and function in breast cancer cells

Table 1.1 Summary of known human CC, CXC, C and CX₃C chemokine/receptor families (Tanaka *et al.* 2005; Zlotnik and Yoshie 2000)

Systematic nomenclature	Original names (other names may exist)	Major receptor(s)
CC family		
CCL1	I-309	CCR8
CCL2	MCP-1 (monocyte chemoattractant protein 1)	CCR2
CCL3	MIP-1 α (macrophage inflammatory protein 1 α)	CCR1, CCR5
CCL4	MIP-1 β (macrophage inflammatory protein 1 β)	CCR5
CCL5	RANTES (regulated on activation, normally T cell expressed and secreted)	CCR1,CCR3,CCR5
CCL6	Unknown	CCR1,CCR2, CCR3
CCL7	MCP-3 (monocyte chemoattractant protein 3)	CCR1,CCR2,CCR3
CCL8	MCP-2 (monocyte chemoattractant protein 2)	CCR2,CCR3,CCR5
CCL9/10	Unknown	CCR1
CCL11	Eotaxin-1	CCR3
CCL12	Unknown	CCR2
CCL13	MCP-4 (monocyte chemoattractant protein 4)	CCR1,CCR2,CCR3
CCL14	HCC-1 (hemofiltrate CC chemokine)	CCR1
CCL15	Lkn-1 (leukotactin 1)	CCR1,CCR3
CCL16	LEC (liver expressed chemokine)	CCR1
CCL17	TARC (thymus and activation regulated chemokine)	CCR4
CCL18	PARC (pulmonary and activation regulated chemokine), macrophage inflammatory protein-4 (MIP-4)	Unknown
CCL19	ELC (Epstein-Barr virus induced receptor ligand chemokine)/macrophage inflammatory protein-3 β (MIP-3 β)	CCR7
CCL20	LARC (liver and activation regulated chemokine/ macrophage inflammatory protein-3 α (MIP-3 α))	CCR6
CCL21	SLC(secondary lymphoid tissue chemokine)/6CKine/exodus-2	CCR7
CCL22	MDC (macrophage derived chemokine)	CCR4
CCL23	MPIF-1 (myeloid progenitor inhibitory factor 1)	CCR1
CCL24	MPIF-2 (myeloid progenitor inhibitory factor 2)	CCR3
CCL25	TECK (thymus expressed chemokine)	CCR9, CCR11
CCL26	Eotaxin-3	CCR3
CCL27	Eskine/CTACK	CCR10
CCL28	MEC (mucosae-associated epithelial chemokine)	CCR10,CCR3

(Continued over page)

Table 1.1 Summary of known human CC, CXC, C and CX₃C chemokine/receptor families, continued (Tanaka *et al.* 2005; Zlotnik and Yoshie 2000)

Systematic nomenclature	Original names (other names may exist)	Major receptor(s)
CXC family		
CXCL1	GRO α (growth related oncogene α)	CXCR2>CXCR1
CXCL2	GRO β (growth related oncogene β)	CXCR2
CXCL3	GRO γ (growth related oncogene γ)	CXCR2
CXCL4	PF-4 (platelet factor 4)	unknown
CXCL5	ENA-78 (epithelial cell-derived neutrophil activating factor 78)	CXCR2
CXCL6	GCP-2 (granulocyte chemoattractant protein 2)	CXCR1,CXCR2
CXCL7	NAP-2 (neutrophil activating protein 2)	CXCR1,CXCR2
CXCL8	IL-8 (interleukin 8)	CXCR1,CXCR2
CXCL9	MIG (monokine induced by interferon- γ)	CXCR3
CXCL10	IP-10 (γ interferon inducible protein 10)	CXCR3
CXCL11	I-TAC (interferon inducible T cell α -chemoattractant)	CXCR3
CXCL12	SDF-1 α/β (stromal cell derived factor-1 α/β)	CXCR4
CXCL13	BCA-1 (B cell activating chemokine 1)	CXCR5
CXCL14	BRAK (breast and kidney chemokine)	Unknown
CXCL15	Unknown	Unknown
CXCL16	SR-PSOX (scavenger receptor that binds phosphatidylserine and oxidized lipoprotein)	CXCR6
C family		
XCL1	Lymphotactin- α	XCR1
XCL2	Lymphotactin- β	XCR1
CX₃C family		
CX ₃ CL1	Fractalkine	CX ₃ CR1
Most chemokines and their respective receptors identified in humans are listed here. Four major chemokine subfamilies are shown together with their original names and their major receptors for each chemokine. This table is modified from (Tanaka <i>et al.</i> 2005; Zlotnik and Yoshie 2000) and (Proudfoot 2002).		

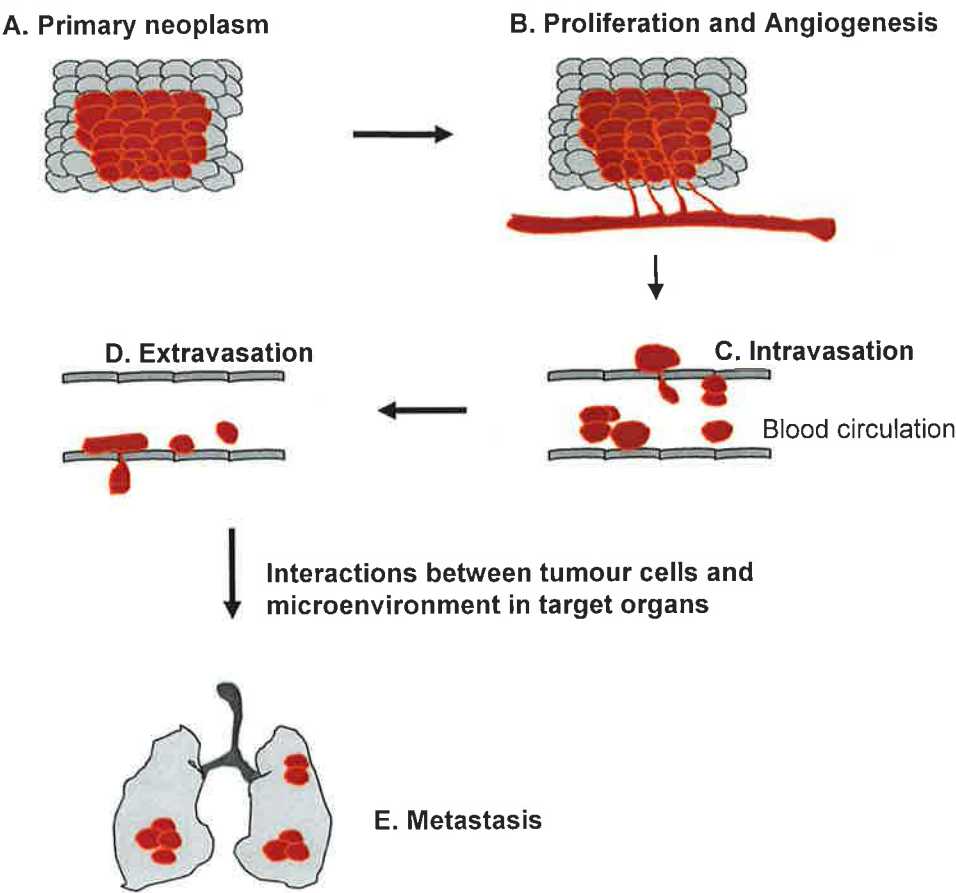


Figure 1.1 *The multi-step process of tumour metastasis*

Cellular transformation occurs at the primary sites (A). The growth of neoplasm is supported initially by simple diffusion of nutrients in an expanding tumour mass. Vascularisation must initiate if a tumour mass is to exceed 1-2 mm³ in diameter. The synthesis and secretion of angiogenic factors establishes a vascular network from the surrounding stroma, the process called angiogenesis (B). Local invasion of some tumour cells to the host stroma occurs and the new blood vessels can provide the routes by which the cells can leave the tumour, and enter the blood circulation, which is termed intravasation (C). Tumour cells might also enter the circulation indirectly via the lymphatic system. The circulating cells arrest selectively in blood vessels of distant target organs where the cells may migrate into the surrounding tissue, termed extravasation (D). In target organs, the cells are exposed to different types of homeostatic factors provided in the microenvironment, resulting in formation and growth of secondary tumours, metastases (E). The figure is adapted from references (Fidler 2003) and (Mundy 2002). Grey cells represent normal cells whereas red cells represent neoplastic cells.

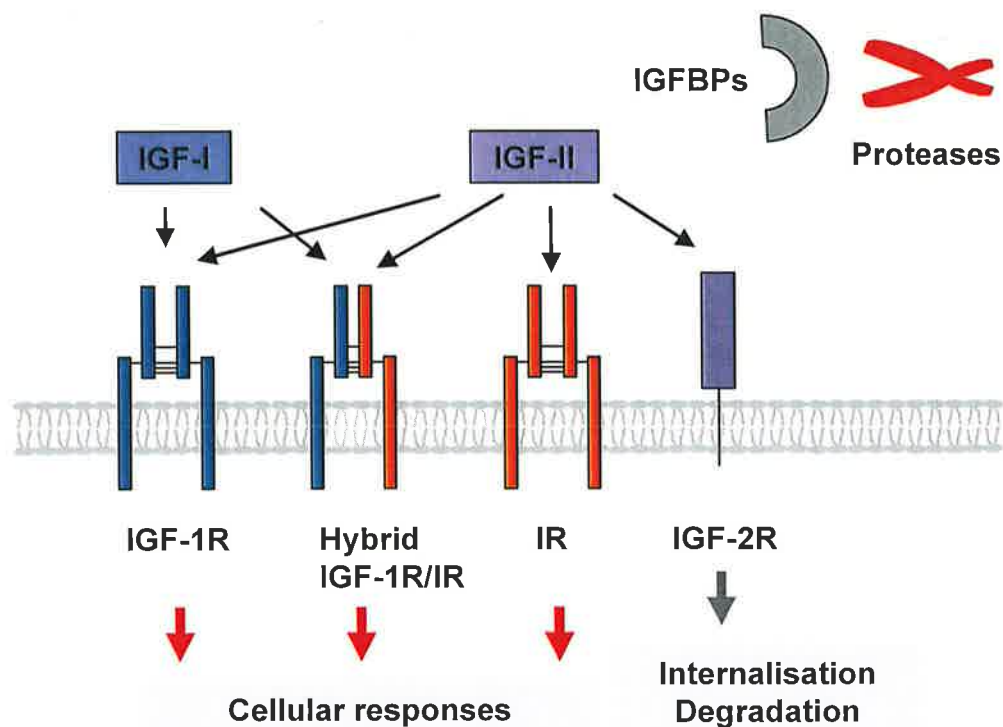


Figure 1.2 Schematic representation of the IGF system

The IGF system comprises a complex network of ligands (IGF-I and IGF-II), their cognate receptors (IGF-1R, IR, IGF-1R:IR hybrid and IGF-2R), six high affinity IGFBPs and IGFBP proteases. IGFs are found circulating mainly in a complex containing IGFs and IGFBPs. Release of IGFs from the complex occurs upon IGFBP proteolysis or extracellular matrix (ECM) binding. This allows IGFs to bind to multiple cognate receptors with different affinities, mediating various biological responses (Denley *et al.* 2005). For more details, see the text. This figure is modified from references (Adams *et al.* 2000; Sachdev and Yee 2001) and (Denley *et al.* 2005). Black arrows indicate the respective receptors whereas red and grey arrows indicate activation pathways to biological responses.

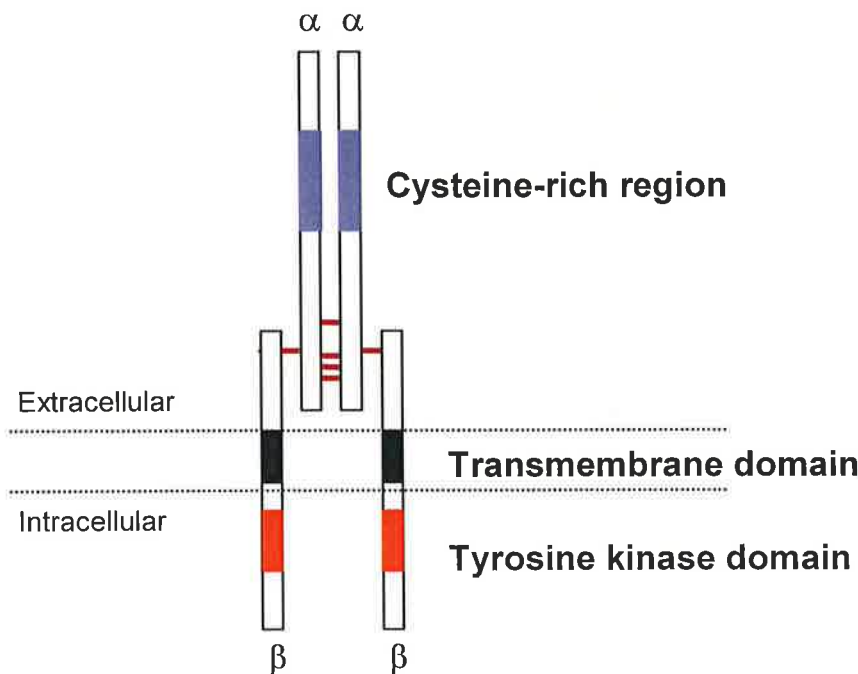


Figure 1.3 Schematic representation of structure of the IGF-1R

IGF-1R is a member of the receptor tyrosine kinase family, composed of two extracellular α subunits and two-membrane spanning β subunits. These subunits form a β - α - α - β arrangement and are held together by the α - α dimer and α - β disulfide bonds at the locations indicated based on reference (Adams *et al.* 2000). Red lines indicate disulphide bridges. The major ligand binding determinants are located within the α subunits. The intrinsic kinase domain, containing multiple sites of tyrosine phosphorylation, is located in the cytoplasmic portions of the β subunits. This figure is modified from reference (Adams *et al.* 2000).

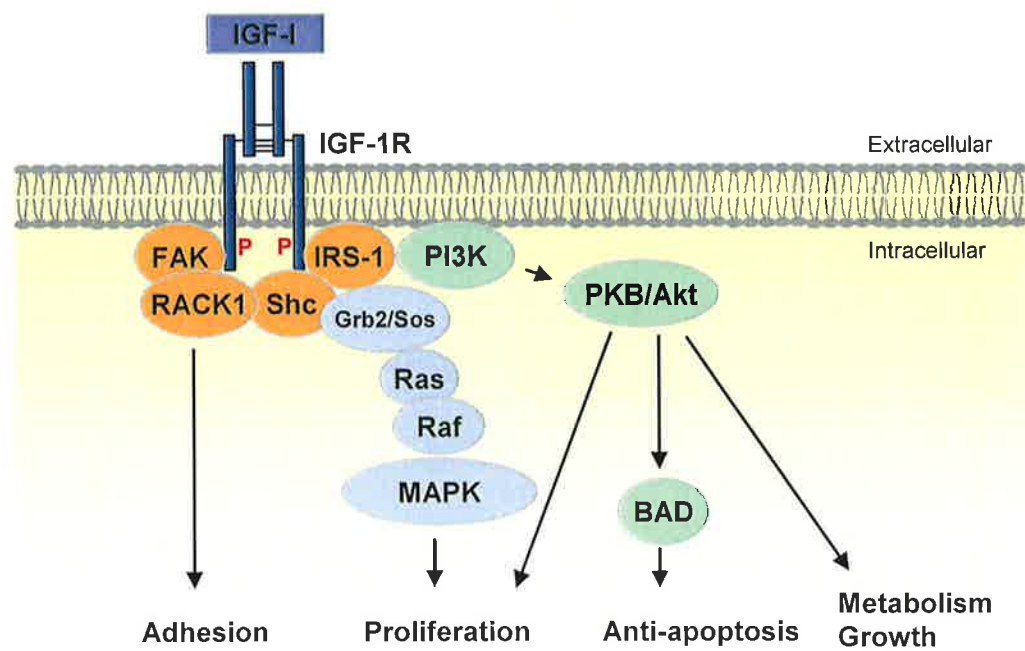


Figure 1.4 Summary of the major signal transduction pathways downstream of IGF-1R.

This schematic depicts some of the established signalling molecules activated following the activation of IGF-1R (Foulstone *et al.* 2005; Sachdev and Yee 2001). The binding of IGF-1 to IGF-1R results in induction of intrinsic tyrosine kinase activity of the receptor. The phosphorylation of multiple tyrosine residues in the cytoplasmic portion of the receptor creates binding sites for several substrates including IRS-1, Shc, FAK and RACK1, which link IGF-1R to downstream signal transduction systems including PI3K and MAPK pathways, leading to various cellular activities. For more details, see the text. Arrows indicate ‘activated downstream’. P indicates multiple phosphorylation of the IGF-1R. Abbreviations: insulin receptor substrate-1 (IRS-1); src-homology 2/2 α -collagen-related (Shc); focal adhesion kinase (FAK); receptor for the activated C kinase 1 (RACK1); growth factor receptor-bound protein 2 (Grb2); son of sevenless (Sos); Ras activated factor (Raf), mitogen-activated protein kinases (MAPK), phosphatidylinositol 3-kinase (PI3K); protein kinase B (PKB/Akt); bcl-associated death promoter (BAD).

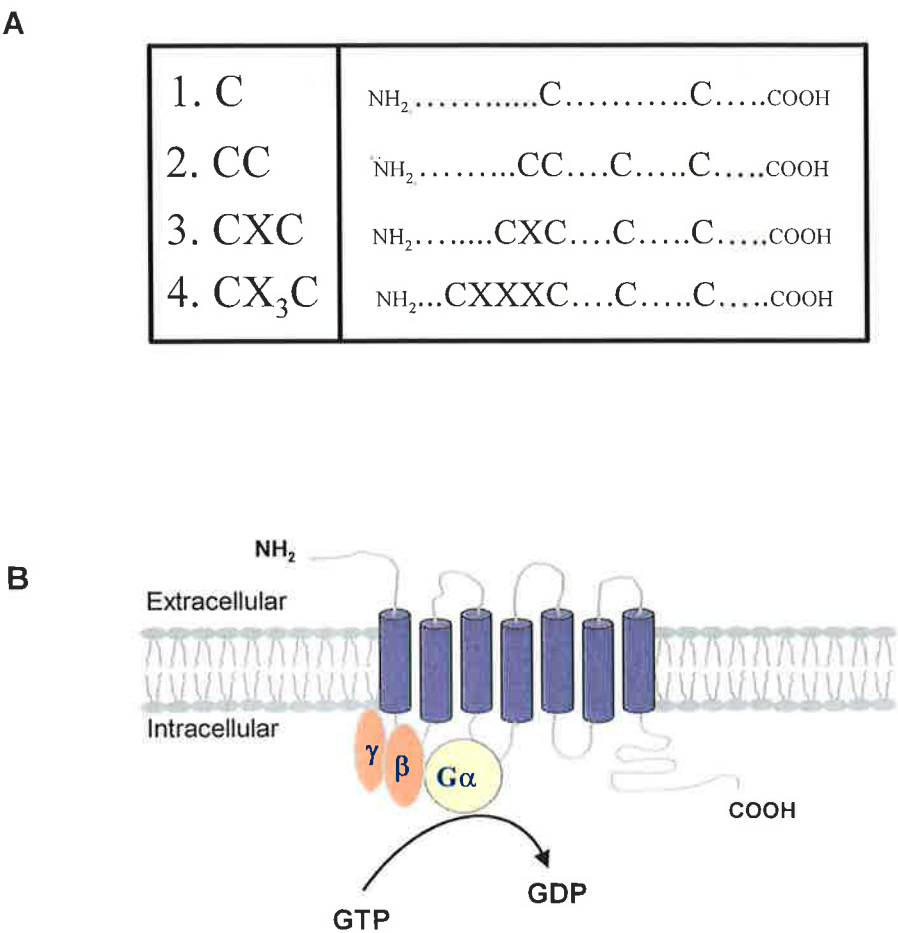


Figure 1.5 Schematics representing the structural classification of chemokines (A) and the typical structure of chemokine receptors (B)

A, Four major subfamilies of chemokines are classified based on the number and arrangement of the conserved cysteines (indicated as C). X indicates an amino acid other than cysteine. Amino and carboxy tails are marked NH₂ and COOH respectively. B, Chemokine receptors are seven-transmembrane receptors, comprising seven helical membrane-spanning regions (purple cylinders) connected by extramembranous loops (grey lines). The NH₂-terminus, together with one or more extracellular loops, is involved in chemokine binding. The COOH-terminus and intracellular loops collaborate to induce the chemokine signal. Heterotrimeric G-proteins, Gα and the complex Gβγ, can couple to the receptor at the second intracellular loop. The Gα subunits, containing GTPase activity, bind to GDP at the resting stage. The binding of chemokines induces conformational changes of the receptor that activate the heterotrimeric G-proteins, causing a dissociation of GDP and its replacement by GTP on the Gα subunits. The GTP-bound Gα subunits and the complex Gβγ are released from the receptor and each activates a variety of intracellular signalling pathways resulting in the different cellular functions. A black arrow indicates replacement of GDP by GTP. These simplified figures are based on references (Allen *et al.* 2007; Horuk 2001) and (Rajagopalan and Rajarathnam 2006).

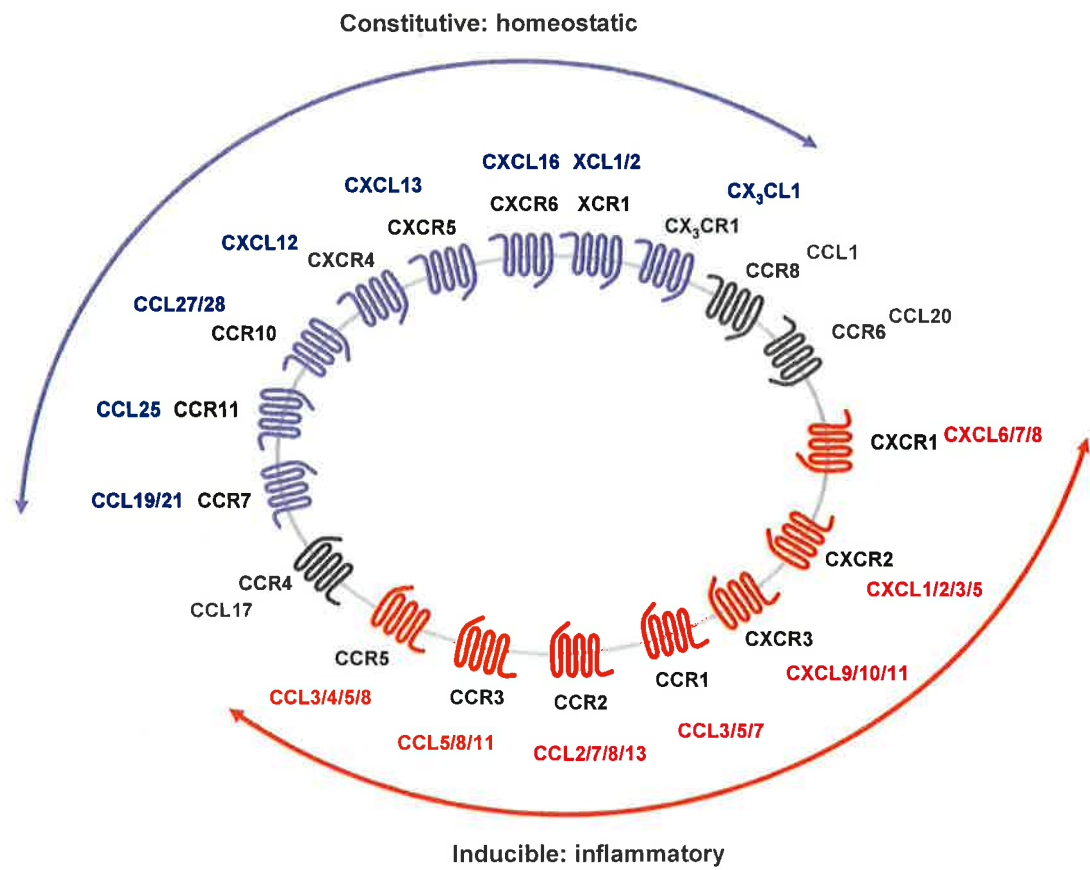


Figure 1.6 Schematic representing functional classification of chemokine system

Chemokine receptors can be expressed constitutively (purple receptors) or inducibly (orange receptors). Chemokines can be classified into two main groups, constitutive (developmentally regulated) (blue ligands) and inducible (inflammatory) (orange ligands) with some exceptions for “dual- function” chemokines (grey ligands). The receptors for “dual-function” chemokines are shown as grey receptors. Chemokines and chemokine receptors for which expression patterns have not been characterised are not included. This figure is produced based on references (Proudfoot 2002) and (Moser *et al.* 2004).

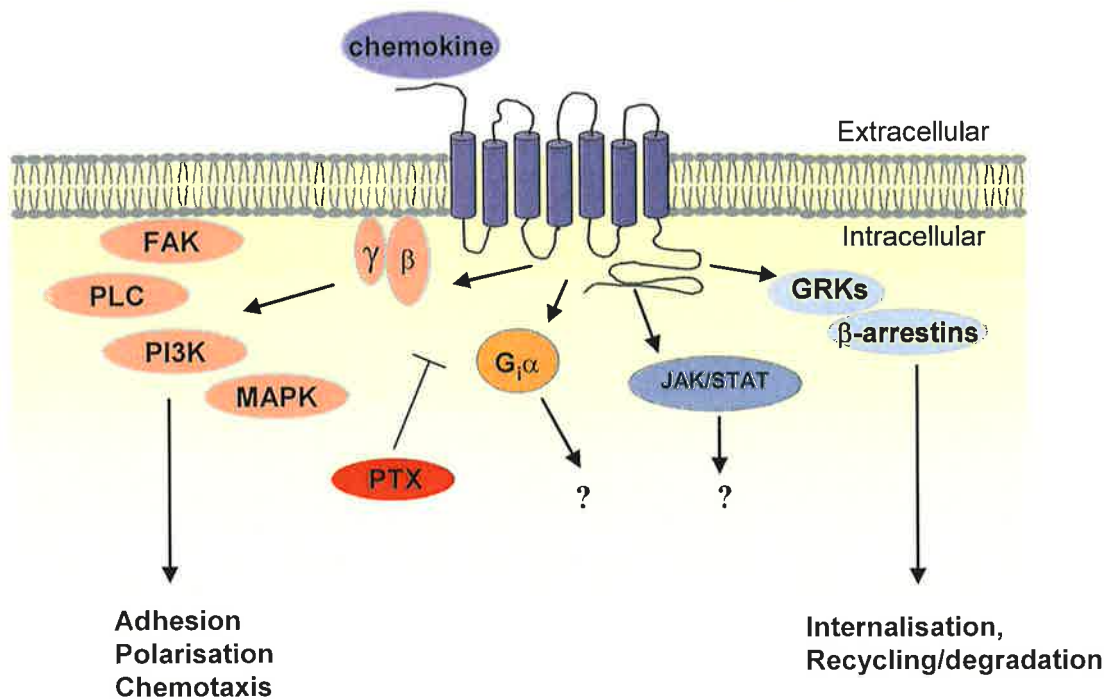


Figure 1.7 Summary of major signal transduction pathways downstream of chemokine receptors

This simplified figure depicts signalling molecules activated following chemokine receptor activation. The binding of chemokines to their respective receptors results in the activation of three main downstream signalling pathways to various cellular responses. The classical G-protein pathway activates various downstream systems including PLC, PI3K, FAK and MAPK, leading to cell adhesion, polarisation and chemotaxis. This pathway can be inhibited by inactivating G_iα using PTX (red oval). The activation of the JAK/STAT pathway being initiated following the tyrosine phosphorylation of activated chemokine receptors has been documented (Mellado *et al.* 2001). The signalling pathways initiated by phosphorylation at serine/threonine residues on the COOH-terminus region, involve the activities of GRKs and β-arrestins. This pathway is responsible for internalisation, recycling/degradation of the activated receptors. For more details, see the text. Black arrows indicate ‘activated downstream’. Question marks indicate unclear downstream signalling pathways. This simplified figure is modified from (Mellado *et al.* 2001) and (Thelen 2001). Abbreviations: focal adhesion kinase (FAK), phospholipase C (PLC), phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), pertussis toxin (PTX), Janus kinase/Signal transducer and activator of transcription (JAK/STAT), G-protein-coupled receptor kinases (GRKs).

CHAPTER 2

Materials and Methods

CHAPETR 2

Materials and Methods

2.1 General Materials

2.1.1 Synthetic Peptides and Inhibitors

CXCL12, CCL19 and CCL21 were obtained from Biomedical Research Centre, University of British Columbia, Vancouver, Canada. Synthetic IGF-I was obtained from GroPrep Pty Ltd, Adelaide, SA, Australia. Pertussis toxin (PTX) was purchased from Sapphire Bioscience, NSW, Australia. Ly294002 and PD980590 were provided by Calbiochem, San Diego, CA, USA.

2.1.2 General Chemicals, Solutions and Buffers, and Antibodies

General chemicals and reagents provided from various main suppliers or manufacturers are listed in Table 2.1 while those specialised are stated in the relevant place in the text. Table 2.2 shows preparation of general solutions and buffers used throughout this study. The list of Abs, suppliers and application in flow cytometry, immunoprecipitation and Western blot was shown in Tables 2.3 and 2.4.

2.2 Cell Culture

2.2.1 Cell Lines

Human leukemic Jurkat T cell and myelomonocytic U937 cell lines, and human breast cancer cell lines, the non-metastatic MCF-7 and highly metastatic MDA-MB-231, were obtained from the American Type Culture Collection (ATCC; Manassa, VA, USA). P6 cells, BALB/c3T3 cells overexpressing the human IGF-1R, and R⁻ cells, mouse 3T3-like cells with a targeted ablation of the IGF-1R gene, were kindly provided by Professor Renato Beserga, Philadelphia, PA, USA. R⁺IR-B cells (R⁻ cells expressing human insulin receptor isoform B) were made by Eric R. Bonython, The University of Adelaide, Adelaide, Australia. B300-19/huCXCR4 cells, murine pre-B cells overexpressing human CXCR4, were kindly provided from Professor Ian Clark-Lewis, Biomedical Research Centre, University of British Columbia, Vancouver, Canada. A 4T1.2 cell line, derived from Balb/cf3H mouse mammary carcinoma, was a kind gift from Dr Robin Anderson, Peter MacCallum Cancer Institute, Melbourne, Australia and 4T1.2-CXCL12 cells, 4T1.2 cells expressing human CXCL12, were made by Sharon Hampton-Smith, The University of Adelaide, Adelaide, Australia.

2.2.2 Cell Culture Maintenance

All basic solutions for cell culture were provided by suppliers or manufacturers listed in Table 2.5. All cell lines used throughout this study were treated according to standard tissue culture procedure, including thawing, subculturing and freezing. The cells in vials stored in liquid nitrogen were thawed rapidly at 37° C in water bath followed by dilution in 10 ml of media, centrifugation (300 × g for 4 minutes), resuspension in related growth

medium shown in Table 2.6 and culture at 37°C in 5% (v/v) CO₂ atmosphere. To subculture adherent cells, confluent monolayer cells were washed twice with PBS. A sufficient amount of 1% trypsin was added to cover cell monolayers for 3-5 minutes at room temperature or 37°C in the incubator if necessary. Detached cells were resuspended in medium containing 10% fetal calf serum (FCS). Cell suspensions containing appropriate number of cells was transferred to fresh growth medium. To passage suspension cells, culture medium containing an appropriate number of cells was transferred to fresh growth medium for further culturing. In general, at the splitting ratio of 1:10, subculture was done once a week for MCF-7, P6, R⁺ and R⁻IR-B cells and twice a week for MDA-MB-231 cells. U937 and B300-19/huCXCR4, 4T1.2 and 4T1.2-CXCL12 cells were subcultured every 2-3 days. For long term storage, cells in exponential phase were washed, harvested and resuspended in freezing medium (Table 2.6) before being transferred to cryogenic vials. The vials were placed in a cryogenic container containing isopropanol at -80°C overnight, and subsequently transferred to a liquid nitrogen tank. Generally, an appropriate number of the cells was required for subculturing, freezing and subjecting to experiments. Viable cell counts were therefore determined by using trypan blue staining. The cells were diluted in 0.8% trypan blue in PBS before counting on a hemacytometer (Improved Neubauer, Weber, UK) and calculated as cells/ml.

2.3 Molecular Techniques

2.3.1 RNA Extraction

A cell suspension was spun down and the cell pellet was subjected to extraction of total RNA. The pellet was mixed with Trizol (Life Technologies, Gilbertsville, PA, USA) (1 ml

per $5-10 \times 10^6$ cells) and left at room temperature for 5 minutes before addition of chloroform (200 μ l per 1 ml of the mixture). The mixture was shaken vigorously by hand for 15 seconds and incubated for 2-3 minutes at room temperature prior to centrifugation at $12,000 \times g$ for 15 minutes at 4°C . An upper aqueous phase containing RNA was transferred to a fresh microcentrifuge tube. The RNA was then precipitated by addition of 500 μ l of isopropanol followed by incubation at room temperature for 10 minutes. The precipitate was then spun down at $12,000 \times g$ for 10 minutes at 4°C . The supernatant was then discarded, and a gel-like pellet containing RNA was left. The RNA pellet was washed by adding 1 ml of 75% ethanol and spinning down at $7,500 \times g$ for 5 minutes at 4°C . The supernatant was subsequently drained and the pellet was air-dried for 5-10 minutes. The RNA precipitate was dissolved in 20 μ l of DEPC-treated water and incubated at $55-60^\circ \text{C}$ for 10 minutes. The purity of RNA was determined by measuring optical density at 260 nm and 280 nm and calculated using the following formula. [Purity = $\text{OD}_{260}/\text{OD}_{280}$] A recommended purity is over 1.5. The concentration of RNA was calculated using the following formula. [RNA concentration ($\mu\text{g}/\mu\text{l}$) = $\text{OD}_{260} \times \text{dilution factor} \times 0.04$]

2.3.2 Synthesis of cDNA by Reverse-transcriptase Enzyme

Prior to synthesis of cDNA from isolated RNA, the RNA was treated by DNase I to remove contaminating chromosomal DNA. Each reaction was set up using RNase-free DNase I and buffers provided by Promega, Madison, WI, USA. Briefly, 5 μg of RNA was diluted to a final volume of 17 μ l in DEPC-treated water to which 2 μ l of 10x reaction buffer and 1 μ l of DNase were added. The reaction was performed at 37°C for 1 hour and

terminated by addition of 2 µl of 10x stop buffer and heat-inactivation at 65° C for 20 minutes. Generation of first strand cDNA from RNA was conducted using Superscript II reverse-transcriptase and the associated buffer as provided (Life Technologies, Gilbertsville, PA, USA). 2.5 µg of extract RNA in 11 µl was combined with 1 µl of oligo (dT) or random primers (500 µg/ml) and heated to 70° C for 5 minutes. After immediate cooling the mixture down to 4° C, the following reagents were added: 4 µl of first strand buffer (5x), 2 µl of DTT (DL-Dithiothreitol) (0.1 M) and 1 µl of deoxynucleoside triphosphate (dNTP) mixture (10 mM each dATP, dTTP, dCTP and dGTP diluted in DEPC-treated water (Amersham Pharmacia Biotech)), and incubated at 42° C for 2 minutes. Finally, 1 µl of Superscript II (200 units/µl) was added and reverse transcription was allowed to proceed at 42° C for 50 minutes before final inactivation at 70° C for 15 minutes. The cDNA products were stored at -20° C until further use.

2.3.3 Amplification of Target Sequences using Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was performed using Dynazymes DNA polymerase and supplied buffer (Finnzymes, UK). Specific sequences for CXCL12 and GAPDH were amplified using following primers (Geneworks, Adelaide, SA, Australia); CXCL12: forward primers 5' GGA ATT CGC CAC CAT GGA CGC CAA GGT CGT CG 3' and reverse primers 5' GAC TAG TTC AGT GAT GGT GAT GGT GGT GCA TCT TGA GCC TC 3'; GAPDH: forward primers 5' TCC TTG GAG GCC ATG TAG GCC AT 3' and reverse primers 5' TGA TGA CAT CAA GAA GGT GGT GAA G 3'. In general, each PCR was set up in a 25 µl reaction mix containing 5 µl of forward primers (5 pmole/µl), 5 µl of reverse primers (5 pmole/µl), 2.5 µl of 10x Mg²⁺-free EXT buffer and 1

µl (for CXCL12) or 1.25 µl (for GAPDH) of 50 mM MgCl₂ (Finnzymes, Espoo, Finland), 0.5 µl of 10 mM dNTP mixture (Amersham Biosciences, Buckinghamshire, England, UK), 0.25 µl of DyNAzyme EXT polymerase (Finnzymes) and 1.25 µl of template DNA. The reactions were cycled in a hot-bonnet thermal cycler (MJ Research Inc., Waltham, MA, USA). The PCR condition for CXCL12 amplification was set up as follows: 95 °C for 10 minutes, 35 cycles of 95° C for 30 seconds, 60° C (for CXCL12) or 54° C (for GAPDH), for 30 seconds and 72° C for 30 seconds, and a final 5 minutes extension at 72° C. All reactions were held at 4° C until analysed.

2.3.4 Agarose Gel Electrophoresis

Agarose gels (2% w/v) were prepared by dissolving in 1xTAE buffer. After heating, the gels were allowed to settle in a horizontal gel apparatus. The gels were submerged in 1xTAE buffer in an electrophoresis tank. DNA samples and markers were mixed with 6x DNA loading buffer (Table 2.2) to a final concentration of 1x and loaded onto the gels. The marker was 100 bp DNA markers provided by Invitrogen, Life Technologies, USA. The gels were electrophoresed in 1xTAE buffer at 100 mA. Following electrophoresis, gels were stained with 5 µg/ml ethidium bromide in TAE buffer for 5-10 minutes, visualised and analysed on a Molecular Imager FX and Quantity One software package.

2.3.5 Whole Cell Lysate Preparation and Protein Concentration Determination

Approximately 5×10^6 cells were lysed at 4° C for 15 minutes in 500-800 µl of Triton-X100 lysis buffer (Table 2.2) supplemented with inhibitors (2 mM Na₃VO₄, 50 mM NaF, 10 mM phenylmethylsulfonyl fluoride (PMSF) and 1:100 protease inhibitor cocktail

(Sigma-Aldrich). Cell lysates were then centrifuged at 14,000 rpm at 4° C for 10 minutes to remove insoluble material and the supernatants were collected. The total protein concentrations in cell lysates were determined using a colorimetric method, utilising a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology Inc, Rockford, USA) as recommended by the manufacturer. Bovine serum albumin (Grade V, Sigma), ranging from 0-2,000 µg/ml, was used to produce a standard curve. The lysates were diluted (1 in 10) and 10 µl of the samples were assayed in 96-well flat bottom tray. BCA reagent (200 µl) was mixed with each of sample, and the plate was incubated at 37° C for 30 minutes. Absorbance was measured at 570 nm using a microplate reader (Amersham Biotrack reader II) and analysed for protein concentration (µg/ml). For protein analysis in SDS-PAGE, the whole cell lysates were prepared by boiling at 95° C for 5 minutes in SDS reducing sample buffer (Table 2.2). Generally, the lysates were subjected to analysis in Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) at 50 µg per well.

2.3.6 Co-immunoprecipitation Using Magnetic Separation Column System

Cell lysates (1 mg of total proteins) were incubated with 1 µg of test Abs or control IgG (Table 2.3) at 4° C overnight. Immunocomplexes were precipitated with protein G-coated microbeads at 4° C for 1 hour before being purified on magnetic microcolumns as recommended by the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, the columns were rinsed by 200 µl of lysis buffer and the cell lysates were loaded onto the column. The columns were then washed 4 times with 200 µl lysis buffer freshly supplemented with inhibitors (2 mM Na₃VO₄, 50 mM NaF, 10 mM phenylmethylsulfonyl

fluoride (PMSF) and 1:100 protease inhibitor cocktail (Sigma-Aldrich) followed by a final wash with 200 µl low salt wash buffer (20 mM Tris-HCl, pH 7.5). The bound proteins were eluted from the column in preheated 1x SDS reducing sample buffer (Table 2.2). The immunoprecipitates were then subjected to SDS-PAGE followed by Western blot analysis.

2.3.7 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis

SDS-PAGE was conducted according to the procedure provided by the manufacturer (Bio-rad, CA, USA). Polyacrylamide gels consisting of 4% stacking gel and 12% or 15% resolving gel compartments (Table 2.2) were prepared using a gel pouring apparatus. Protein samples and protein markers were loaded on the polyacrylamide gel in 1x electrode buffer (Table 2.2) and electrophoresis was performed at 170 V in the stacking gels and at 200 V in the running gel until the ion front reached the bottom of the gels. Western blot protein markers were BenchMark™ Pre-stained and MagicMark™ XP ECL markers obtained from Invitrogen, Life Technologies, USA. Proteins in the gel were transferred onto PDVF membrane (Hybond™ P, Amersham Pharmacia Biotech) by wet transfer system (Bio-rad, CA, USA) in 1x working Western transfer buffer (Table 2.2) and analysed by Western blotting. Briefly, membranes were blocked in 1% (v/v) blocking reagent (10% purified casein in maleic acid buffer, Roche Molecular Biochemicals, Mannheim, Germany) in TBS-T for 1 hour at room temperature. After 15 minute wash in TBS-T for 3 times, the membrane was then incubated with primary Abs (Table 2.3) at 4° C overnight. The membranes were washed for 3 times in TBS-T followed by incubation with horseradish peroxidase (HRP)-conjugated secondary IgG (Table 2.4) in TBS-T

containing 0.1% blocking reagent at room temperature for 1 hour. Another series of washes in TBS-T was performed and proteins on the membranes were detected by an ECL detection solution kit (Amersham Pharmacia Biotech) and visualised by exposure to X-ray film, developed using a CURIX[®]60 x-Ray developer.

2.3.8 Retroviral-mediated RNAi 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 into the siRNA-expressing construct. The target site for CXCR4 was 21 nucleotides at the position of 470 to 490 of human CXCR4 cDNA sequences (5' GGT GGT CTA TGT TGG CGT CTG 3'). The oligonucleotides containing specific sequences for *Renilla* luciferase were used to produce the expression vector for the negative control (5' AAA CAU GCA GAA AAU GCU G 3') (Elbashir *et al.* 2001).

To produce retroviral supernatants, 293T packaging cells were transfected with 10 µg of specific or control expression vectors, 8 µg of pVPack-VSV-G, 8 µg of pVPack-GP (Stratagene) and 60 µl of LipofectAMINE 2000 reagent (Life Technologies, Inc.) in 100 mm-tissue culture dishes in Opti-MEM medium (Life Technologies, Inc.) without FCS and antibiotics, essentially as recommended by the supplier. The medium was replaced 16 hours later, and virus-containing supernatants were harvested at 48 hours 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. MDA-MB-231 cells were plated in 60 mm-tissue culture dish at

approximately 40% confluency; 24 hours later, cell medium was removed, and 5 ml of specific or control viral supernatants were added. The supernatant was replaced by cell-growing medium after 6 hours of infection. The infected cells were then incubated for an additional 24 hours at 37 °C before being plated at 1:20 dilution for the selection of individual clones in media containing 5 ng/ml puromycin. After 1 week individual clones were picked and expanded for further analysis.

2.4 Immunoassays for Protein Detection

2.4.1 Immunostaining and Flow Cytometric Analysis

Cells were trypsinised and suspended to 5×10^6 cells/ml in staining buffer (Table 2.2). The cells were fixed with 3.7% paraformaldehyde in PBS (Table 2.2) at room temperature for 10 minutes followed by washing with PBS and resuspension in staining buffer. The cell suspension was then incubated with purified human IgG (Sigma) (10 µg per 10^6 of cells) at room temperature for 30 minutes to block Fc receptors on the cell surface. 50 µl of cell suspension containing 2.5×10^5 cells were aliquoted to each round-bottom tube and labelled with either test Abs or their isotype-matched IgG control (10 µl of 50 µg/ml of Abs) (all Abs used are listed in Tables 2.3 and 2.4) as described below. The cells were directly stained with Ab-conjugated with fluorescein isothiocyanate (FITC) at 4° C for 30 minutes. Alternatively, the cells were incubated with unlabelled primary Abs at 4° C for 30 minutes and washed with staining buffer followed by incubation with secondary Abs conjugated with either FITC or phycoerythrin (PE), at 4° C for 30 minutes. The stained cells were then washed with staining buffer followed by PBS and then analysed on a Becton Dickinson FACScan using CellQuest Pro software (BD Biosciences).

For all analyses, the 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 Abs. All 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 Abs). In each flow cytometric analysis, two parameters were determined for assessing expression of surface receptor proteins; first, the statistical percentages of positive events presenting the percentages of positive-staining cells and second, the geometric mean of fluorescence intensity (MFI) indicating the quantity of receptor expression per cell.

2.4.2 Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was conducted to detect CXCL12 in cell supernatants and lysates. Cells, 2.5×10^5 cells per well, were grown in a 24-well tissue culture plate overnight. The cells were starved in serum-free medium for 2-3 hours before being treated with 10 nM IGF-I or left untreated. At specific time points, cell supernatants were collected and mixed with a 1:200 dilution of protease inhibitor cocktail (Sigma) to prevent protein degradation. Cell lysates were prepared by adding PBS containing protease inhibitor cocktail onto a cell layer followed by 3 cycles of freezing and thawing. All cell lysates were kept in -20°C until analysed.

The wells of a 96-well ELISA plate (Costar) were coated overnight with 100 μl /well of anti-CXCL12 capture Abs (monoclonal mouse anti-human/mouse CXCL12, R&D systems, Minneapolis, MN, USA) (prepared at 2 $\mu\text{g}/\text{ml}$ in 0.1 M NaHCO_3 buffer). Wells were washed 3 times with PBS-T followed by blocking with 300 μl per well of PBS

containing 1% BSA (Sigma) and 5% sucrose for 1-2 hours. Wells were washed and 100 µl/well of standard dilutions of recombinant mouse CXCL12 (Peprotech, Rocky Hill, NJ, USA), culture supernatants and cell lysates were added and the plate was incubated for 2 hours at room temperature. Wells were washed before the addition of 100 µl of biotinylated anti-CXCL12 detection Abs (polyclonal goat anti-human CXCL12, R&D systems, Minneapolis, MN, USA) prepared at 200 ng/ml and subsequent incubation at room temperature for 2 hours. Wells were washed and streptavidin-conjugated HRP (Rockland, Gilbertsville, PA, USA) was added at 100 µl/well. The tray was incubated for 30 minutes prior to addition of o-phenylenediamine (OPD) solution, prepared according to the manufacturer's recommendation (Sigma), at 200 µl/well. The tray was then incubated in the dark for 20 minutes to develop the colour reaction before 50 µl of 3 M HCl was added to each well to stop the reaction. Absorbance was read at 490 nm on a Biotrak II Plate Reader (GE Healthcare, NSW, Australia).

2.5 Assays for Assessment of Receptor Function

2.5.1 Calcium Mobilisation

Approximately 1×10^5 cells in 1x HBSS (Table 2.2) were pre-incubated for 15 minutes with 2 µM of Fura-2AM (Molecular Probes, Eugene, OR) at 37° C followed by washing with 1x HBSS. The cells were treated with either PBS, 100 nM CXCL12 or 10 nM IGF-I and intracellular calcium related-fluorescence changes were monitored for 100 seconds on a luminescence spectrophotometer (SLM 8000-Amino-Bowman Series 2, Urbana, IL). The data was presented as a fold increase relative to PBS treated cells. To standardise results, cells were lysed with 10 µl of 10% TritonX-100 to obtain a maximum value of

free calcium, and consequently 75 μ l of 100 mM EGTA and 10 μ l of 2M NaOH were added to the cuvette to chelate free calcium ions in order to obtain a minimum value. Experimental data were calculated by the formula $Y = 224 \times (Y - Y_{\min}) / (Y_{\max} - Y)$ where $Y_{\min}$ was the value obtained from addition of NaOH and EGTA, $Y_{\max}$ was the value obtained after addition of 10% TritonX-100 and Y was the value obtained from the experimental samples. Intracellular calcium ion mobilisation was presented in nM.

2.5.2 Kinase Receptor Activation Assay (KIRA)

The KIRA assay was performed with modifications to a previously described protocol (Chen *et al.* 2003; Denley *et al.* 2004; Sadick *et al.* 1999). P6, MCF-7 and MDA-MB-231 cells (2.5×10^5 cells /well) were cultured in 24-well flat-bottom culture plates for overnight. The culture medium was replaced by serum-free medium (RPMI-1640 (GibcoBRL, Grand Island, NY, USA) with 0.5% bovine serum albumin (BSA)) for 4-5 hours before being incubated with various concentrations of stimulants, either CXCL12 or IGF-I. After 10 minutes of stimulation, cell lysates were prepared by addition of Triton-X100 lysis buffer pH 7.5 (20 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1mM EGTA, 10% glycerol and 1% Triton-X100) containing 2 mM Na_3VO_4 , 10 mM NaF and 1:100 protease inhibitor (Sigma), and then dispensed into 96 well-white polystyrene plates (Lumitrac 600, Greiner Bio-one, Frickenhausen, Germany) which were precoated with anti-IGF-1R Abs (mAb 24-31) diluted in 50 mM $NaHCO_3/Na_2CO_3$, pH 9.6 (0.25 μ g/well) and subsequently blocked with 0.5% BSA in TBS-T. After overnight incubation, the plates were washed with TBS-T and the activated receptor complex formed was detected by incubating with europium-labelled anti-phosphotyrosine PY20 (PerkinElmer, Turku, Findland) diluted in ligand binding buffer (Table 2.2) (10 ng/well) for 2 hours at room

temperature. After washing the plates 5 times with distilled water, 100 μ l of DELFIA enhancement solution (PerkinElmer, Turku, Finland) were added per well. Time-resolved fluorescence was measured using 340 nm excitation and 610 nm emission filters on a BMG Lab Technologies PolarstarTM Fluorometer.

2.5.3 Chemotaxis Assay

Chemotaxis was measured in a modified Boyden chamber using polycarbonate filters (8 μ M for MDA-MB-231 cells and 12 μ M for MCF-7 cells, Neuroprobe, Gaithersburg, MD, USA) coated with 25 μ g/ml Collagen type I (Sigma) in 10 mM acetic acid. The cells were assayed in serum-free conditions. Cell suspensions in serum-free medium (RPMI-1640) containing 0.5% BSA were preincubated with calcein-AM (1 μ g/ml of final concentration, Molecular Probes, Eugene, OR, USA) for 30 minutes before being loaded in the upper chamber ($\sim 5 \times 10^4$ viable cells/well), whereas the lower chamber contained various concentrations of stimulants (CXCL12, CCL19, CCL21 or IGF-I), diluted in serum-free medium. After the chamber was incubated in a tissue culture incubator at 37°C, for 4 hours for MDA-MB-231 cells and 6 hours for MCF-7 cells, membranes were taken out, and cells remaining on the upper surface were removed. Fluorescence intensity of the transmigrated cells on the lower surface were measured using a Molecular Imager[®] Fx (BioRad Laboratories, USA), and expressed as a migration index, representing the fluorescent signal of stimulated cells compared with that of non-stimulated cells.

2.6 Statistics

Data were analysed by unpaired student's t-test using Graphpad Prism Software (San Diego, CA, USA). In all analyses, p values less than 0.05 were considered statistically significant.

Table 2.1 General chemicals and reagents

Name	Main Suppliers or Manufacturers
Acrylamide/Bis (37.5:1) (40%)	Bio-rad, Hercules, CA, USA
Agarose powder, electrophoresis grade	Heidelberg, Germany
Ammonium persulfate (APS)	Sigma, St. Louis, MO, USA
Bovine serum albumin (BSA)	Sigma, St. Louis, MO, USA
Chloroform	BDH Chemicals, Kilsyth, Vic, Australia
Diethylenetriamine pentaacetic acid (DTPA)	Sigma, St. Louis, MO, USA
Diethylpyrocarbonate (DEPC)	Sigma, St. Louis, MO, USA
Disodium hydrogen orthophosphate (Na_2HPO_4)	BDH Chemicals, Kilsyth, Vic, Australia
DL-Dithiothreitol (DTT)	Sigma, St. Louis, MO
Ethylendiaminetetra-acetic acid (EDTA)	BDH Chemicals, Kilsyth, Vic, Australia
Ethylene glycol-bis (β -aminoethyl ether) N, N, N', N'-tetraacetic acid ($\text{C}_{14}\text{H}_{24}\text{N}_2\text{O}_{10}$) (EGTA)	Sigma, St. Louis, MO, USA
Ethanol	BDH Chemicals, Kilsyth, Vic, Australia
Ethidium bromide	Molecular Probes, Oregon, USA
Glacial acetic acid	BDH Chemicals, Kilsyth, Vic, Australia
Glycerol	Ajax Chemicals, Auburn, NSW, Australia
Glycine	Sigma, St. Louis, MO, USA
Hydrochloric acid (HCl)	BDH Chemicals, Kilsyth, Vic, Australia
HEPES ($\text{C}_8\text{H}_{18}\text{N}_2\text{O}_4\text{S}$)	BDH Chemicals, Kilsyth, Vic, Australia
Isopropanol	BDH Chemicals, Kilsyth, Vic, Australia
Magnesium Chloride (MgCl_2)	Ajax Chemicals, Auburn, NSW, Australia
N, N, N', N'-tetramethyl ethylenediamine (TEMED)	Sigma, St. Louis, MO, USA
Paraformaldehyde (PFA)	BDH Chemicals, Kilsyth, Vic, Australia
Polyoxyethylene sorbitan monolaurate (Tween-20)	Sigma, St. Louis, MO, USA
Potassium Chloride (KCl)	Ajax Chemicals, Auburn, NSW, Australia
Potassium dihydrogen orthophosphate (KH_2PO_4)	BDH Chemicals, Kilsyth, Vic, Australia
Sodium azide (NaN_3)	Ajax Chemicals, Auburn, NSW, Australia
Sodium carbonate (Na_2CO_3)	Ajax Chemicals, Auburn, NSW, Australia
Sodium chloride (NaCl)	BDH Chemicals, Kilsyth, Vic, Australia
Sodium dodecyl sulfate (SDS)	Sigma, St. Louis, MO, USA
Sodium hydrogen carbonate (NHCO_3)	Ajax Chemicals, Auburn, NSW, Australia
Sodium hydroxide (NaOH)	BDH Chemicals, Kilsyth, Vic, Australia
Tris (hydroxymethyl) aminomethane	Amresco, Solon, Ohio
Triton-X100	Sigma, St. Louis, MO, USA

Table 2.2 General solutions and buffers

Name	Content
Ammonium persulfate (APS) (10% w/v)	10% (w/v) APS (freshly prepared)
Coomassie Blue R stain solution	0.1% (w/v) Coomassie brilliant blue R, 30% (v/v) methanol and 10% (v/v) acetic acid
DEPC-treated milliQ water	0.1% v/v DEPC in MilliQ water
DNA loading buffer (6x)	30% (w/v) sucrose, (w/v) 0.35% Orange G
Electrode (Running) buffer (10x)	30.3 g/l Tris base, 144.0 g/l glycine and 10.0 g/l SDS
Hank's balanced salt solution (HBSS) (stock 10x)	80 g/l NaCl, 4 g/l KCl, 0.32 g/l Na ₂ HPO ₄ , 0.6 g/l KH ₂ PO ₄ and 10 g/L D-glucose
Hank's balanced salt solution (HBSS) (1x working buffer)	1x HBSS supplemented with 0.01 M HEPES pH 7.4 and 1.6 mM CaCl ₂ (Freshly prepared)
Ligand binding buffer	100 mM HEPES, 100 mM NaCl, 0.05% Tween20 and 2 μ M DTPA, pH8.0
Paraformaldehyde (PFA), 3.7% (w/v)	3.7% (w/v) in PBS pH 7.4 (dissolved at 55°C for approximately 30 minutes)
Phosphate buffered saline (PBS)	0.137 M NaCl, 2.7 mM KCl, 1.46 mM KH ₂ PO ₄ , 8.1 mM Na ₂ HPO ₄ , PH 7.4
PBS-T	PBS containing 0.05% (v/v)Tween20
Resolving gel (12% or 15%)	30% or 38% (v/v) of 40% Acrylamide/Bis, 25% (v/v) resolving gel buffer, 1% (v/v) of 10% SDS, 0.05% (v/v) TEMED and 0.5% of 10% APS in distilled water
Resolving gel buffer	1.5 M Tris-HCl, pH 8.8
SDS reducing (sample) buffer (1x)	50 mM Tris-HCl (pH 6.8), 50 mM DTT, 1% SDS, 0.005% bromphenol blue, 10% glycerol
Stacking gel (4%)	6% (v/v) of 40% Acrylamide/Bis, 25% (v/v) stacking gel buffer, 0.02% (v/v) 10% SDS, 0.05% (v/v) TEMED and 0.5% of 10% APS in distilled water
Stacking gel buffer	0.5 M Tris-HCl, pH 6.8

(Continued over page)

Table 2.2 General solutions and buffers (continued)

Name	Content
Staining buffer (immunostaining for FACS)	PBS containing 1% (w/v) BSA, 0.04% NaN ₃
TBS	25 mM Tris pH 7.4, 137 mM NaCl, 2.7 mM KCl
TBS-T	TBS containing 0.1% (v/v) Tween-20
Tris/acetic acid/EDTA (TAE)	40 mM Tris, 40 mM Glacial acetic acid, 1 mM EDTA (pH 8.0)
Triton-X100 lysis buffer	50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM MgCl ₂ , 1 mM EGTA, 0.5 mM EDTA, 10% glycerol, 1% TritonX-100
Western transfer buffer (10x stock)	30.3 g/L Tris base, 144.0 g/L glycine
Western transfer buffer (1x working buffer)	1x Western transfer buffer with 20% ethanol
All solutions and buffers shown in this table were made with milli-Q water and sterilised by autoclaving and filtering through 0.45 µm filters when necessary. Abbreviations: FACS; fluorensence-activated cell-sorting, KIRA; kinase receptor activation assay	

Table 2.3 Primary Abs used in flow cytometry, Immunoprecipitation and Western blot

Name	Conc/dilution	Application	Sources
FITC-conjugated anti-huCXCR4 (mu monoclonal IgG _{2a})	0.5 µg/reaction	FACS	R&D systems, Minneapolis, MN, USA
anti-huCCR7 (rat monoclonal IgG _{2a})	0.5 µg/reaction	FACS	BD Pharmingen™, San Diego, CA, USA
anti-huIGF-1R (7C2) (mu monoclonal IgG)	0.5 µg/reaction	FACS	Mehrnaz Keyhanfar, The University of Adelaide, SA, Australia
anti-IR clone 83-7 (mu monoclonal IgG)	0.5 µg/reaction	FACS	Professor Kenneth Siddle, Cambridge, UK, USA
FITC-conjugated mulgG ₁ (X63)	0.5 µg/reaction	FACS	Home-made
rat IgG (GL113)	0.5 µg/reaction	FACS	Home-made
mu IgG ₁ (X63)	0.5 µg/reaction	FACS	Home-made
anti-huCXCR4 (12G5) (mu monoclonal IgG _{2a})	1 µg/reaction 0.5 µg/reaction	IP FACS	R&D systems, Minneapolis, MN, USA
anti-huIGF-1R (24-31)(200 µg/ml) (mu monoclonal IgG)	1 µg/reaction	IP	Professor Kenneth Siddle, Cambridge, UK
anti-hemagglutinin (F-7)(200 µg/ml) (mu monoclonal IgG _{2a})	1 µg/reaction	IP	Santa-cruz, CA, USA
anti-IGF-1Rβ (C-20)(200 µg/ml) (rab polyclonal IgG)	1:500	WB	Santa-cruz, CA, USA
anti-huCXCR4 (1mg/ml) (rab polyclonal IgG)	1:1,000	WB	Chemicon, CA, USA
anti-huCCR7 (rab monoclonal IgG)	1:1,000	WB	Epitomics, CA, USA
anti-huG _i α ₂ (T-19)(200 µg/ml) (rab polyclonal IgG)	1:500	WB	Santa-cruz, CA, USA
anti-huGβ ₍₁₋₄₎ (M-14)(200 µg/ml) (rab polyclonal IgG)	1:500	WB	Santa-cruz, CA, USA
anti-β-muactin (rab polyclonal IgG)	1:5,000	WB	Sigma, St. Louis, MO, USA
Abbreviations: Conc; concentration, FITC; fluorescence isothiocyanate, hu; human, mu; mouse, rab; rabbit, IP; immunoprecipitation, FACS; fluorescence-activated cell-sorting, WB; Western blot.			

Table 2.4 Secondary Abs used in flow cytometry and Western blot

Name	Conc/dilution	Application	Sources
PE-conjugated goat anti-mouse IgG (1 mg/ml)	1:150	FACS	Rockland, Gilbertsville, PA, USA
FITC-conjugated donkey anti-rat IgG (1 mg/ml)	1:150	FACS	Jackson ImmunoResearch, PA, USA
HRP-conjugated goat anti-mouse IgG (2 mg/ml)	1: 10,000	WB	Rockland, Gilbertsville, PA, USA
HRP-conjugated donkey anti-rabbit IgG (2 mg/ml)	1: 10,000	WB	Rockland, Gilbertsville, PA, USA
Abbreviations: Conc; concentration, PE; phycoerythrin, FITC; fluorescence isothiocyanate, HRP; Horseradish peroxidase, WB; Western blot, FACS; fluorescence-activated cell-sorting			

Table 2.5 Basic reagents and medium used in cell culture

Name	Suppliers
Alpha MEM (Minimum Essential Medium)	GibcoBRL , Grand Island, NY, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, St Louis, MO, USA
Dulbecco's Modified Eagle Medium (DMEM) with 20 mM HEPES	Infectious Disease Laboratories Media Production Unit (IMVS), SA, Australia
Fetal calf serum (FCS) (heat inactivated at 56° C for 1 hour)	JRH Bioscience Ltd, Hampshire, UK
G418, 100 mg/ml	Life Technologies, Gilbertsville, PA, USA
HEPES (C ₈ H ₁₈ N ₂ O ₄ S), 1M	Infectious Disease Laboratories Media Production Unit (IMVS), SA
L-glutamine, 0.2 M	Infectious Disease Laboratories Media Production Unit (IMVS), SA, Australia
β-mercaptoethanol, 27 mM	Sigma-Aldrich, St Louis, MO, USA
Non-essential amino acid, 10 mM	Gibco-BRL, Grand Island, NY, USA
Penicillin/Gentamycin	Infectious Disease Laboratories Media Production Unit (IMVS), SA, Australia
Puromycin, 25 µg/ml	Calbiochem, San Diego, CA, USA
RPMI (Roswell Park Memorial Institute) medium 1640	Infectious Disease Laboratories Media Production Unit (IMVS), SA, Australia
Sodium pyruvate, 100 mM	Sigma-Aldrich, St.Louis, MO, USA
Trypan blue	Sigma-Aldrich, St.Louis, MO, USA
Trypsin, 1M	Infectious Disease Laboratories Media Production Unit (IMVS), SA, Australia

Table 2.6 Summary of related growth medium to cell lines and freezing medium

Cell lines	Related growth medium and freezing medium
MCF-7	DMEM with 20 mM HEPES, 1% penicillin/gentamycin and 10% FCS
R-	DMEM with 20 mM HEPES, 1% penicillin/gentamycin and 10% FCS
P6	DMEM with 20 mM HEPES, 1% penicillin/gentamycin, 250 µg/ml G418 and 10% FCS
R-IR-B	DMEM with 20 mM HEPES, 1% penicillin/gentamycin, 400 µg/ml G418 and 10% FCS
MDA-MB-231, Jurkat	RPMI 1640 with 10 nM HEPES, 1% penicillin/gentamycin, 1 mM sodium pyruvate and 10% FCS
B300-19/huCXCR4	RPMI 1640 with 10 mM HEPES, 1% penicillin/gentamycin, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM non-essential amino acid, 0.054 mM β-mercaptoethanol and 10% FCS
U937	RPMI 1640 with 1% penicillin/gentamycin and 10 % FCS
4T1.2	Alpha MEM with 1% penicillin/gentamycin and 10 % FCS
4T1.2-CXCL12	Alpha MEM with 1% penicillin/gentamycin, 6 µg/ml puromycin and 10% FCS
Freezing medium	Serum-free medium with 20% DMSO and 30% FCS

CHAPTER 3

**Characterisation of Expression and Function
of Chemokine Receptors, CXCR4 and CCR7,
and IGF-1R in Breast Cancer Cell Lines**

CHAPTER 3

Characterisation of Expression and Function of Chemokine Receptors, CXCR4 and CCR7, and IGF-1R in Breast Cancer Cell Lines

3.1 Introduction

IGF-I and more recently the chemokine, CXCL12 and CCL21, have been implicated in metastasis and invasion of breast carcinoma (Dunn *et al.* 1998; Muller *et al.* 2001; Sachdev *et al.* 2004). Accumulating data suggest that the binding of these molecules to their corresponding receptors, IGF-1R and, CXCR4 and CCR7, respectively, results in a cascade of events leading to cellular responses that are essential for the metastatic and invasive potential of breast cancer (Doerr and Jones 1996; Muller *et al.* 2001). The molecular mechanisms underpinning breast cancer metastasis and invasion by these molecules are poorly understood and must be further investigated.

In the past decades, numerous breast cancer cell lines, ranging from non-transformed, malignant to highly invasive cells, have been established and widely used as *in vitro* models for breast cancer research (Burdall *et al.* 2003). A panel of the cell lines has been characterised in terms of invasive behaviour using *in vitro* chemoinvasion and chemotaxis assays, and *in vivo* in nude mice (Burdall *et al.* 2003; Culty *et al.* 1994; Thompson *et al.* 1992). As summarised in Table 3.1, it is suggested that the ER⁺ breast cancer cell lines such as ZR-75-1, MCF-7 and T47D exhibit a non-invasive phenotype whereas the ER⁻ lines including MDA-MB-231, MDA-MB-435 and MDA-MB-436 show invasive characteristics. Our laboratory has also conducted *in vivo* assays in SCID mice to examine

the metastatic potential of such breast cancer cell lines. The cell lines were injected intravenously and observed for their ability to form metastases in the lung. Our data indicate that the non-transformed MCF10A cells, and the malignant MDA-MB-453 and MDA-MB-134 cells, are non-metastatic, whereas the MDA-MB-231 and BT-549 cells are highly metastatic in SCID mice (Holland 2007).

The overall aim of this thesis is to explore potential cross-talk between the chemokine receptors CXCR4, CCR7 and the growth factor receptor IGF-1R. However, in order to achieve this aim, a basic characterisation of these receptor systems in breast epithelial carcinoma cells was necessary. Therefore, the aim of the experiments outlined in this chapter was to characterise expression and function of CXCR4, CCR7 and IGF-1R in the breast cancer cell lines. In this research, the non-metastatic ER⁺ MCF-7 and the highly metastatic ER⁻ MDA-MB-231 cells were selected because there is a considerable difference in the degree of invasive and metastatic ability and their responsiveness to IGF-I (Bartucci *et al.* 2001; Doerr and Jones 1996). However, the response to the aforementioned chemokines and chemokine receptors has only been partially defined (Muller *et al.* 2001). In this chapter, the expression of the chemokine receptors and IGF-1R was examined by conducting flow cytometric and Western blot analysis. The functionality of the receptors was determined by testing chemotactic responses of the cells to their corresponding ligands. Receptor activation was also studied by determining the activation of signalling molecules downstream of IGF-1R, and chemokine receptors, CXCR4 and CCR7.

3.2 Results

3.2.1 Expression of the chemokine receptors, CXCR4 and CCR7, and the growth factor receptors, IGF-1R and IR, by MCF-7 and MDA-MB-231 cells

The expression of the chemokine receptors, CXCR4 and CCR7, and the growth factor receptors, IGF-1R and IR, was examined in the non-metastatic MCF-7 and highly metastatic MDA-MB-231 lines. The cells were labelled with Abs specific for each receptor or an isotype-matched control IgG and analysed by fluorescent-activated cell sorting (FACS) for receptor surface expression. The cell lines B300-19/huCXCR4, U937, P6 and R⁺IR-B were used as positive control cell lines expressing CXCR4, CCR7, IGF-1R and IR respectively. As shown in figures 3.1 and 3.2, and Table 3.2, the data indicate that both MCF-7 and MDA-MB-231 cells expressed high levels of CXCR4 (93.72 $\pm$ 4.24 % of positive cells with a geometric mean of 69.14 $\pm$ 18.65 for MCF-7 cells and 95.43 $\pm$ 1.16 % of positive cells with a geometric mean of 83.44 $\pm$ 17.43 for MDA-MB-231 cells). The two cell lines also expressed CCR7, which was found to be markedly higher in MCF-7 cells than in MDA-MB-231 cells (61.37 $\pm$ 15.98 % of positive cells with a geometric mean of 92.78 $\pm$ 51.47 for MCF-7 cells and 47.62 $\pm$ 9.66 % of positive cells with a geometric mean of 84.73 $\pm$ 18.16 for MDA-MB-231 cells). MCF-7 cells also expressed IGF-1R at a high level (98.60 $\pm$ 20.62 % of positive cells with a geometric mean of 25.44 $\pm$ 8.11) whereas MDA-MB-231 cells expressed lower levels of IGF-1R (24.49 $\pm$ 10.51 % of positive cells with a geometric mean of 14.06 $\pm$ 3.54). In addition, both cell lines expressed low levels of IR (11.46 $\pm$ 3.18 % of positive cells with a geometric mean of 10.1 $\pm$ 0.36 for MCF-7 and 5.37 $\pm$ 1.28 % of positive cells with a geometric mean of 14.42 $\pm$ 0.11 for MDA-MB-231 cells).

The cellular content of CXCR4, CCR7, and IGF-1R was also analysed in lysates from MCF-7 and MDA-MB-231 cells. Because of the low level of expression of IR, no further experimentation was conducted on this receptor. The lysates, containing equal quantities of total protein, were subjected to Western blot using Abs specific to CXCR4, CCR7, and IGF-1R. In agreement with the data from flow cytometric analysis, both cell lines expressed CXCR4, CCR7 and IGF-1R as demonstrated in figure 3.3A. The presence of CXCR4 and CCR7 was also observed in the positive control lysates prepared from human Jurkat cells (T cell leukaemia) and myelomonocytic U937 cell line respectively, which have been shown to express these receptors in other studies (Humrich *et al.* 2006; Ott *et al.* 2005; Sotsios *et al.* 1999) while IGF-1R was detectable in the lysate from the mouse fibroblast P6 line overexpressing huIGF-1R but not in that from a cell line lacking IGF-1R (R⁻) expression (Pietrzkowski *et al.* 1992; Sell *et al.* 1993).

In addition, because they have been previously implicated in signal transduction involving chemokine receptors, and to a lesser extent, by IGF-1R, the expression of the G-protein subunits, G_iα₂ and Gβ was examined in MCF-7 and MDA-MB-231 cells. Cell lysates from the two breast cancer cells, each of which contained an equal amount of total protein, were analysed in Western blot assays. As demonstrated in figure 3.3B, the level of G_iα₂ in the lysates of MCF-7 cells was markedly lower than that of MDA-MB-231 cells whereas the levels of Gβ were similar in both cell lines. The expression of both G-protein subunits was also observed in the positive control cell lysates from a Jurkat cell line as previously demonstrated in other studies (Lippert *et al.* 2000; N'Diaye and Brown 2003). Western blotting for β-actin in lysates of Jurkat, MCF-7 and MDA-MB-231 cells demonstrated equal protein loading.

3.2.2 The chemotactic responses of MCF-7 and MDA-MB-231 cells to chemokines CXCL12, CCL19 and CCL21, and to IGF-I

To determine the functionality of chemokine receptors and IGF-1R in MCF-7 and MDA-MB-231 cells, the cells were tested for their ability to migrate in response to their ligands, CXCL12 (for CXCR4), CCL19 and CCL21 (for CCR7), and IGF-I (for IGF-1R) by performing a modified Boyden chamber chemotaxis assay. Even though the expression of CXCR4 and CCR7 was similar on MDA-MB-231 and MCF-7 cells, only the former cells responded to the ligands for those receptors (Figure 3.4 A-D). The optimal migration of MDA-MB-231 cells in response to CXCL12, CCL19 and CCL21 was 100 nM, 10 nM and 50 nM respectively. In contrast, both cell lines migrated in response to IGF-I, at the optimal concentration of 10 nM for both MCF-7 and MDA-MB-231 cells. However, in keeping with the lower level of IGF-1R expression, the degree of IGF-I-mediated chemotaxis in MDA-MB-231 was lower than that observed in MCF-7 cells at all concentrations of IGF-I tested (Figure 3.4 E-F). Additional experiments were conducted in MDA-MB-231 cells to determine the effect of stimulation with combined CXCL12 and IGF-I. The results of these experiments indicated an additive effect of those ligands on chemotaxis of MDA-MB-231 cells (Figure 3.5).

3.2.3 Activation of the IGF-1R complex in response to IGF-I in MCF-7 and MDA-MB-231 cells

It has been demonstrated in several cellular systems that the binding of IGF-I by IGF-1R results in the formation of a tyrosine-phosphorylated IGF-1R complex, an early event in IGF-1R-mediated signalling transduction (Chen *et al.* 2003; Denley *et al.* 2004; Sadick *et*

al. 1999). The activity of IGF-1R in MCF-7 and MDA-MB-231 cells was therefore determined by performing a kinase receptor activation assay (KIRA) to detect the level of tyrosine-phosphorylated IGF-1R complex formed following the activation by different concentrations of IGF-I, 1, 10 and 100 nM. The previous studie (Denley *et al.* 2004) and preliminary experiments performed in the current study indicate maximum levels of phosphorylated IGF-1R complex present at 10 mins after 10 nM IGF-I stimulation in the fibroblast P6 cells overexpressing huIGF-1R (data not shown). Therefore, in subsequent experiments, the breast cancer cell lines were stimulated with different concentrations of IGF-I for 10 mins. As shown in Figure 3.6, the formation of activated IGF-1R complex in response to IGF-I stimulation was demonstrated in P6, MCF-7 and MDA-MB-231 cells. However, the levels of activated IGF-1R complex formed in MDA-MB-231 were lower than those in MCF-7 cells, correlating with the lower levels of IGF-1R expression observed in the flow cytometric and Western blot analysis.

3.2.4 The effect of pertussis toxin on chemotaxis of MDA-MB-231 cells in response to CXCL12, CCL19 and CCL21

As described in section 3.2.2, only MDA-MB-231 cells, chemotactically responded to the chemokines CXCL12, CCL19 and CCL21. The activation of the chemokine receptors was therefore further analysed in this cell line by determining the involvement of G-protein activity in their chemotactic responses to CXCL12, CCL19 and CCL21. The cells were pretreated with pertussis toxin (PTX), a specific inhibitor of G_iα subunits, and then tested for their ability to migrate toward various concentrations of each chemokine. Figure 3.7A demonstrates that PTX (used at 10, 100 and 1,000 ng/ml), completely blocked the chemotactic response of the cells to CXCL12 at all concentrations tested. Similar

experiments were conducted to test the chemotactic responses to CCL19 and CCL21 using PTX at a concentration of 100 ng/ml. Figure 3.7B shows that the pre-treatment of PTX resulted in the complete inhibition of chemotaxis in response to CCL19 and CCL21 in MDA-MB-231 cells. These data indicate that $G_i\alpha$ activity is required for the chemotactic responses of MDA-MB-231 cells to the chemokines CXCL12, CCL19 and CCL21.

3.2.5 CXCR4 and G-proteins are constitutively associated in both MCF-7 and MDA-MB-231 cells, however uncoupling of G-proteins from CXCR4 in response to CXCL12 only occurs in MDA-MB-231 cells.

Significant previous data indicate that the interaction between activated GPCRs including chemokine receptors, and G-proteins is a key event in signal transduction (Hein *et al.* 2005; Thelen 2001). In the present study, co-immunoprecipitation and Western blot assays were performed to investigate the association between chemokine receptors, initially focusing on CXCR4 and the G-protein subunits, $G_i\alpha_2$ and $G\beta$, in MCF-7 and MDA-MB-231 cells (Figure 3.8). Cell lysates (with equal quantities of total protein) were subjected to immunoprecipitation with anti-CXCR4 or control mouse IgG, followed by sequential Western blot using anti-CXCR4, anti- $G_i\alpha_2$ and anti- $G\beta$. Control IgG failed to precipitate CXCR4, $G_i\alpha_2$ and $G\beta$. In contrast, the immunoprecipitation with anti-CXCR4 led to coprecipitation of the three proteins in both MCF-7 and MDA-MB-231 cells, indicating the coupling of both G-protein subunits to CXCR4 receptors in both cell lines. Of note, as shown in Figure 3.8, the levels of $G_i\alpha_2$ in immunoprecipitates of CXCR4 receptor are markedly higher in MDA-MB-231 cells than in MCF-7 cells, consistent with the expression levels detected in whole lysates of both cell lines (Figures 3.8 and 3.3B). The

levels of G β in the immunocomplex of CXCR4 are also markedly higher in MDA-MB-231 than in MCF-7 cells even though the expression levels present in the whole lysates from the two cells are similar (Figures 3.8 and 3.3B). This observation suggests that the binding of G β to the receptor may be dependent on the presence of G α in the CXCR4 receptor complex.

The kinetics of G-protein uncoupling from CXCR4 were studied by conducting co-immunoprecipitations and Western blots to observe the binding of G-proteins to the receptor following stimulation of the cells with 100 nM CXCL12. Cell lysates prepared from both MCF-7 and MDA-MB-231 cells stimulated with CXCL12 for different periods of time were subjected to immunoprecipitation with specific Abs to CXCR4 and Western blots to detect the presence of G α_2 and G β . The results of these experiments indicate the uncoupling of G α_2 and G β from CXCR4 at 15 and 30 seconds after CXCL12 stimulation in MDA-MB-231 cells (Figure 3.9A). In contrast, no dissociation of G α_2 and G β from CXCR4 was observed in MCF-7 cells under similar conditions (Figure 3.9B). These data indicate that CXCL12-mediated G-protein release from CXCR4 receptors occurred only in the highly metastatic MDA-MB-231 not in the non-metastatic MCF-7 cells.

Similar experiments were conducted to examine the association of G α_2 and G β with CCR7 as well as the kinetics of the G-protein uncoupling from the receptor. However, due to technical difficulties, mainly variability with respect to anti-CCR7 antibodies, this issue could not be clarified at this point of time.

3.3 Discussion and Conclusion

In experiments described in this chapter, analysis of expression and function of chemokine receptors and IGF-1R in breast cancer cells was addressed in order to characterise the cell lines for investigation into potential cross-talk between the two types of receptors. The studies in this part of the thesis documenting these basic characteristics of the well established non-metastatic MCF-7 and highly metastatic MDA-MB-231 cells have provided a solid basis for further experimentation. The data shown here also support the important roles of these receptors in migration of breast cancer cells, which is mandatory for metastatic and invasive behaviour of breast cancer cells.

Accumulating studies have demonstrated the expression of IGF-1R and its potential roles in the metastasis and invasion of breast cancer (Bartucci *et al.* 2001; Dunn *et al.* 1998; Sachdev *et al.* 2004). As outlined in section 1.3.2.2, it is well documented that the binding of IGF-1R by IGF-I result in autophosphorylation of tyrosine residues on the receptor that recruits several adaptor proteins such as IRS and RACK to the receptor. Subsequently, this results in the activation of downstream signal transduction systems, leading to several cellular responses including cell migration (O'Connor 2003; Rubin *et al.* 1983; Sasaki *et al.* 1985). In the current study, as demonstrated by flow cytometric and Western blot analysis, both MCF-7 and MDA-MB-231 cells exhibit a significant level of IGF-1R expression. The activity of IGF-1R was demonstrated by detecting the formation of tyrosine-phosphorylated IGF-1R complex using a KIRA assay and the ability of the cells to migrate in response to IGF-I in a chemotaxis assay. The data indicate that upon the stimulation with IGF-I, the tyrosine-phosphorylated IGF-1R complex is formed, resulting in chemotactic responses in both cell types. This supports the existence of active IGF-1R-

mediated signalling leading to cell migration in these cells. The capability of IGF-I to induce migrational signal transduction via IGF-1R is supported by prior evidence in several cellular systems indicating that specific antibodies to IGF-1R such as α -IR3, 7C2 and 9E11 competitively inhibit the ability of IGF-I to induce cell migration (Doerr and Jones 1996; Keyhanfar *et al.* 2007; Puglianiello *et al.* 2000). Blockade of IGF-1R signalling by dominant-negative expression of C-terminally-truncated IGF-1R also causes inhibition of IGF-I-mediated cell motility in MDA-MB-435 cells (Sachdev *et al.* 2004). This observation is also correlated well with a previous report, indicating that, even though IGF-I has proliferative and anti-apoptotic effects on MCF-7, but not on MDA-MB-231 cells, it shows the ability to induce migrational responses in both cell lines (Bartucci *et al.* 2001).

Although IGF-I mediates biological activities mainly through the tyrosine kinase of IGF-1R, the insulin receptor (IR), which shares a high level of structural homology with IGF-1R, is also able to mediate signal transduction which is found to be similar to IGF-1R-mediated signalling systems (Denley *et al.* 2005; Kim and Accili 2002; Morgan *et al.* 1987; Rubin *et al.* 1983). Several lines of evidence indicate that, despite the similarity in their structure and biological effects in cultured cells, IR and IGF-1R exhibit distinct physiological outcomes. IR regulates metabolic responses such as glucose uptake into muscle and fat cells whereas IGF-1R appears to function primarily as a growth mediator (Fujita-Yamaguchi *et al.* 1986; Kim and Accili 2002; Steele-Perkins *et al.* 1988; Ullrich *et al.* 1986). It is suggested that the physiological effects of both receptors can be modulated by many factors including, but not limited to, the number of receptors on cell surface and tissue distribution (De Meyts *et al.* 1995; Kim and Accili 2002). In view of the potential interaction between IGF-I and both IGF-1R and IR, an analysis of IR expression in MCF-

7 and MDA-MB-231 cells was undertaken in this study. The data presented in this study indicate that both MCF-7 and MDA-MB-231 cells express IR at a very low level. The low level of IR observed in both cells, together with the fact that IGF-I only binds to IR at a very low affinity (Denley *et al.* 2005), led to the conclusion that it was unlikely that IR played a significant role in the response of the cell lines to IGF-I. Therefore, no further functional analysis of IR was conducted in this study.

Several lines of evidence have indicated a potential correlation between the low expression levels of IGF-1R as well as its major downstream signalling protein, IRS, and the progression to metastatic and invasive stage of breast carcinoma (Nakamura *et al.* 2004; Schnarr *et al.* 2000; Surmacz 2000). Of interest, the quantitative analysis of IGF-1R expression performed in this study indicates that the level of IGF-1R expression in MDA-MB-231 cells was significantly lower than that in MCF-7 cells. This leads to the lower levels of formation of the tyrosine-phosphorylated IGF-1R complex and of chemotactic response, following the stimulation of IGF-I, in MDA-MB-231 cells. These data support several previous studies indicating high levels of IGF-1R expression in ER⁺ breast cancer cells and lower expression levels of the receptor in the more aggressive ER⁻ cells (Bartucci *et al.* 2001; Guvakova and Surmacz 1997; Sepp-Lorenzino *et al.* 1994). A recent study has also demonstrated that the reduction of IGF-1R expression in MCF-7 cells results in a more metastatic phenotype of those cells (Pennisi *et al.* 2002). Nonetheless, at this point of time, the significance of this observation in breast cancer is not yet certain.

Due to the recent discovery of involvement of the chemokine receptor, CXCR4 and to a lesser extent, CCR7, in the progression of breast cancer metastasis, subsequent studies have begun to investigate the expression and functional aspects of the receptors to

determine their roles in the metastatic and invasive potential of breast cancer cells (Holland *et al.* 2006; Lee *et al.* 2004; Muller *et al.* 2001; Tanaka *et al.* 2005). Consistent with a previous report demonstrating the expression of CXCR4 and CCR7 mRNA in a range of breast cancer cell lines and primary tumours (Muller *et al.* 2001), as clearly demonstrated by flow cytometry and Western blot in this study, both MCF-7 and MDA-MB-231 cells expressed both CXCR4 and CCR7 at a high level. Several previous studies have documented the expression of CXCR4 and its function (assessed by the induction of calcium mobilisation and chemotaxis of the cells in response to CXCL12) in certain invasive breast cancer cell lines including MDA-MB-231, MDA-MB-361 and DU4475 cells (Lee *et al.* 2004; Muller *et al.* 2001). In the present study, in spite of the expression of CXCR4 and CCR7 being observed in both MDA-MB-231 and MCF-7 cells, the chemotactic response respectively to the chemokines, CXCL12, CCL19 and CCL21, was found only in the former cells. The data clearly indicates the functionality of CXCR4 and CCR7 in MDA-MB-231, but not MCF-7 cells. This finding is strongly supported by a recent study conducted in our laboratory indicating the existence of functionally active CXCR4 in a range of the highly invasive but not in non-metastatic breast cancer cell lines (Holland *et al.* 2006). That study demonstrated that, upon the stimulation with CXCL12, chemotaxis and intracellular responses including calcium mobilisation, actin polymerisation and phosphorylation of a range of signalling molecules were detected only in the invasive breast cancer cell lines. Together with the observations from the current study, this strongly supports the notion that functional activation of CXCR4 is associated with the metastatic potential of breast cancer cells, even though the molecular mechanisms for this evidence are still to be fully elucidated.

As is the case with other GPCRs, the heterotrimeric G-proteins, G_{α} and the $G\beta\gamma$ complex, are well-known as key regulators of chemokine receptor-mediated signal transduction (Mellado *et al.* 2001; Thelen 2001). In order to define the signalling downstream of CXCR4 and CCR7 in MDA-MB-231 cells, the present study focused on the activation of G-proteins following the ligation of the chemokine receptors by their ligands. It was demonstrated that pretreatment with PTX, a specific inhibitor of $G_i\alpha$ subunits, completely inhibited chemotaxis induced by the chemokines, CXCL12, CCL19 and CCL21. This evidence is absolutely consistent with a number of reports with respect to various chemokine receptors in leukocytes (Damaj *et al.* 1996; Frade *et al.* 1997; Murphy 1994; Vila-Coro *et al.* 1999). Those reports, together with the data shown here, support the essential role of the activity of PTX-sensitive G-proteins, $G_i\alpha$, in chemotaxis induced by chemokines in breast epithelial carcinoma cells. As mentioned in section 1.3.3.2, the classical view is that chemokine receptor-mediated signalling requires a dissociation of GTP- $G_i\alpha$ and the $G\beta\gamma$ complex from the activated receptors (Mellado *et al.* 2001; Thelen 2001). Although a direct role of $G_i\alpha$ in cell migration is still uncertain, the activation of $G_i\alpha$ is required for the sequestration of the $G\beta\gamma$ complex from the activated receptors. This has been shown to be an essential step for the activation of the migrational signalling pathways by chemokine receptors (Arai *et al.* 1997; Neptune and Bourne 1997; Neptune *et al.* 1999). Accumulating studies with respect to different chemokine receptors in leukocytes indicate that a physical association of $G_i\alpha$ with the receptors is necessary for the induction of chemotaxis by chemokines (Damaj *et al.* 1996; Mellado *et al.* 1998; Rodriguez-Frade *et al.* 1999; Vila-Coro *et al.* 1999). Those studies demonstrated that, in various types of leukocytes, $G_i\alpha$ does not precouple to chemokine receptors and the ligation of the receptors results in tyrosine phosphorylation of the receptors followed by

the rapid coupling of $G_i\alpha$ to the activated receptors. Nonetheless, the results of experiments investigating G-protein and chemokine receptor interactions in breast cancer cells shown in this study indicate a significant difference in this aspect. Co-immunoprecipitation studies revealed that, unlike the case in leukocytes in the aforementioned studies, $G_i\alpha_2$ and $G\beta$ subunits are constitutively associated with CXCR4 in both MCF-7 and MDA-MB-231 cells. Furthermore, the data from these experiments clearly show that stimulation of MDA-MB-231 cells with CXCL12 led to uncoupling of $G_i\alpha_2$ and $G\beta$ from CXCR4, a finding that is consistent with the chemotactic response of the cells to CXCL12. It is also noteworthy that uncoupling of $G_i\alpha_2$ and $G\beta$ from CXCR4 did not occur in MCF-7 cells which were refractory to chemotaxis in response to CXCL12.

The distinction between MCF-7 and MDA-MB-231 cells with respect to CXCR4 function is potentially of significance. Despite CXCR4 and CCR7 expression being observed in MCF-7 cells, the receptors are not functionally active as clearly demonstrated by a lack of chemotaxis and no uncoupling of $G_i\alpha_2$ and $G\beta$ from CXCR4 in response to CCL19 and CCL21. Although the discrepancy between expression and function of chemokine receptors is uncommon in the literature, it has been previously observed with respect to CXCR4 in the human hepatocyte cell line HepG2 (Mitra *et al.* 2001), and other chemokine receptors in a range of cell types (Bajetto *et al.* 1999; Johnston *et al.* 2003; Liao *et al.* 1999; Trentin *et al.* 2004). The molecular basis for this non-functional phenotype, at least with respect to cell migration, was not determined in those studies. However, based on the analysis of G-protein expression and kinetics of G-protein binding to CXCR4 shown in this study, at least two possible explanations are proposed. First,

MCF-7 cells exhibit the relatively lower level of $G_i\alpha_2$ expression. This affects the levels of both $G_i\alpha_2$ and $G\beta$ constitutively bound to CXCR4. As clearly demonstrated, the levels of both $G_i\alpha_2$ and $G\beta$ in CXCR4 immunoprecipitates are markedly lower in MCF-7 cells. Second, following the ligation of CXCR4 by CXCL12, $G_i\alpha_2$ and $G\beta$ are unable to dissociate from the activated CXCR4, in this cell line. In addition to the observations in this study, a report by Holland *et al* has demonstrated that, in the non-metastatic breast cancer cells, $G_i\alpha_2$ and $G\beta$ fail to form a functional heterotrimeric complex which is suggested to be important for mediating the signalling by GPCRs (Holland *et al.* 2006; Rahmatullah and Robishaw 1994). Either or both of these mechanisms may lead to less optimal signal transduction and consequently no chemotaxis by the chemokines in MCF-7 cells.

In summary, the studies in this chapter have provided evidence for the differential expression and function of the chemokine receptors, CXCR4 and CCR7, and IGF-1R in the non-metastatic MCF-7 and highly metastatic MDA-MB-231 cells. As discussed above, both cell lines express chemokine receptors, CXCR4 and CCR7, and IGF-1R. However, the MDA-MB-231 cells exhibit the significantly lower level of IGF-1R expression and function and, despite the expression of CXCR4 and CCR7 observed in both cells, these receptors are only functionally active in MDA-MB-231 cells. Notably, both receptor types are associated with the migration of breast cancer cells and the stimulation of combined CXCL12 and IGF-1 lead to an additive migrational effect in the metastatic MDA-MB-231 cells. These data support the hypothesis that the cellular phenotypes found only in the highly metastatic breast cancer cells such as MDA-MB-231 cells are potentially obligatory for an establishment of metastatic and invasive ability of breast cancer cells, although this suggestion still requires much more clarification. In

addition, the data also validate the two cell lines for an investigation of migrational signal transduction downstream of the chemokine receptors and IGF-1R, particularly the cross-talk between the two activation systems.

Table 3.1 Invasive characteristic of human breast cancer cell lines ⁽¹⁾

Cell lines	Tissue source	ER ⁽²⁾	Invasion ⁽³⁾	Chemotaxis ⁽⁴⁾	Nude mouse ⁽⁵⁾
ZR-75-1	Ascites	+	+	+	P (0/10/10)
MCF-7	Pleural effusion	+	++	++	P (0/20/20)
T47D	Pleural effusion	+	+	+	N
SK-Br-3	Pleural effusion	-	+	+	N
MDA-MB-231	Pleural effusion	-	+++++	+++++	LI (9/16/16)
MDA-MB-435	Pleural effusion	-	+++	++	LI (10/20/20)
MDA-MB-436	Pleural effusion	-	+++	++++	LI (4/6/10)
MDA-MB-468	Pleural effusion	-	+	+++	P (0/3/10)
BT549	Primary	-	+++++	+++++	N
MCF-7ADR	MCF-7	-	+++	+++	P (0/9/11)
⁽¹⁾ The various characteristics of human breast cancer cell lines described in this table are from (Thompson <i>et al.</i> 1992).					
⁽²⁾ The absence or presence of estrogen receptor are indicated by – and + respectively.					
^{(3) (4)} The invasion and chemotaxis assays were determined by assessing the migrational ability of cells in Boyden chamber assays using fibroblast conditioned medium as a chemoattractant. To study invasiveness of cells, the cells were assessed for their ability to penetrate a uniform matrigel barrier in order to transverse a porous polycarbonate filter in response to a chemotactic gradient. To determine the directed migration of cells, the filters were coated with a thin layer of collagen IV that promotes cell attachment and allows the free migration of the cells toward the gradient of conditioned medium. Activity in the Boyden chamber chemoinvasion assay and in Boyden chamber chemotaxis toward fibroblast-conditioned medium, graded as % of the MDA-MB-231: +, 0-20%, ++, 20-40%; +++, 40-60%; +++++, 60-80%; ++++++, >80%.					
⁽⁵⁾ Activity in the athymic nude mouse (NCr nu/nu): N, non-tumourigenic; P, primary tumour formation, no local invasiveness or metastasis; LI, Local invasion through the peritoneum, colonisation of visceral organs. Numbers in parentheses indicate number of animals with LI/ number of tumours/number of injection sites.					

Table 3.2 Flow cytometric analysis of chemokine receptors and growth factor receptors on MCF-7 and MDA-MB-231 breast cancer cells ^(e)

Cell lines	% Positive Cell	Geometric mean
CXCR4 Expression		
B300-19/huCXCR4 ^(a)	43.45±24.11	36.46±7.10
MCF-7	93.72±4.24	69.14±18.65
MDA-MB-231	95.43±1.16	83.44±17.43
CCR7 Expression		
U937 ^(b)	36.67±15.7	90.65±49.3
MCF-7	61.37±15.98	92.78±51.47
MDA-MB-231	47.62±9.66	84.73±18.16
IGF-1R Expression		
P6 ^(c)	98.59±1.05	166.99±32.19
R-IR-B ^(d)	1.45±0.42	16.95±4.61
MCF-7	98.6±20.62	25.44±8.11
MDA-MB-231	24.49±10.51	14.06±3.54
IR Expression		
P6	1.45±0.57	14.18±4.80
R-IR-B ^(d)	97.5±0.44	47.3±22.67
MCF-7	11.46±3.18	10.1±0.36
MDA-MB-231	5.37±1.28	14.42±0.11
^(a) B300-19/huCXCR4 positive control cell line for huCXCR4 ^(b) U937 positive control cell line for huCCR7 (Humrich <i>et al.</i> 2006; Ott <i>et al.</i> 2005) ^(c) P6 positive control cell line for huIGF-1R (Pietrkowski <i>et al.</i> 1992) ^(d) R-IR-B positive control cell line for human insulin receptor B (Denley <i>et al.</i> 2004) ^(e) Data are pooled from at least three separate experiments and presented as mean ± SD of % positive cells and geometric mean.		

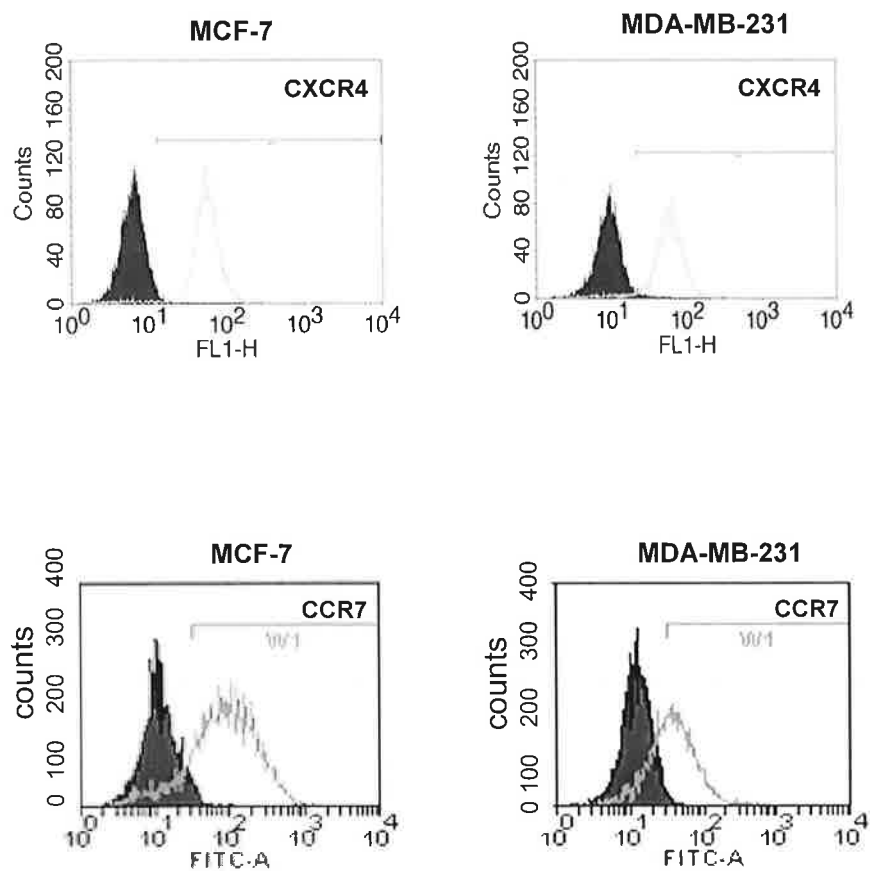


Figure 3.1 Expression of chemokine receptors, CXCR4 and CCR7, on MCF-7 and MDA-MB-231 breast cancer cells

The level of expression of the receptors was assessed by flow cytometry using specific Abs to CXCR4 (FITC-conjugated monoclonal anti-CXCR4) and CCR7 (rat monoclonal anti-CCR7). The histogram of either CXCR4 or CCR7 expression on MCF-7 and MDA-MB-231 cells is representative of three others performed with similar results. The filled and blank histograms are the isotype control Ab and CXCR4 or CCR7 Ab staining, respectively.

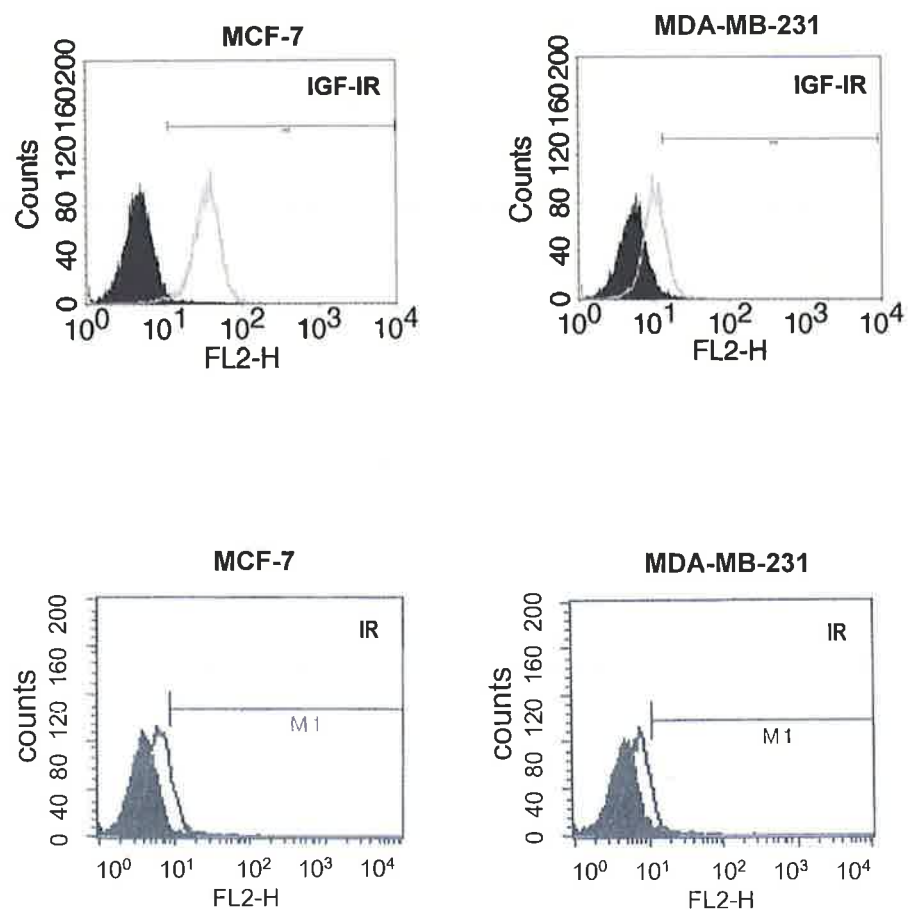


Figure 3.2 Expression of growth factor receptors, IGF-1R and IR, on MCF-7 and MDA-MB-231 breast cancer cells

The level of IGF-1R and IR was assessed by flow cytometry using specific Abs to IGF-1R (hybridoma supernatant clone 7C2) and IR (anti-IR clone 83-7). The histogram of either IGF-1R or IR expression on MCF-7 and MDA-MB-231 cells is representative of three others performed with similar results. The filled and blank histograms are the isotype control Ab and IGF-1R or IR Ab staining respectively.

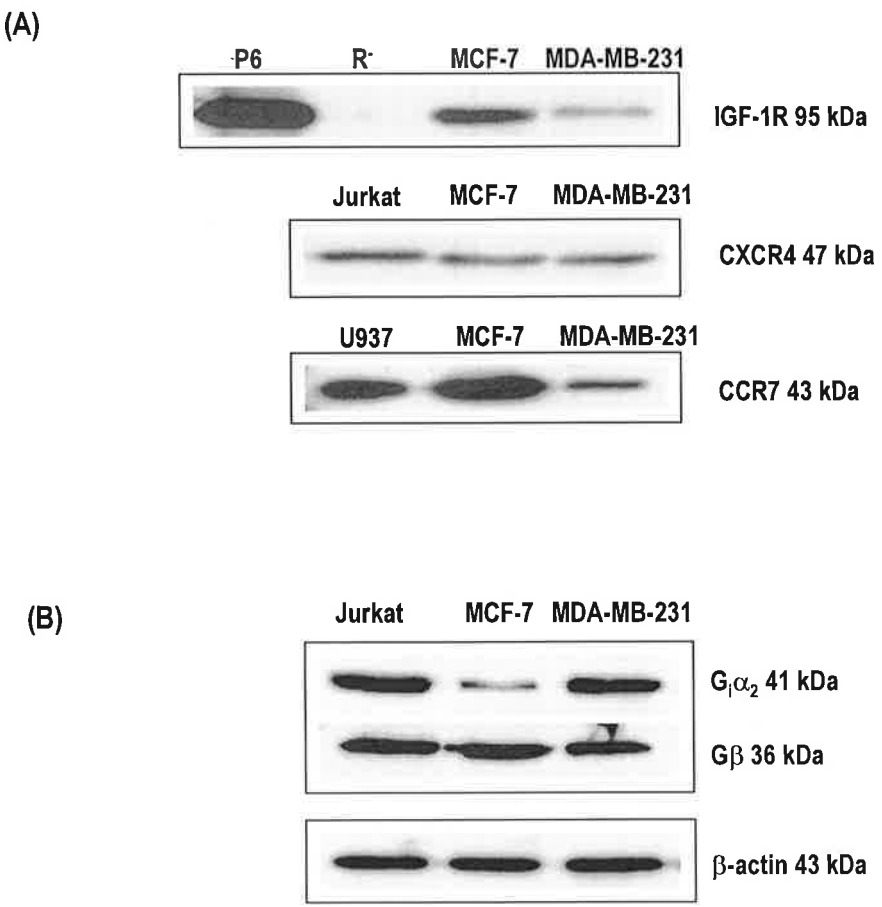


Figure 3.3 Analysis of expression of growth factor and chemokine receptors as well as G-protein subunits in MCF-7 and MDA-MB-231 cells by Western blot analysis

Lysates from MCF-7 and MDA-MB-231 cells were subjected to SDS-PAGE and Western blot to detect IGF-1R, CXCR4, CCR7 as well as the G-protein subunits, G_iα₂ and Gβ, using specific Abs. Total protein in lysates was measured by BCA assay and 50 μg of total protein was subjected to analysis. The level of expression of IGF-1R, CXCR4 and CCR7 in the two breast cancer cells is shown in (A) whereas that of G_iα₂ and Gβ is shown in (B). The mouse fibroblast P6 line is a positive cell line expressing IGF-1R whereas the R⁻ line is a cell line lacking IGF-1R expression (negative control). The Jurkat cell line is known to express CXCR4 as well as G_iα₂ and Gβγ while U937 cells have previously been shown to express CCR7 by flow cytometry. The level of β-actin in all lysates was used as a loading control. These data are representative of at least three separate experiments performed with similar results.

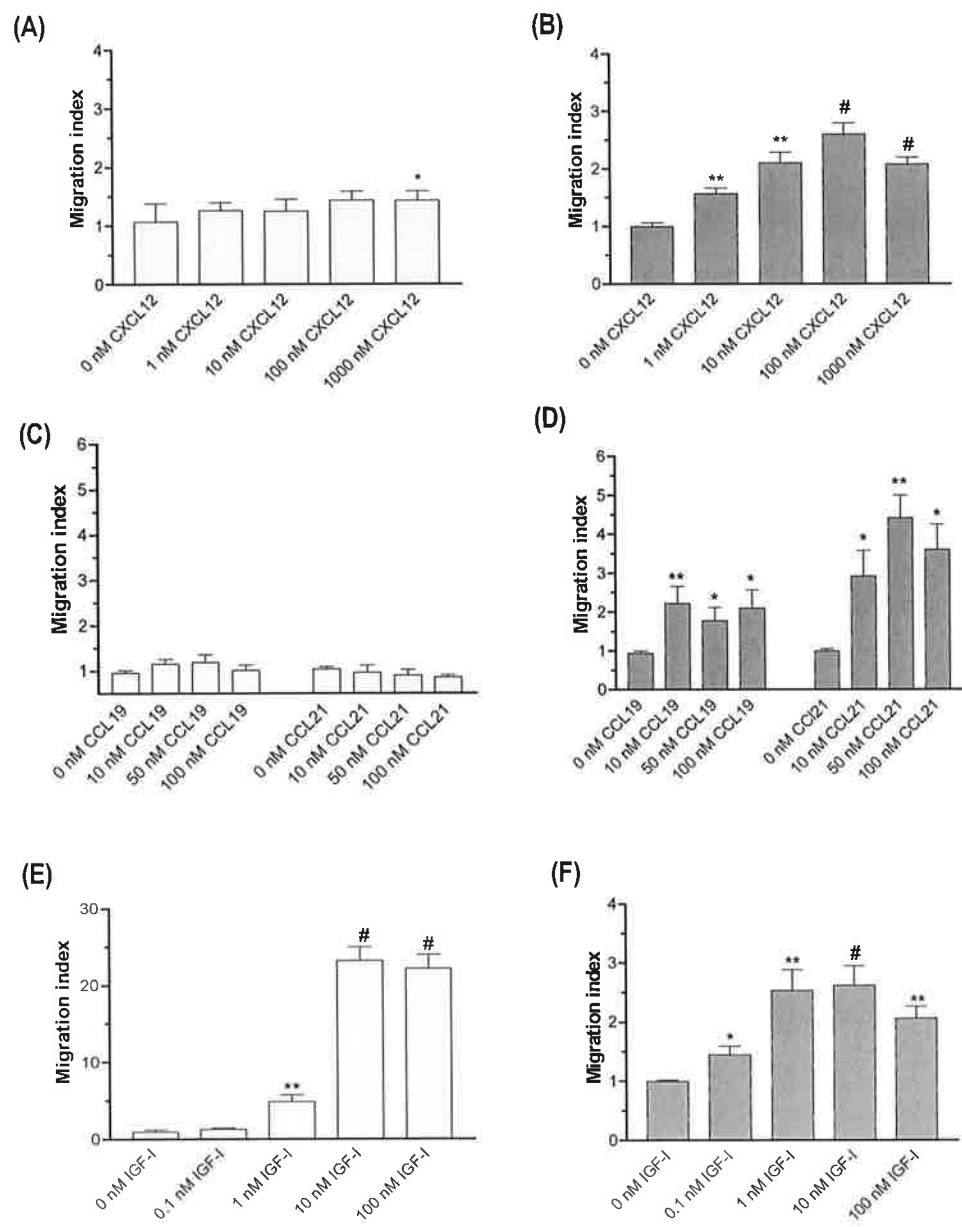


Figure 3.4 The chemotactic response of the breast cancer cell lines, MCF-7 and MDA-MB-231, to chemokines and growth factors

The ability of MCF-7 and MDA-MB-231 cells to migrate in response to different concentrations of ligands for CXCR4 (CXCL12), CCR7 (CCL19 and CCL21) and IGF-1R (IGF-I) was tested using a modified Boyden chamber assay. A, C and E demonstrate chemotactic response to CXCL12, CCL19 and CCL21, and IGF-I respectively in MCF-7 cells whereas B, D and F show those in MDA-MB-231 cells. The migration index represents the fluorescent signals of stimulated cells compared with those of unstimulated cells. All panels are expressed as mean $\pm$ S.E.M. of migration index from at least three separate experiments each performed in triplicate. Asterisks indicate values statistically different from control values (student's unpaired t test) at *, $p < 0.05$; **, $p < 0.005$; #, $p < 0.0001$.

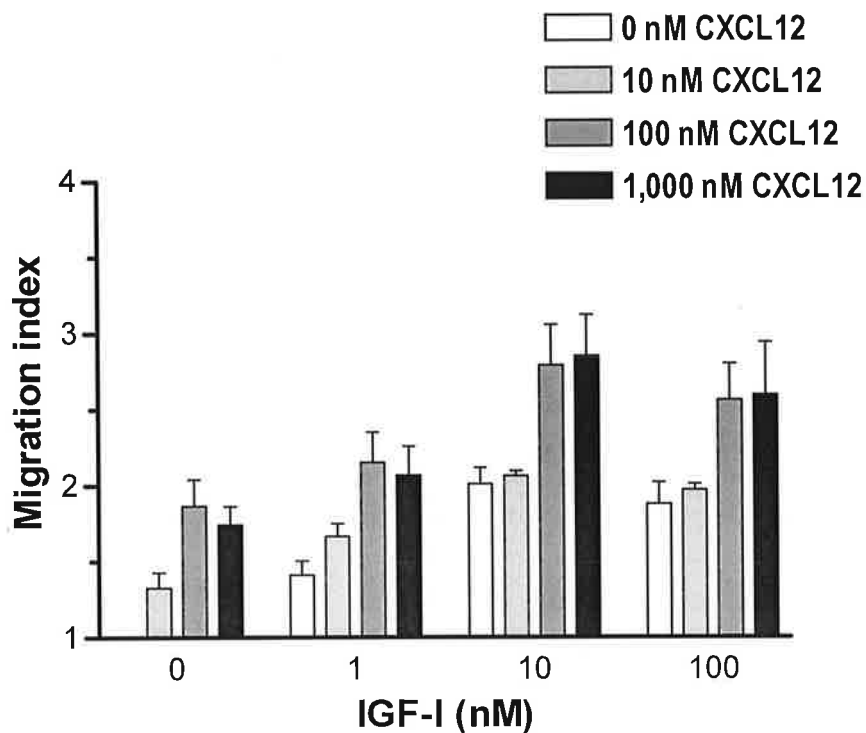


Figure 3.5 *An additive chemotactic response of MDA-MB-231 cells to a combination of CXCL12 and IGF-I*

The cells were assessed for their chemotactic ability in response to a mixture of CXCL12 and IGF-I using the modified Boyden chamber assay. The migration index represents the fluorescent signals of stimulated cells compared with those of unstimulated cells. All panels are expressed as mean $\pm$ S.E.M. of migration index from at least three separate experiments each performed in triplicate.

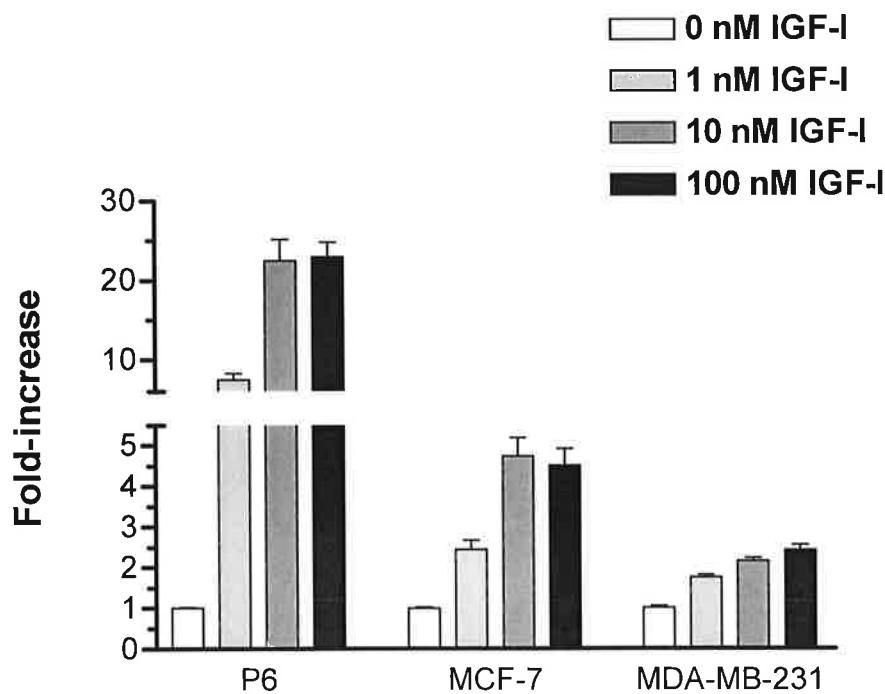


Figure 3.6 *The formation of the activated IGF-1R complex in response to IGF-I in breast cancer MCF-7 and MDA-MB-231 cells*

A KIRA assay was performed to measure the levels of tyrosine-phosphorylated IGF-1R complex formed following the incubation of the cells with different concentrations of IGF-I. Fold-increase represents the level of phosphorylated receptor complex formed in stimulated cells compared with unstimulated cells. The fibroblast cell line P6 that overexpresses human IGF-1R was used as a positive control. Data are presented as the mean $\pm$ S.E.M. from at least three independent experiments each performed in triplicate.

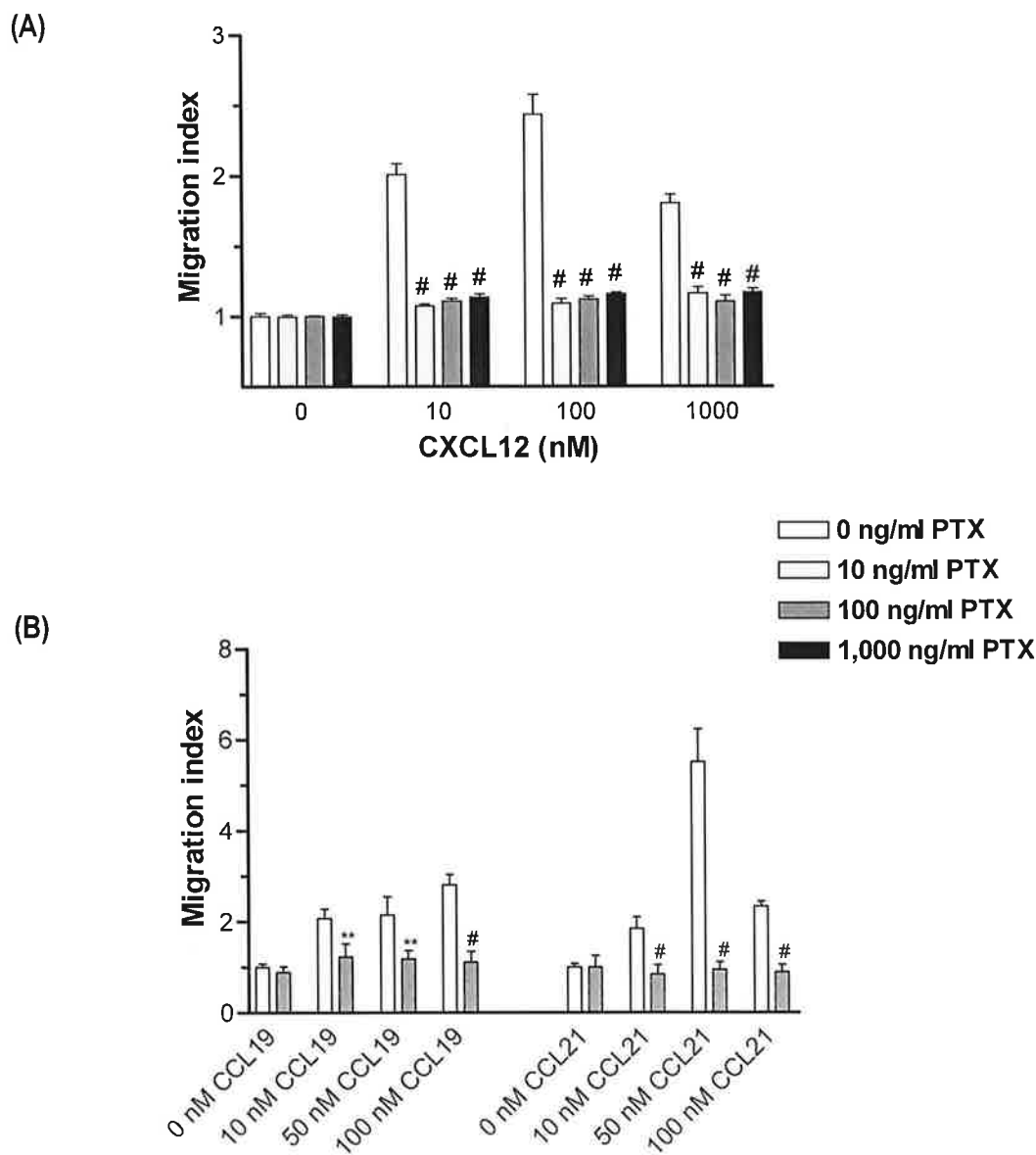


Figure 3.7 Effect of pertussis toxin on the chemotactic responses of MDA-MB-231 cells to CXCL12, CCL19 and CCL21

The cells were left untreated or pretreated with various concentrations of PTX for 16-18 hours prior to evaluating their ability to migrate toward different concentrations of CXCL12, CCL19 and CCL21 using a modified Boyden chamber assay. The chemotaxis of untreated and PTX-treated cells in response to CXCL12 (A), and CCL19 and CCL21 (B) is shown. The migration index represents the fluorescent signals of stimulated cells compared with those of unstimulated cells. All panels are expressed as mean $\pm$ S.E.M. of migration index from at least three separate experiments each performed in triplicate. ** ($p < 0.005$) and # ($p < 0.0001$) indicate statistically significant inhibition of chemotaxis in response to the chemokines (student's unpaired t test).

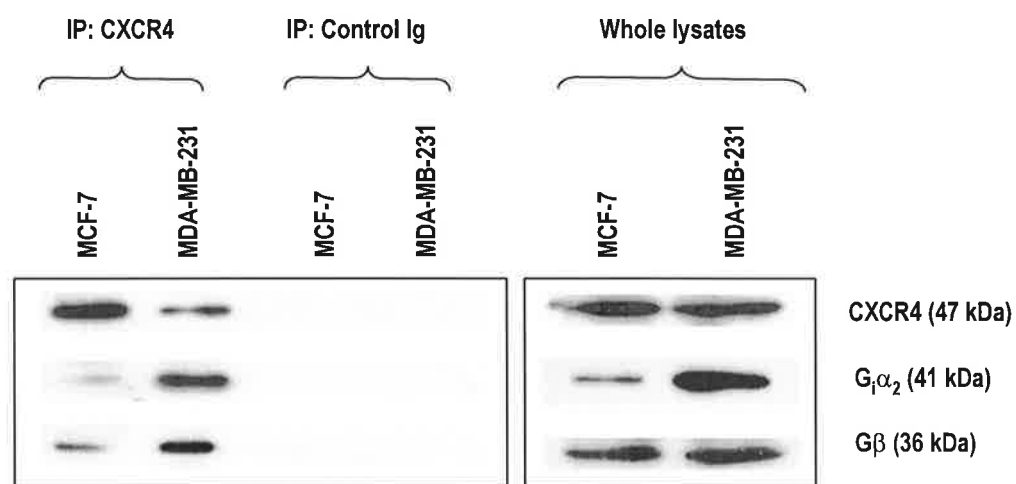


Figure 3.8 *Constitutive association of G-proteins, $G_i\alpha_2$ and $G\beta$, with CXCR4 in MCF-7 and MDA-MB-231 cells*

Lysates prepared from either MCF-7 or MDA-MB-231 cells were subjected to immunoprecipitation with either control IgG (monoclonal anti-hemagglutinin clone F-7) or anti-CXCR4 (monoclonal anti-CXCR4 clone 12G5) followed by Western blot to detect CXCR4, $G_i\alpha_2$ and $G\beta$. Alternatively, whole cell lysates were subjected to Western blot analysis for CXCR4, $G_i\alpha_2$ and $G\beta$. These data from the same Western blot are representative of 3-5 experiments performed with similar results.

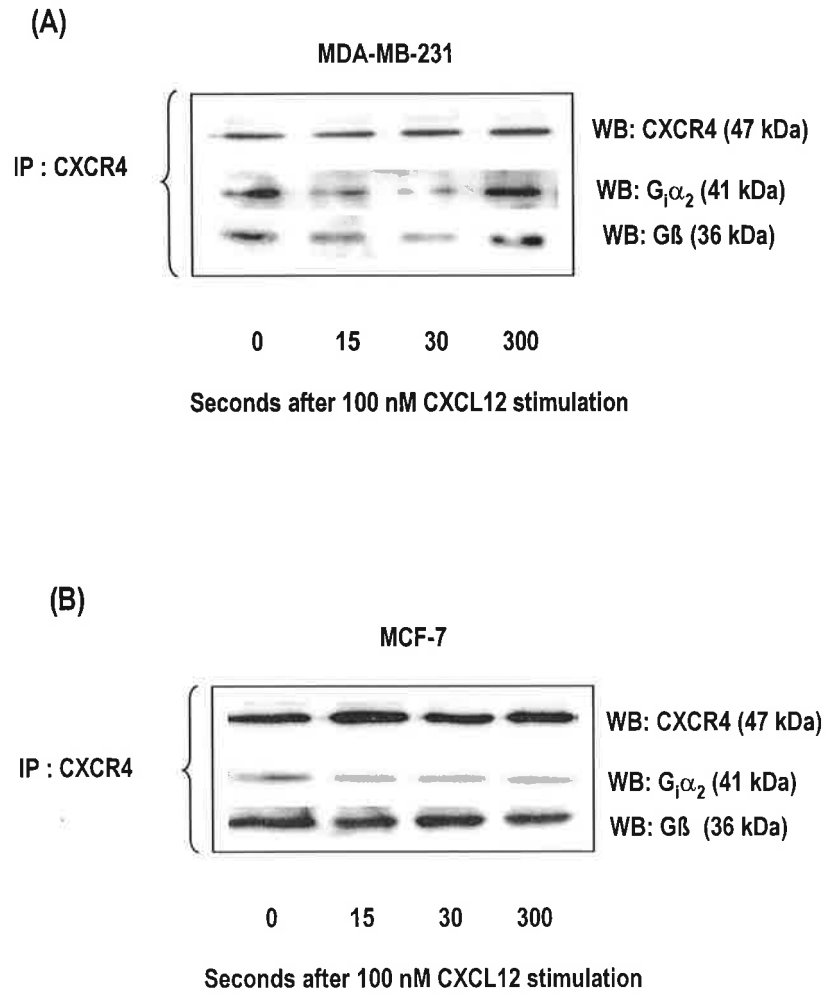


Figure 3.9 *Dissociation of G-proteins from CXCR4 following the stimulation with CXCL12 in MDA-MB-231 (A) and MCF-7 (B) cells*

MDA-MB-231 and MCF-7 cells were starved in serum-free medium for 2-3 hours before being stimulated with 100 nM CXCL12 for increasing period of time 0, 15, 30 and 300 seconds. Cell lysates were prepared and subjected to immunoprecipitation with anti-CXCR4 (monoclonal anti-CXCR4 clone 12G5) followed by Western blot to detect CXCR4, G_iα₂ and Gβ. Loading control is shown by the level of CXCR4 expression in immunoprecipitates. These data from the same Western blot are representative of 3-5 experiments performed with similar results.

CHAPTER 4

Transactivation between CXCR4 and IGF-1R Signal Transduction Pathways in Breast Cancer Cells

CHAPTER 4

Transactivation between CXCR4 and IGF-1R Signal Transduction Pathways in Breast Cancer Cells

4.1 Introduction

Different receptors on the surface of a cell mediate signal transduction from different extracellular stimuli such as hormones, growth factors and cytokines leading to cellular activities involved in a variety of physiological and pathological conditions (Krause and Van Etten 2005; Spiegel and Weinstein 2004). A number of recent studies have suggested the existence of cross-activation or cross-talk between the signalling pathways utilised by different types of receptors, particularly between growth factor receptor tyrosine kinases (RTKs) and GPCRs, thereby adding significant complexity to downstream signalling networks. For example, EGFR is tyrosine-phosphorylated in response to CCL11, a ligand for the GPCR CCR3, leading to MAPK activation and IL-8 production in bronchial epithelial cells (Adachi *et al.* 2004). In rat aortic vascular smooth muscle cells, both PDGFR and EGFR are phosphorylated by sphingosine 1-phosphate (S1P), a lipid mediator that is a ligand for the S1PR family of GPCRs, leading to activation of effectors downstream of PDGFR and EGFR including Shc, and the p85 regulatory subunit of the class IA PI3K (Tanimoto *et al.* 2004). In contrast, examples of transactivation of GPCRs by RTKs are less abundant, although recently it has been shown that IGF-I stimulates phosphorylation of CCR5 in MCF-7 breast cancer cells. In that study, chemotaxis induced by IGF-I was inhibited by a neutralising anti-CCL5 antibody, indicating that

transactivation of CCR5 by IGF-I is indirect, requiring production of a CCR5 ligand (Mira *et al.* 2001).

IGF-I and chemokines including CXCL12 and CCL21 exert their biological effects by inducing cellular signal transduction (Mellado *et al.* 2001; Thelen 2001; Zhang and Yee 2000). The binding of these molecules to their respective receptors, the receptor RTK IGF-1R and the GPCRs CXCR4 and CCR7, respectively, induces a network of signal transduction, which still requires further investigation. The data from the previous chapter clearly demonstrate the existence of functional chemokine receptors, CXCR4 and CCR7, and functional IGF-1R in the highly metastatic MDA-MB-231 cells. The investigation of potential interactions between these two classes of receptors is important for our understanding of the mechanisms by which breast cancer cells process multiple signalling inputs and translate them into activity. Therefore, the potential cross-talk between the signal transduction downstream of the chemokine receptors and IGF-1R in MDA-MB-231 cells was examined. Since, at the time this project was conducted, the role of CXCR4 in the metastatic potential of breast cancer had been more clearly established than that of CCR7, as shown in several previous reports (Chen *et al.* 2003; Liang *et al.* 2005; Muller *et al.* 2001), specific emphasis was placed on the interaction between CXCR4 and IGF-1R.

In this chapter, two possible types of the cross-talk between CXCR4- and IGF-1R-mediated signal transduction were considered: IGF-1R may transactivate the CXCR4 signalling pathway or conversely, CXCR4 may transactivate the IGF-1R signalling pathway. The potential of cross-activation between the two receptors in MDA-MB-231 cells was examined in both directions.

4.2 Results

4.2.1 Investigation into the transactivation of CXCR4 by IGF-I/IGF-1R

4.2.1.1 Effect of PTX treatment on IGF-I-induced chemotaxis and IGF-1R activation in MDA-MB-231 and MCF-7 cells

GPCRs mediate signal transduction through heterotrimeric G-proteins (Hein *et al.* 2005; Thelen 2001) and the activity of G-proteins, in particular, $G_i\alpha_2$, following CXCL12-mediated activation of CXCR4 in breast cancer cells has been demonstrated previously (Chapter 3, Sections 3.2.4 and 3.2.5). As an initial probe to determine whether IGF-1R transactivates CXCR4, the involvement of G-protein activity in IGF-I-mediated chemotaxis in MDA-MB-231 cells was examined. The cells were treated with various concentrations of PTX, an inhibitor of $G_i\alpha$, prior to testing their ability to migrate in response to different doses of IGF-I. As shown in figure 4.1A, PTX at concentrations of 10, 100 and 1,000 ng/ml partially blocked the chemotactic responses to all doses of IGF-I in MDA-MB-231 cells. Similar experiments performed in MCF-7 cells, which were previously shown to exhibit non-functional CXCR4 and CCR7 in chapter 3, indicate that the pretreatment of MCF-7 cells with PTX, at all concentrations tested, had no effect on IGF-I-induced chemotaxis at any of the three doses of IGF-I (Figure 4.1B). These results clearly demonstrate the contribution of G-protein activity to IGF-I-mediated chemotaxis only in MDA-MB-231 cells, suggesting that GPCRs are required for the migration of the cells in response to IGF-I.

To test the possibility that blocking $G_i\alpha$ activity with PTX inhibits the activation of IGF-1R induced by IGF-I, the lysates of cells untreated or treated with different doses of PTX

were assayed for the level of tyrosine-phosphorylated IGF-1R complex formed in response to IGF-I, at various concentrations, in a KIRA assay. PTX, at the doses of 10 and 100 ng/ml, failed to alter the level of IGF-1R activation induced by the stimulation of IGF-I, at all concentrations tested, in either MDA-MB-231 or MCF-7 cells (Figure 4.2A and B respectively). These results indicate that $G_{i\alpha}$ activation is not involved in IGF-I-induced formation of the activated IGF-1R complex in these cell lines.

4.2.1.2 Effect of RNAi-mediated CXCR4 knockdown on IGF-I-induced chemotaxis and IGF-1R activation in MDA-MB-231 cells

The involvement of CXCR4 in IGF-I-mediated chemotaxis of MDA-MB-231 cells was examined by employing RNAi to knockdown CXCR4 expression. To produce the CXCR4 knockdown cells, MDA-MB-231 cells were infected with a retrovirus expressing either RNAi specific to CXCR4 or target sequences for *Renilla* luciferase as a negative control. Individual clones were isolated and characterised for CXCR4 surface expression by flow cytometry. CXCR4 function was determined by assessing the common events downstream of CXCR4 activation, including intracellular calcium mobilisation and chemotaxis, in response to CXCL12. Compared with wild-type MDA-MB-231 cells and the negative control clone, RNAi clones 11, 21, and 27 displayed a significant reduction of surface CXCR4 expression (Figure 4.3A, shown only for clone 11) and of the level of transient increase of calcium concentration in response to CXCL12 (Holland 2007). The surface expression of IGF-1R was not affected by RNAi CXCR4 knockdown in any of the clones as demonstrated in figure 4.3B (shown only for clone 11). The chemotaxis assay showed that RNAi clones 11, 21, and 27 exhibited a complete inhibition of chemotactic responses to CXCL12 (Figure 4.3C). Together, these data indicate that siRNA-mediated CXCR4 knockdown specifically inhibited the expression and function of CXCR4, but did not

affect the expression of IGF-1R, in any of the clones tested. To determine the contribution of CXCR4 to IGF-I-mediated chemotaxis in MDA-MB-231 cells, the CXCR4 knockdown clones were assessed their ability to chemotactically migrate toward different concentrations of IGF-I in the chemotaxis assay. The data, shown in figure 4.4, indicate that RNAi clones 11, 21 and 27 exhibit a significant reduction in the degree of chemotaxis in response to IGF-I at all concentrations tested, suggesting an essential role for CXCR4 in IGF-I-mediated chemotaxis in MDA-MB-231 cells.

The effect of RNAi-mediated CXCR4 knockdown on IGF-I-mediated IGF-1R activation in MDA-MB-231 cells was also determined. Using the KIRA assay, the lysates prepared from CXCR4 knockdown, wide-type and negative clones were tested for the levels of tyrosine-phosphorylated IGF-1R complex formed in response to various concentrations of IGF-I. Figure 4.5 showed that there was no significant difference between the levels of activated IGF-1R in the lysates from the CXCR4 knockdown clones, 11, 21 and 27, and in those from wide type and negative clones, at all doses of IGF-I tested. The results indicate that CXCR4 is not involved in the IGF-I-mediated formation of activated IGF-1R complex in MDA-MB-231 cells.

4.2.1.3 IGF-I does not induce CXCL12 production in MDA-MB-231 cells.

To determine whether the involvement of CXCR4 receptors activation in the IGF-I-induced chemotaxis requires the production of their corresponding ligand CXCL12, the ability of IGF-I to produce CXCL12 was investigated. MDA-MB-231 cells were treated with 10 nM IGF-I for increasing periods of time. Expression of CXCL12 mRNA was examined by a reverse transcriptase-polymerase chain reaction (RT-PCR) (Figure 4.6) and CXCL12 protein synthesis was assessed by an enzyme-linked immunoadsorbent assay

(ELISA) (Figure 4.7). Figure 4.6 demonstrates that there was no CXCL12 mRNA synthesised in unstimulated cells or cells incubated with 10 nM IGF-I (lane 3 and 4-6 respectively) at any of the time points tested, in spite of the presence of CXCL12 expression in positive control cDNA prepared from 4T1.2 cells stably transfected with CXCL12 (lane 2). The production of CXCL12 protein was examined in both cell supernatants and lysates as shown in figure 4.7 (A) and (B) respectively. The supernatants from the culture of 4T1.2 cells, lacking CXCL12 synthesis, and 4T1.2-CXCL12 cells, stably expressing CXCL12, were included as negative and positive controls respectively. The results from these experiments suggest that there was no significant production of CXCL12 in the cell supernatants or lysates of either resting or IGF-I-stimulated cells, at any time point tested. These data indicate that there is no constitutive production of CXCL12 in MDA-MB-231 cells and that IGF-I stimulation does not induce the production of CXCL12 in this cell line. Therefore, the activity of CXCL12 is not required for an IGF-I-mediated chemotactic response in these cells.

4.2.1.4 IGF-1R, CXCR4 and G-proteins are physically associated in MDA-MB-231 and MCF-7 cells, however transactivation of CXCR4/G-protein signal transduction by IGF-I only occurs in MDA-MB-231 cells.

The nature of the interaction between IGF-1R and CXCR4/G-proteins in MCF-7 and MDA-MB-231 cells was investigated. Immunoprecipitation was performed on cell lysates using anti-IGF-1R antibodies, followed by Western blot for either CXCR4, $G_{i\alpha_2}$ or $G\beta$ (Figure 4.8). Immunoprecipitation of IGF-1R in both cell types under resting conditions led to coprecipitation of all three proteins, indicating the existence of a constitutive complex between IGF-1R, CXCR4, $G_{i\alpha_2}$ and $G\beta$ in these cell lines. In contrast, immunoprecipitation with control IgG failed to coprecipitate CXCR4, $G_{i\alpha_2}$ and $G\beta$,

whereas these proteins were readily detectable in whole cell lysates subjected directly to Western blot. Notably, consistent with the data demonstrating an association of G-proteins with the immunoprecipitated complex of CXCR4 in chapter 3 (Chapter 3, Section 3.2.5 and, Figures 3.8 and 3.3), the levels of $G_{i\alpha_2}$ subunits in immunoprecipitates of IGF-1R were markedly higher in MDA-MB-231 than in MCF-7 cells, correlating with the expression levels detected in the whole lysates from the two cells. In addition, despite the similar levels of $G\beta$ in the whole lysates from both cell lines, the levels of $G\beta$ in immunoprecipitates of IGF-1R were consistently higher in MDA-MB-231 than in MCF-7 cells (Figure 4.8).

As described in chapter 3 (Chapter 3, Section 3.2.5), CXCL12-mediated CXCR4 activation induces the uncoupling of G-protein subunits, $G_{i\alpha_2}$ and $G\beta$, from CXCR4 in MDA-MB-231 cells. The effect of stimulation with IGF-I on the association of $G_{i\alpha_2}$ and $G\beta$ with CXCR4 was therefore examined. MCF-7 and MDA-MB-231 cells were stimulated with 10 nM IGF-I for increasing periods of time. Cell lysates were prepared and subjected to immunoprecipitation with anti-CXCR4 followed by Western blot with anti-CXCR4, anti- $G_{i\alpha_2}$ or anti- $G\beta$ (Figure 4.9, top panel). Equal quantities of proteins in each of the lysates used in these experiments were confirmed by Western blot analysis for the level $G_{i\alpha_2}$ and $G\beta$ (Figure 4.9, bottom panel). The results from these experiments showed that stimulation of MCF-7 cells with IGF-I failed to release either $G_{i\alpha_2}$ or $G\beta$ from CXCR4 at any of the time points tested. In contrast, $G_{i\alpha_2}$ and $G\beta$ were released from CXCR4 after stimulation with IGF-I in MDA-MB-231 cells, indicating the activation of CXCR4 following IGF-I stimulation in this cell line. These results demonstrate that, despite the constitutive formation of a complex between IGF-1R, CXCR4 and G-proteins

observed in both resting MCF-7 and MDA-MB-231 cells, the transactivation of CXCR4 signalling by IGF-I as determined by the uncoupling of G-proteins from CXCR4 in response to IGF-I, was detected only in MDA-MB-231 cells.

4.2.2 Investigation into transactivation of IGF-1R by CXCL12/CXCR4

4.2.2.1 CXCL12 does not induce IGF-1R signal transduction in MDA-MB-231 cells.

To investigate potential transactivation of IGF-1R by CXCL12, IGF-1R activation downstream of CXCR4 was determined. A KIRA assay was performed to detect formation of tyrosine-phosphorylated IGF-1R after stimulation with the ligand for CXCR4, CXCL12. Based on the preliminary data indicating that formation of the activated IGF-1R complex is detected within 10 mins following IGF-I stimulation, the cells were treated with a range of doses of CXCL12 for 10 mins and cell lysates were subjected to the KIRA assay to detect the levels of activated IGF-1R complex. The assay demonstrated that CXCL12 failed to activate the formation of activated IGF-1R complex in P6 (positive control cell line), MCF-7 and MDA-MB-231 cells at any of the concentrations tested (Figure 4.10) despite the fact that IGF-I-mediated IGF-1R activation was observed in all cells as shown in chapter 3 (Chapter 3, Figure 3.6). Thus, the results indicate that there was no activation of IGF-1R by CXCL12 in these cell lines.

4.2.2.2 Pretreatment of PTX and CXCR4 knockdown do not affect formation of activated IGF-1R complex in MDA-MB-231 cells.

As previously described in sections 4.2.1.1 and 4.2.1.2, the involvement of G-proteins and CXCR4 in IGF-I-mediated IGF-1R activation was studied by examining the effect of

pertussis toxin (PTX), a specific inhibitor of $G_{i\alpha}$, and siRNA-mediated CXCR4 knockdown on the activation of IGF-1R signalling pathway induced by IGF-I in breast cancer cells using the KIRA assay. Figure 4.2 demonstrated that, pretreatment of the cells with PTX did not affect the IGF-I-mediated IGF-1R activation in both MCF-7 and MDA-MB-231 cells. RNAi of CXCR4 in MDA-MB-231 cells also did not have any effect on the level of IGF-I-induced IGF-1R activation (Figure 4.4). Taken together, the data support that there was no involvement of CXCR4/G-protein signalling in the IGF-1R-induced activation pathway in breast cancer MDA-MB-231 cells.

4.3 Discussion and Conclusion

The studies described in this chapter have provided evidence for transactivation between CXCR4 and IGF-1R in the highly metastatic MDA-MB-231 cells. The nature of this cross-talk appears to be unidirectional. While there is no evidence of activation of IGF-1R by CXCR4, it is clearly shown that IGF-1R transactivates CXCR4 utilising the classical G-protein signal transduction for the induction of cell migration in the cells. The basis of this transactivation appears to be dependent on a physical association between CXCR4 and IGF-1R but does not involve the action of the CXCR4 ligand, CXCL12. The cross-talk shown in this study potentially has an impact on our understanding of the intracellular signalling of these two important receptors both in normal and pathological conditions, particularly breast cancer metastasis.

As mentioned in the introduction to this chapter (Chapter 1, Section 1.3.4), several types of transactivation between GPCRs and RTKs have been reported in the literature. First, RTKs can be transactivated by GPCRs. For instance, EGFR is phosphorylated in response

to CCL11, a ligand for the GPCR CCR3, leading to MAPK activation and IL-8 production in bronchial epithelial cells (Adachi *et al.* 2004). Second, GPCRs can be transactivated by RTKs. For example, IGF-I stimulates phosphorylation of CCR5 in MCF-7 cells. This appears to be indirect, requiring the production of CCL5, a ligand for CCR5 (Mira *et al.* 2001). Finally, bidirectional transactivation between the two receptor systems has also been observed. PDGFR is phosphorylated by S1P, a ligand for GPCR S1P receptors (S1PRs), leading to activation of downstream effectors including Shc, and the p85 regulatory subunit of class 1A PI3K (Tanimoto *et al.* 2004) and PDGF has been demonstrated to transactivate the S1P receptors (Waters *et al.* 2003).

It has been previously well documented that RTK signalling can be activated by GPCRs as demonstrated in several growth factor receptor systems including EGFR, PDGFR, and IGF-1R and it has been suggested that the phosphorylation of RTKs by GPCR ligands is the major characteristic of this type of transactivation (Adachi *et al.* 2004; Daub *et al.* 1996; Herrlich *et al.* 1998; Rao *et al.* 1995). However, the data generated in the present study demonstrate that incubation of MDA-MB-231 cells with CXCL12 did not induce the formation of the tyrosine-phosphorylated IGF-1R complex that has been shown as an early detectable event following the binding of IGF-I by IGF-1R (see Chapter 3). Additional data also show that neither pretreatment of the cells with PTX nor RNAi of CXCR4 inhibited IGF-I-induced formation of the activated IGF-1R complex, indicating that CXCR4 and G-proteins are not involved in this aspect of IGF-1R-mediated signal transduction. Together, these data indicate a lack of transactivation of IGF-1R by CXCL12/CXCR4 in MDA-MB-231 cells.

In contrast, this study demonstrates transactivation of CXCR4 by IGF-I in MDA-MB-231 cells. Blockade of $G_i\alpha$ activity by prior treatment with PTX, which was shown to completely inhibit CXCL12-induced chemotaxis of the cells (see Chapter 3), significantly inhibited the chemotactic response of the cells to IGF-I. Moreover, RNAi-mediated CXCR4 knockdown, which was demonstrated to efficiently inhibit the expression and function of CXCR4 in MDA-MB-231 cells by flow cytometric analysis, and calcium mobilisation and chemotaxis assays, respectively, also significantly blocked IGF-I-induced chemotaxis of MDA-MB-231 cells. Of interest, PTX failed to inhibit IGF-I-mediated migration in MCF-7 cells which lack functionally active CXCR4 (see Chapter 3). Together, these data strongly suggest that IGF-I-induced chemotaxis is dependent on functional expression of CXCR4 and the subsequent activation of G-protein subunits.

The mechanism by which IGF-I transactivates CXCR4 in MDA-MB-231 cells was investigated further. At least one previous study demonstrates that IGF-I can induce chemokine production which then activates its receptor to participate in IGF-I-induced migrational signalling (Mira *et al.* 2001). However, the data shown in the present study indicate that there is no involvement of CXCL12 in IGF-I-induced chemotaxis of the cells. This is supported by the observation that the cells did not synthesise or release CXCL12, the only known ligand for CXCR4, either constitutively or in response to IGF-I as determined by reverse-transcriptase-PCR and ELISA. These data therefore rule out an autocrine or sequential function of CXCL12 in the induction of chemotaxis by IGF-I in the cells.

Nonetheless, this study reveals aspects of the nature of the interaction between IGF-1R and CXCR4/G-protein subunits in both MCF-7 and MDA-MB-231 cells. Co-

immunoprecipitation studies indicated the existence of a constitutive complex containing IGF-1R, CXCR4 and the G-protein subunits, $G_{i\alpha_2}$ and $G\beta$, in both cell lines. As discussed in Chapter 3, the uncoupling of $G_{i\alpha_2}$ and $G\beta$ from CXCR4 following the activation of CXCR4 by CXCL12 is a key event for mediating chemotactic signalling by CXCL12 in MDA-MB-231 cells. It is apparent that a dissociation of both $G_{i\alpha_2}$ and $G\beta$ from CXCR4 was also observed following IGF-I stimulation in MDA-MB-231 cells, indicating activation of CXCR4 signalling in response to IGF-I in this cell line. Taken together, these data suggest that IGF-1R and CXCR4 form a complex via physical association that allows IGF-I to activate migrational signal transduction through CXCR4 and G-protein subunits, $G_{i\alpha_2}$ and $G\beta$, in MDA-MB-231 cells.

Interestingly, a physical interaction between IGF-1R and $G_{i\alpha}$ or $G\beta$ subunits has previously been reported (Dalle *et al.* 2001; Hallak *et al.* 2000). In those studies, PTX was also shown to inhibit IGF-I-induced activation of MAPK in neuronal cells (Hallak *et al.* 2000) and IGF-I-mediated mitogenesis of HIRcB cells and 3T3L1 adipocytes (Dalle *et al.* 2001). The requirement of PTX-sensitive G-protein activation in IGF-I-mediated mitogenic signal transduction has also been demonstrated in several other cell types (Kuemmerle and Murthy 2001; Luttrell *et al.* 1995; Lyons-Darden and Daaka 2004). Together, these studies led to the hypothesis that IGF-1R may act as a GPCR that mediates signal transduction through G-protein subunits. However, the data shown in the present study clearly indicate that the association of G-proteins with IGF-1R appears to be indirect, requiring the presence of functional GPCR, specifically CXCR4. Certainly, the data shown here indicate that the presence of CXCR4 is required for G-protein dependent cell migration in response to IGF-I. This observation may explain the previous evidence for the involvement of G-proteins in growth factor-mediated signalling.

It is important to note that the inhibition of IGF-I-induced chemotaxis by PTX and knockdown of CXCR4 in MDA-MB-231 cells was only partial even though CXCL12-induced migration of the cells was completely inhibited. As mentioned earlier, neither prior treatment with PTX nor RNAi-mediated CXCR4 knockdown had any effect on IGF-I-mediated tyrosine-phosphorylation of the IGF-1R complex. It is possible that the residual cell migration observed in response to IGF-I in PTX-treated cells is a result of the activation of tyrosine kinase-dependent pathways through IGF-1R. To the best of our knowledge, one of the signalling systems downstream of RTK activation that potentially works independently of PTX-sensitive G-proteins signalling in response to IGF-I is the class IA PI3K pathway. It has been known that cell migration in eukaryotic cells depends on activation of class IA and IB PI3Ks (Katso *et al.* 2001; Vanhaesebroeck *et al.* 2001). In general, RTKs mediate cell migration via the activation of class IA PI3Ks whereas GPCRs activate class IB PI3Ks to drive cell migration (Procko and McColl 2005; Ward 2004). IGF-I has been clearly shown to activate class IA PI3Ks and this is dependent on tyrosine phosphorylation of IGF-1R (Myers *et al.* 1993; Tartare-Deckert *et al.* 1996). In contrast, based on accumulating data with respect to cell migration of leukocytes, chemokine receptors including CXCR4 are likely to mediate PTX-sensitive cell migration through class IB PI3Ks even though the contribution of the class IA has also been shown in certain circumstances (Ward 2004). Thus, it is possible that the residual cell migration observed in PTX-treated MDA-MB-231 cells and in MDA-MB-231 cells in which CXCR4 had been knocked down in response to IGF-I was due to tyrosine kinase-dependent activation of class IA PI3Ks.

While accumulating data indicate $G_i\alpha$ dependent signal transduction induced by growth factors (Waters *et al.* 2004), a lack of this signalling pathway in response to activation of particular growth factor receptors such as EGFR has also been reported (Freedman *et al.* 2002; Luttrell *et al.* 1995). Luttrell *et al.* have reported that rat fibroblast cells overexpressing human IR showed a more robust MAPK activation but lost $G_i\alpha$ and $G_{\beta\gamma}$ -dependent signals (Luttrell *et al.* 1995). The results of previous studies indicate that the involvement of G-proteins in growth factor receptor signal transduction may be dependent on cell types. In the present study, transactivation of CXCR4/G-proteins by IGF-I was not observed in MCF-7 cells. There was no inhibitory effect of PTX on IGF-I-mediated chemotaxis in this cell line, indicating that the chemotaxis of the cells induced by IGF-I is independent of $G_i\alpha$ activity. It has been previously suggested that a high density of growth factor RTK expressed in a given cell mediates a robust and sustained signal through the RTK and may mask G-protein-mediated signalling pathways in response to growth factors (Waters *et al.* 2004). Certainly, MCF-7 cells express a high level of IGF-1R which correlates with a high level response to IGF-I with respect to both tyrosine phosphorylation of the IGF-1R complex and cell migration (see Chapter 3). It is therefore possible that IGF-I utilises the classical tyrosine kinase-dependent pathways via IGF-1R resulting in a strong signal that over-rides $G_i\alpha$ -mediated signalling in this cell line. On the other hand, an alternative explanation has been clearly defined in this study. MCF-7 cells express the non-functional CXCR4 which exhibits a blockade of G-protein activation, and therefore eliminate CXCR4/G-protein signalling in response to IGF-I. As clearly demonstrated, IGF-1R and CXCR4/G-proteins could be coprecipitated in both MDA-MB-231 and MCF-7 cells, indicating that a lack of transactivation of CXCR4 was not because of a lack of association of IGF-1R and CXCR4/G-proteins in those cells. Rather, the data

indicate that the cross-talk between the two receptors is mediated at the level of G-protein activity. This defect in GPCR function, specifically at the level of G-protein activation in a given cell, as in the case of CXCR4 in these breast cancer cells, may also explain evidence of a lack of involvement of G-protein dependent signalling by growth factors reported in several other studies (Freedman *et al.* 2002; Luttrell *et al.* 1995; Rakhit *et al.* 2001).

In summary, this study has demonstrated unidirectional transactivation of CXCR4 by IGF-I/IGF-1R that is required for a significant proportion of the migrational response to IGF-I in MDA-MB-231 cells. This transactivation does not involve the activation of CXCR4 by its ligand, CXCL12. Rather, it depends on a physical association between IGF-1R and CXCR4/G-proteins. This allows IGF-I to induce the activation of G-proteins that appears to be dependent on the presence of a functional pool of CXCR4. Importantly, the transactivation is not observed in MCF-7 cells due to a defect in CXCR4-mediated G-protein signal transduction in this cell type. The lack of integration of IGF-1R and CXCR4 signalling pathways in the non-metastatic MCF-7 cells suggests a potentially important role of this cross-talk in the metastatic potential of breast cancer cells.

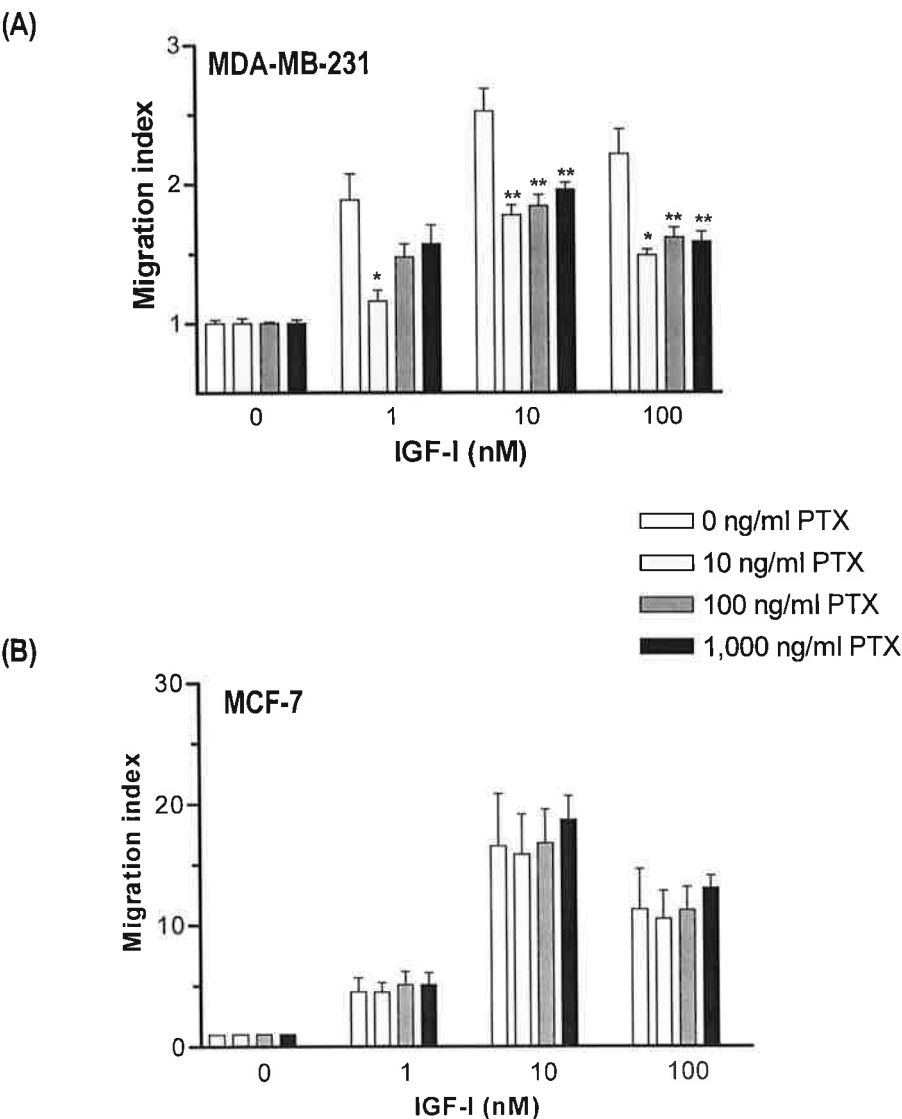


Figure 4.1 Effect of PTX on the chemotactic response of MDA-MB-231 (A) and MCF-7 (B) cells to IGF-I

MDA-MB-231 and MCF-7 cells were left untreated or pretreated with different doses of PTX, 10, 100 and 1,000 ng/ml, for 16-18 hours prior to testing their chemotactic response to various concentrations of IGF-I using a modified Boyden chamber assay. The chemotaxis of untreated and PTX-treated MDA-MB-231 (A) and MCF-7 cells (B) is shown. The migration index represents the fluorescent signals of stimulated cells compared with those of unstimulated cells. All panels are expressed as the mean $\pm$ S.E.M. of the migration index from at least three separate experiments, each performed in triplicate. Asterisks indicate significantly different from control values (student's unpaired t test) at *, $p < 0.05$; **, $p < 0.005$.

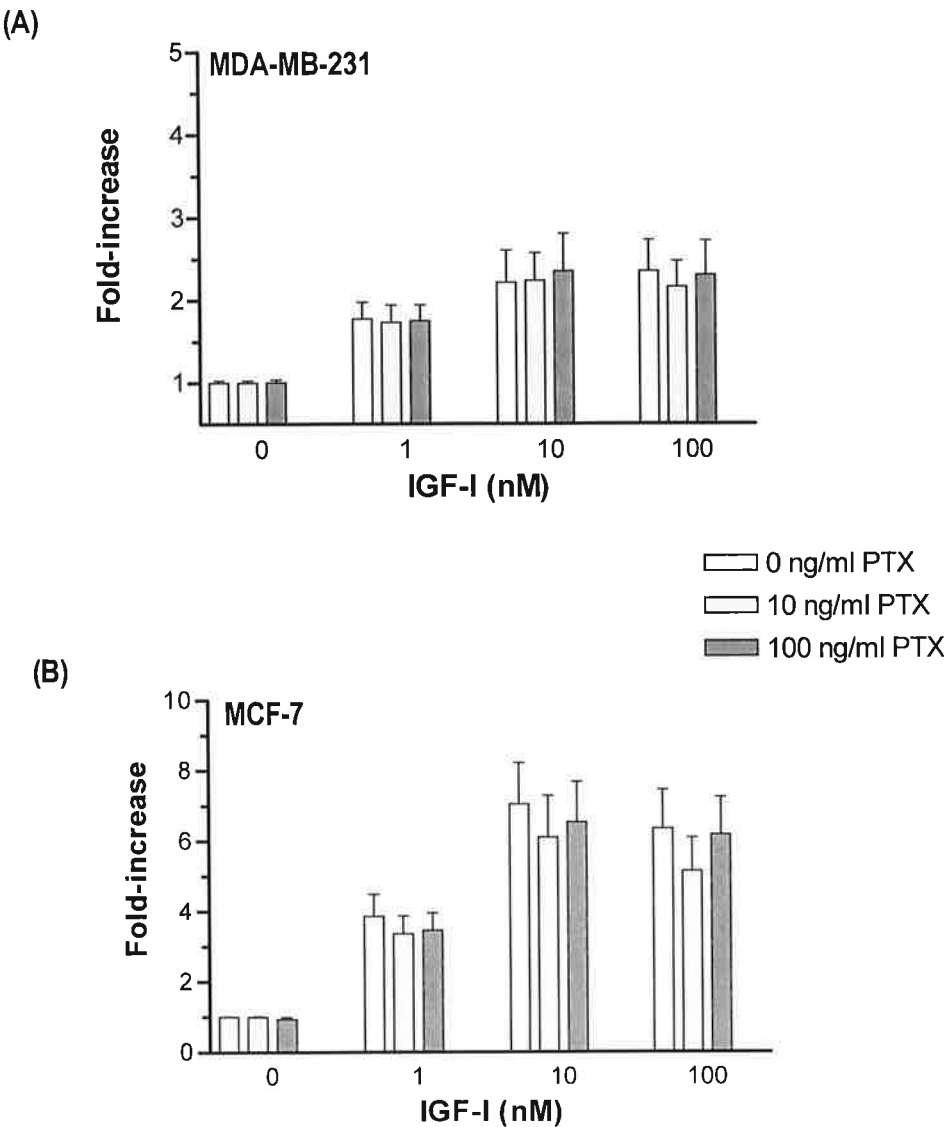


Figure 4.2 Effect of PTX on the activation of IGF-1R complex following stimulation with IGF-I in MDA-MB-231 and MCF-7 cells

The cells were left untreated or pretreated with PTX at concentration of 10 and 100 ng/ml for 16-18 hours and the level of tyrosine-phosphorylated IGF-1R complex formed after the stimulation with different doses of IGF-I was assayed by a KIRA assay. The level of phosphorylated IGF-1R complex formed in untreated and PTX-treated MDA-MB-231 (A) and MCF-7 (B) cells is shown. Fold-increase represents the level of activated IGF-1R complex in the stimulated cells in relation to those observed in the unstimulated cells. Data are presented as the mean $\pm$ S.E.M. from at least three independent experiments each performed in triplicate.

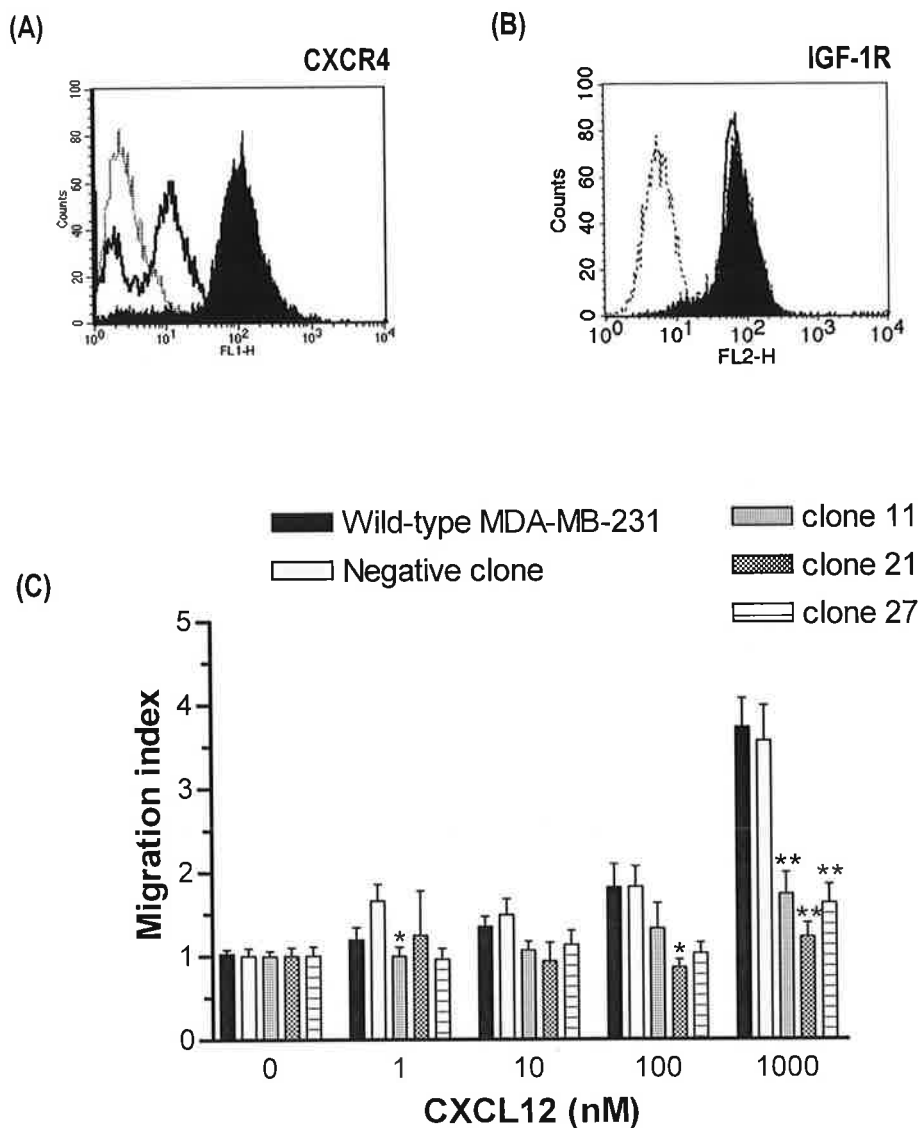


Figure 4.3 The retroviral-mediated siRNA knock down of CXCR4 in MDA-MB-231 cells

Cells were infected with a retrovirus producing either siRNA to knockdown CXCR4 or specific sequences for *Renilla* Luciferase as a negative control. Individual clones were selected in puromycin-containing media and analysed by flow cytometry for the surface expression of CXCR4 and IGF-1R (solid line) together with the wild-type untreated cells (filled histogram). Isotype-matched IgGs were used as a negative control (dotted line). A and B, histograms show the reduction of surface level of CXCR4 and no change in the level of IGF-1R for MDA-MB-231 cells (shown only for clone 11). C, Clones 11, 21 and 27 were assessed for their ability to migrate in response to different concentrations of CXCL12 using a modified Boyden chamber assay. The migration index represents the fluorescent signals of stimulated cells compared with those of unstimulated cells. Data are presented as mean $\pm$ S.E.M. of migration index from 2-5 separate experiments each performed in quadruplicate. Asterisks indicate statistically significantly different from control values (student's unpaired t test) at *, $p < 0.05$ or **, $p < 0.005$.

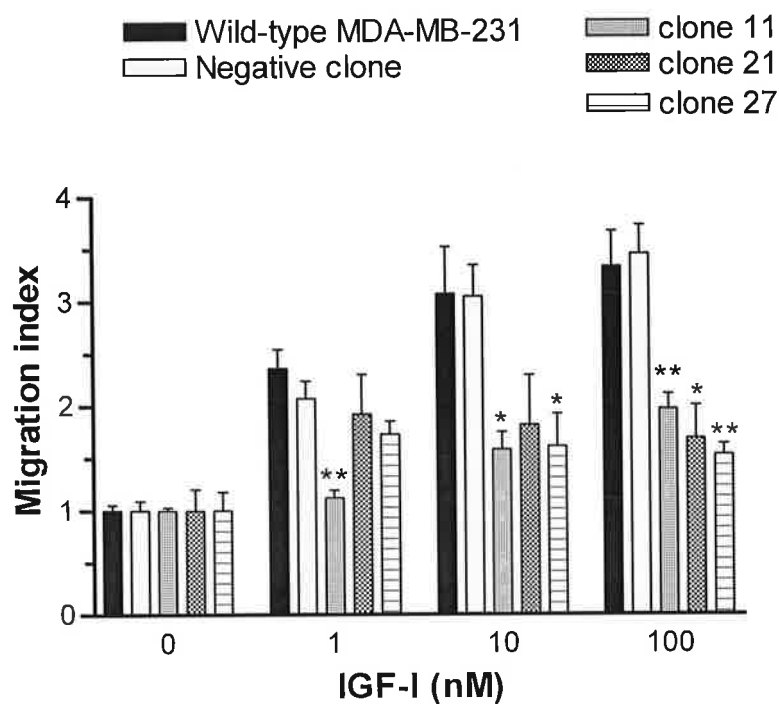


Figure 4.4 Effect of siRNA-mediated CXCR4 knockdown on IGF-I-induced chemotaxis of MDA-MB-231 cells

CXCR4 knockdown clones 11, 21 and 27 were tested for their chemotactic response to different concentrations of IGF-I in comparison with negative control and wild type clones. The migration index represents the fluorescent signals of stimulated cells compared with those of unstimulated cells. Data are presented as mean $\pm$ S.E.M. of migration index from 2-5 separate experiments each performed in quadruplicate. Asterisks indicate statistically significantly different from control values (student's unpaired t test) at *, $p < 0.05$ or **, $p < 0.005$.

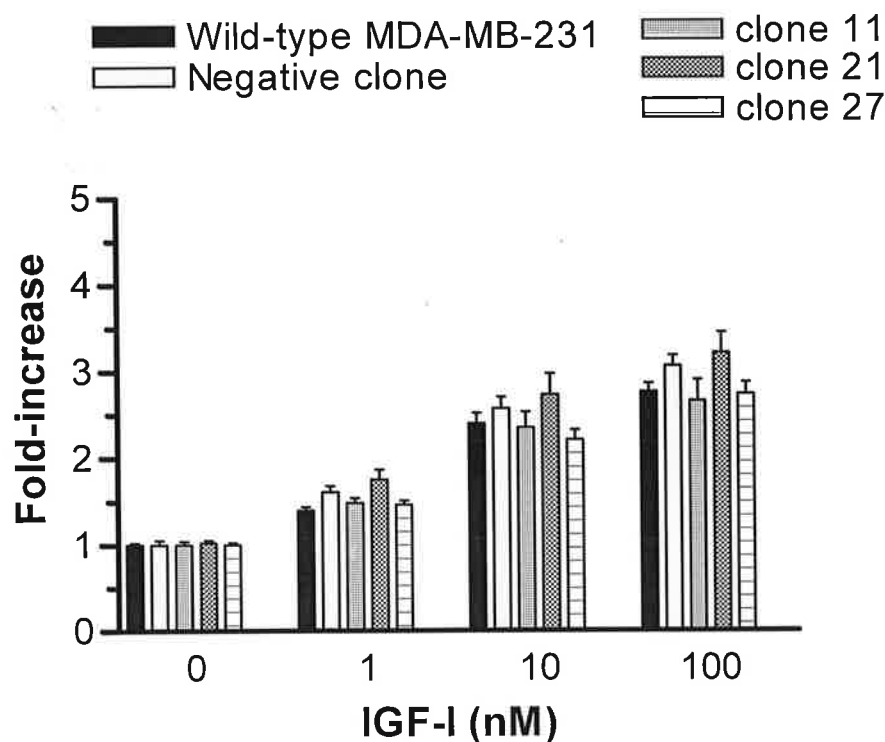


Figure 4.5 *Effect of siRNA-mediated CXCR4 knockdown on IGF-I-mediated IGF-1R activation in MDA-MB-231 cells*

A KIRA assay was performed to examine the level of tyrosine-phosphorylated IGF-1R complex, in response to different concentrations of IGF-I, in CXCR4 knockdown clones 11, 21, and 27 compared with negative control and wild-type cells. Fold-increase represents the level of receptor complex formed in IGF-I-stimulated compared with unstimulated cells. Data are presented as mean $\pm$ S.E.M. of fold-increase from 4 separate experiments each performed in triplicate.

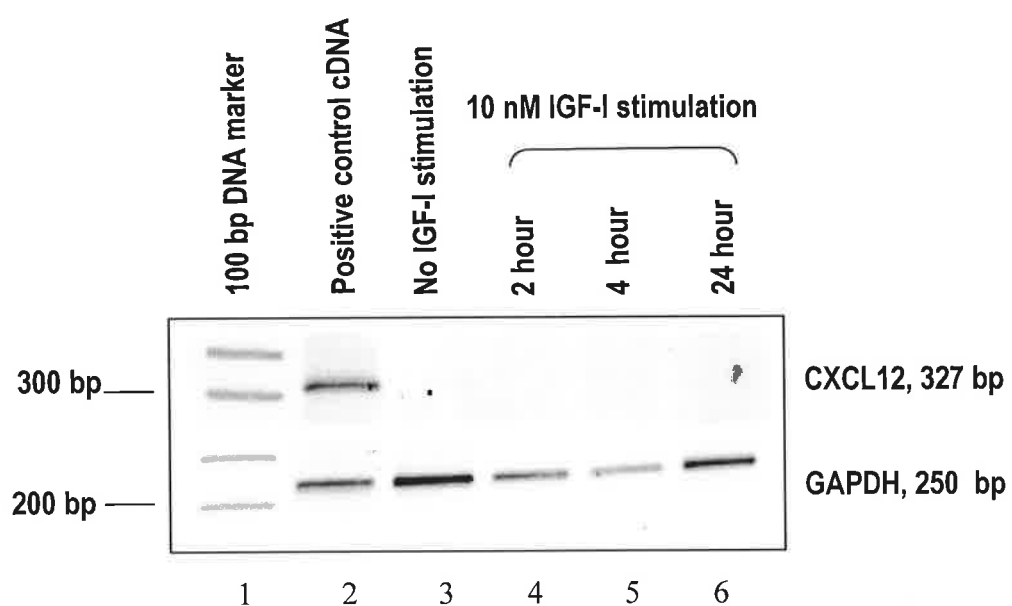


Figure 4.6 Lack of production of CXCL12 mRNA following incubation of MDA-MB-231 cells with IGF-I as demonstrated by a RT-PCR assay

Cells were starved in serum-free medium for 2-3 hours before being stimulated with 10 nM IGF-I for 2, 4 or 24 hours or left unstimulated for 24 hours. The unstimulated and stimulated cells were subjected to RNA extraction, and RT-PCR assay using specific primers to CXCL12 or a control sequence, GAPDH. A representative agarose gel is shown. The top panel shows the presence of CXCL12 mRNA (~327 bp) whereas the bottom panel demonstrates that of control GAPDH mRNA (~250 bp). The cDNA extracted from 4T1.2-CXCL12 cells were used as a positive control. Lane 1: DNA markers, lane 2: positive control cDNA, lane 3: unstimulated MDA-MB-231 cells, lane 4, 5 and 6: MDA-MB-231 cells stimulated with 10 nM IGF-I at 2, 4 and 24 hour respectively.

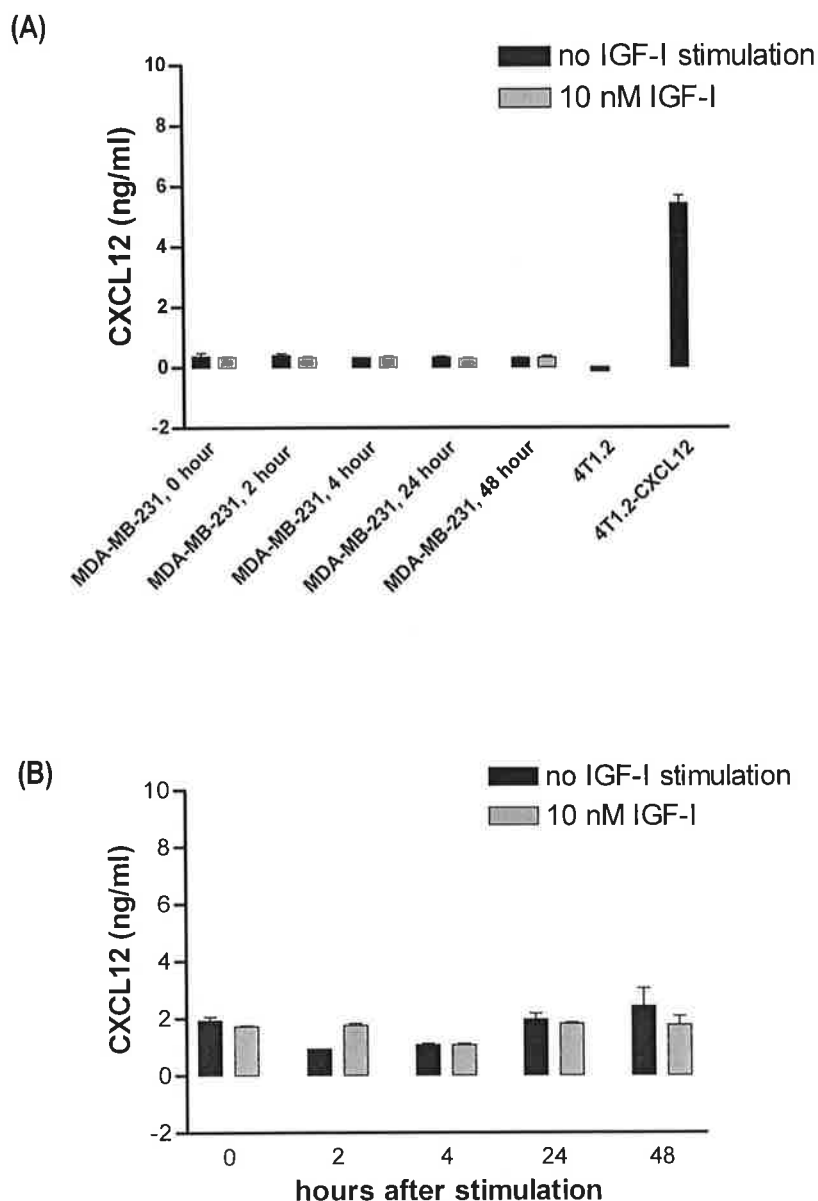


Figure 4.7 Lack of production of CXCL12 protein following IGF-I stimulation of MDA-MB-231 cells analysed by an enzyme-linked immunosorbent assay (ELISA)

MDA-MB-231 cells were starved in serum-free medium for 2-3 hours before being left unstimulated or stimulated with 10 nM IGF-I for 0, 2, 4, 24 or 48 hours. An ELISA was conducted to detect CXCL12 in cell supernatants (A) and lysates (B). The supernatants from 4T1.2 and 4T1.2-CXCL12 cell culture were used as a negative and positive control respectively (A). Data are presented as mean $\pm$ S.E.M. of CXCL12 levels (ng/ml) performed in triplicate. An ELISA was performed by Sharon Hampton-Smith, The University of Adelaide, Adelaide, Australia.

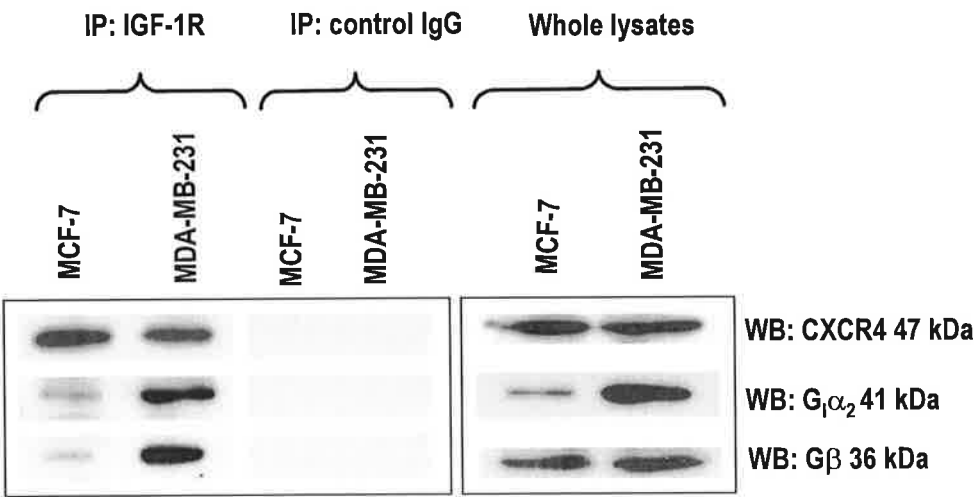


Figure 4.8 *Physical association between IGF-1R, CXCR4 and G-protein subunits in MDA-MB-231 and MCF-7 cells*

Lysates from MDA-MB-231 and MCF-7 cells were subjected to immunoprecipitation using either control IgG (monoclonal anti-hemagglutinin clone F-7) or anti-IGF-1R (monoclonal anti-IGF-1R clone 24-31) followed by Western blot to detect CXCR4, G_iα₂ and Gβ. Alternatively, whole cell lysates were subjected to Western blot detecting CXCR4, G_iα₂ and Gβ. These data are from the same Western blot shown in figure 3.8 and are representative of 3-5 experiments performed with similar results.

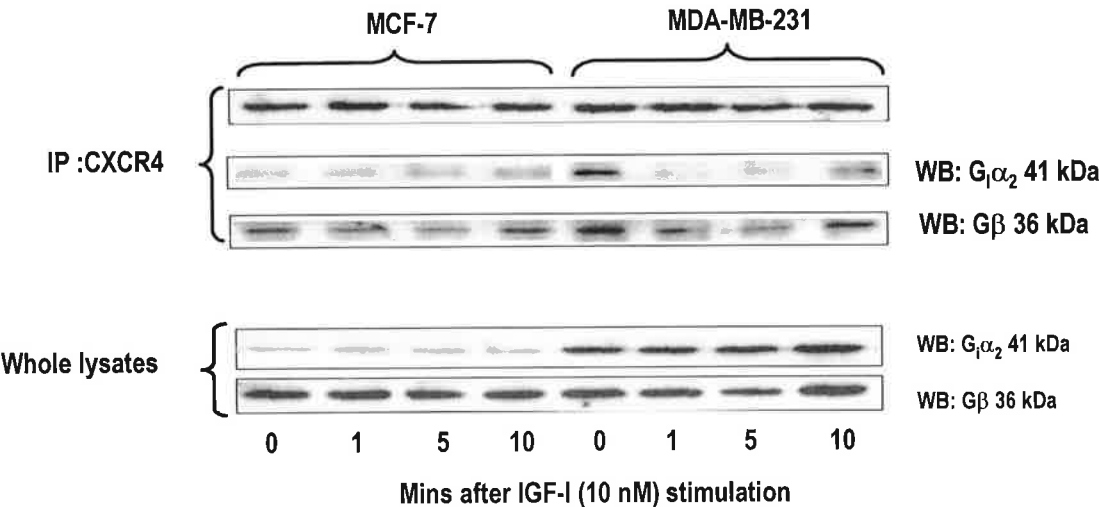


Figure 4.9 *Uncoupling of G-protein subunits from CXCR4 following incubation of MDA-MB-231 and MCF-7 cells with IGF-I*

MDA-MD-231 and MCF-7 cells were incubated in serum-free medium for 2-3 hours before being stimulated with 10 nM IGF-I for increasing period of time 0, 1, 5 and 10 minutes. Cell lystaes were prepared and subjected to immunoprecipitation using anti-CXCR4 (monoclonal anti-CXCR4 clone 12G5) followed by Western blot to detect CXCR4, $G_i\alpha_2$ or $G\beta$. Western blots of the whole lysates are shown for $G_i\alpha_2$ and $G\beta$ as an indication of equal protein loading. These data from the same Western blot are representative of 3-5 experiments performed with similar results.

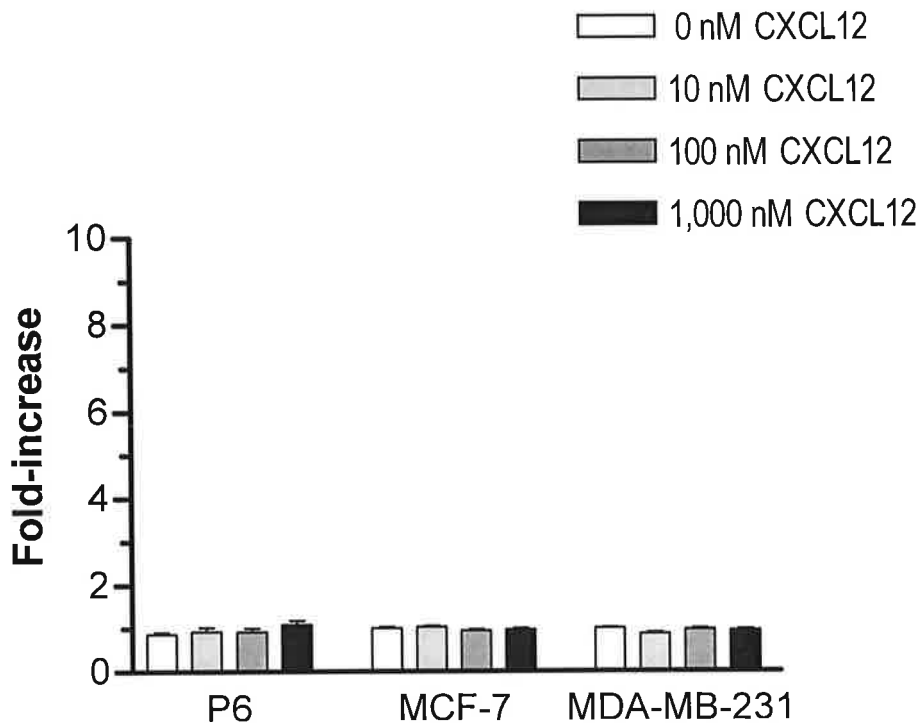


Figure 4.10 Lack of activation of the IGF-1R complex formed in response to CXCL12 in MCF-7 and MDA-MB-231 cells

A KIRA assay was performed to examine the level of tyrosine-phosphorylated IGF-1R complex formed following incubation of the cells with different concentrations of CXCL12. Fold-increase represents the level of receptor complex formed in cells incubated or not, with CXCL12. The cells line P6 that overexpresses human IGF-1R was included since the cells have been characterised as a functional IGF-1R positive cell line. Data are presented as the mean $\pm$ S.E.M. from at least three independent experiments each performed in triplicate.

CHAPTER 5

Coregulation of CXCR4 and IGF-1R Expression and Function in Breast Cancer Cells

CHAPTER 5

Coregulation of CXCR4 and IGF-1R Expression and Function in Breast Cancer Cells

5.1 Introduction

The processes of receptor internalisation and degradation/recycling have long been regarded as key events in the modulation of receptor activity, and aberrant regulation of these processes has been implicated in a number of diseases including cancer (Bache *et al.* 2004). Based on the literature, both CXCR4 and IGF-1R are downregulated following exposure to their ligands, CXCL12 and IGF-I respectively, via classical clathrin-coated pit and ubiquitin-proteasome/lysosomal pathways (Carelli *et al.* 2006; Fernandis *et al.* 2002; Girnita *et al.* 2005; Lapham *et al.* 2002; Marchese and Benovic 2001; Vecchione *et al.* 2003). Although mechanisms for these processes have been increasingly investigated, significant questions remain. Accumulating recent evidence indicates that chemokine receptor expression and function are modulated by growth factors (Bachelder *et al.* 2002; Mira *et al.* 2001; Satyamoorthy *et al.* 2002). Growth factor RTKs such as PDGFR, EGFR and IGF-1R have also been shown to utilise signal transduction pathways downstream of GPCRs which are responsible for desensitisation of the activated receptors (Hupfeld and Olefsky 2007). In the context of the findings described in chapter 4 suggesting the constitutive formation of an IGF-1R, CXCR4 and G-protein complex and the transactivation of CXCR4 by IGF-I in the metastatic breast cancer MDA-MB-231 cells (Akekawatchai *et al.* 2005), this raises the possibility of coregulation of expression of the two receptors, which may have an impact on the activation states of the two receptor

systems in different conditions. The aim of experiments conducted in the present chapter was therefore to investigate the potential coregulation of CXCR4 and IGF-1R expression and function in MDA-MB-231 cells. To accomplish this aim, the internalisation and degradation of the two receptors, as well as molecular mechanisms involved in these processes, were studied in MDA-MB-231 breast cancer cells. As in the previous chapters, the MCF-7 cell line was used as a non-metastatic comparison.

5.2 Results

5.2.1 Effect of IGF-I and CXCL12 on surface expression of IGF-1R, CXCR4 and CCR7 in MDA-MB-231 and MCF-7 cells

Whether exposure of cells to IGF-I or CXCL12 induces internalisation of IGF-1R and CXCR4 was initially investigated in the highly metastatic MDA-MB-231 cells which have been shown to exhibit transactivation of CXCR4 by IGF-I. Flow cytometry was utilised to assess the surface expression of the receptors in the cells following incubation with IGF-I and CXCL12. The level of CCR7, which has been shown to be expressed in the MCF-7 and MDA-MB-231 cells as demonstrated in chapter 3, was also examined in these experiments. Since IGF-I and CXCL12 at the optimum doses of 10 nM and 100 nM respectively are capable of activating chemotactic response in MDA-MB-231 cells, the same doses were used to treat the cells in this experiment (see Chapter 3, Section 3.2.2). Control cells and those incubated with 10 nM IGF-I or 100 nM CXCL12 for increasing period of time, 1, 3, 6 and 24 hours, were labelled with specific Abs to IGF-1R, CXCR4 and CCR7 prior to analysing the level of receptor expression. Figures 5.1 and 5.2 depict histograms and graphs, respectively, showing surface expression of IGF-1R, CXCR4 and

CCR7 on MDA-MB-231 cells following the incubation with IGF-I whereas figures 5.3 and 5.4 show those following the stimulation with CXCL12. The results demonstrated in Figures 5.1 (A, B and C) and 5.2 (A and B) indicate that incubation with IGF-I decreased the number of IGF-1R- and CXCR4-positive cells as well as the level of mean fluorescent intensity (MFI) in both of the cell lines but did not have any effect on the number and MFI of CCR7-positive cells. The optimum reduction of both parameters assessing internalisation of IGF-1R and CXCR4 was observed at 24 hours and 3-6 hours after incubation with IGF-I, respectively. In contrast to the incubation with IGF-I, the data shown in figures 5.3 (A, B and C) and 5.4 (A and B) indicate that CXCL12 stimulation did not affect either the number or MFI of IGF-1R-, CXCR4- or CCR7-positive staining MDA-MB-231 cells. In marked contrast, treatment with CXCL12 at the same concentration resulted in the reduction of CXCR4 surface expression in B300-19/huCXCR4 cells, which stably express CXCR4, demonstrating the functionality of CXCL12 used in these experiments (Figure 5.3 D). Taken together, the data indicate that incubation of the cells with IGF-I caused co-internalisation of IGF-1R and CXCR4, but not CCR7 whereas that with CXCL12 did not have any effect on the surface expression of any of these receptors on MDA-MB-231 cells.

The internalisation of IGF-1R, CXCR4 and CCR7 was also investigated in the non-metastatic MCF-7 cells, which lack cross-activation between IGF-1R and CXCR4. Flow cytometric analysis was conducted to test the expression of IGF-1R, CXCR4 and CCR7 on surface of control cells and those incubated with IGF-I. As demonstrated in figures 5.5 (A, B and C) and 5.6 (A and B), even though the incubation with IGF-I did not cause any change in the number of IGF-1R-, CXCR4- or CCR7-positive cells, it decreased the MFI of IGF-1R, but not that of CXCR4 or CCR7 in MCF-7 cells, with the highest reduction

being observed at 3 hours post incubation. The data indicate that incubation of MCF-7 cells with IGF-I resulted in the internalisation of IGF-1R, but not of CXCR4 or CCR7.

5.2.2 Effect of incubation with IGF-I on the total level of IGF-1R, CXCR4 and CCR7 in MDA-MB-231 cells

To determine whether incubation with IGF-I causes the degradation of IGF-1R, CXCR4 or CCR7 in MDA-MB-231 cells, the cells were left untreated or treated with 10 nM IGF-I for 1, 6, and 24 hours prior to examining the total level of these receptors contained in cell lysates by performing Western blot analysis. Figure 5.7 (A) and (B) indicate that the incubation with IGF-I decreased the total level of IGF-1R, but not of CXCR4 or CCR7, indicating degradation of IGF-1R following incubation with IGF-I. The maximum reduction of the level of IGF-1R was found after 24 hour incubation with IGF-I. Of note, incubation with IGF-I did not result in the degradation of CXCR4, despite the fact that IGF-I induces internalisation of CXCR4 in MDA-MB-231 cells (Figures 5.1 (B) and 5.2 (A) and (B)). In addition, experiments were performed to assess degradation of these receptors in MCF-7 cells. The results also indicate that incubation of the cells with IGF-I caused a reduction of total level of IGF-1R, but not CXCR4 or CCR7 in a similar manner to that observed in MDA-MB-231 cells (data not shown).

5.2.3 Effect of IGF-I on CXCL12-mediated calcium mobilisation in MDA-MB-231 cells

As described earlier in section 5.2.1, incubation of cells with IGF-I resulted in the internalisation of CXCR4 in MDA-MB-231 cells. Additional experimentation was then conducted to determine whether incubation of the cells with IGF-I affects the functionality of CXCR4. In this study, the induction of calcium mobilisation, a common intracellular

response to chemokines (Hendey and Maxfield 1993; Henschler *et al.* 2003; Mandeville and Maxfield 1997), was employed to detect CXCR4 function in response to CXCL12. Initially, the effect of incubation of MDA-MB-231 cells with 100 nM CXCL12 or 10 nM IGF-I, doses that optimally induce chemotaxis in this cell line (Chapter 3, Section 3.2.2), on intracellular calcium ($[Ca^{2+}]_i$) levels was examined. The data showed that the addition of 10 nM IGF-I had no effect on the level of $[Ca^{2+}]_i$ in MDA-MB-231 cells whereas that of 100 nM CXCL12 resulted in a rapid, transient increase in $[Ca^{2+}]_i$ levels (Figure 5.8).

Next, the effect of pretreatment with IGF-I on CXCL12-mediated calcium mobilisation in MDA-MB-231 cells was examined. The cells were left unexposed or exposed to buffer or 10 nM IGF-I for increasing period of times 3, 6 and 24 hours prior to examination of their response to 100 nM CXCL12. The representative data shown in figure 5.9 (A) and (B) indicate that the exposure of the cells to 10 nM IGF-I, for 3 or 6 hours, decreased the magnitude of the response to CXCL12. The experiment examining the effect of 24 hour exposure to IGF-I showed a similar result (data not shown). These data indicate that the stimulation of MDA-MB-231 cells with IGF-I causes a decrease of CXCR4 activity, consistent with the data in section 5.2.1 demonstrating a decrease of surface CXCR4 expression in response to IGF-I.

5.2.4 Signalling pathways involved in the internalisation and degradation of IGF-1R and CXCR4

To investigate signal transduction pathways involved in the internalisation and degradation of IGF-1R and CXCR4 in MDA-MB-231 cells, different inhibitors of downstream signalling molecules, G-proteins, PI3Ks and MAPKs, were employed. The cells were treated with PTX (100 ng/ml), Ly294002 (100 μ M) and PD980590 (20 μ M),

inhibitors of G-proteins, PI3Ks and MAPKs respectively, prior to incubation for 24 hours with or without 10 nM IGF-I. All inhibitors were used at the optimum concentrations, based on previous studies showing their inhibitory effect on IGF-I-mediated chemotactic response in MDA-MB-231 cells (see Chapter 4 and reference therein (Dunn *et al.* 2001)). Control cells and those incubated with IGF-I were then examined in terms of the surface expression of the two receptors by flow cytometry (Figure 5.10) and total receptor protein in whole cell lysates by Western blot analysis (Figure 5.11). The data shown in figure 5.10 indicate that the internalisation of CXCR4 following incubation with IGF-I is partially inhibited by pretreatment of the cells with PTX or PD980590 and completely blocked by that with Ly294002. However, none of the inhibitors altered the level of IGF-I-induced IGF-1R internalisation. These data indicate the involvement of G-proteins, PI3Ks and MAPKs in the process of CXCR4, but not of IGF-1R, internalisation induced by IGF-I in MDA-MB-231 cells. Figure 5.11 (A) and (B) show the effect of these inhibitors on the degradation of IGF-1R in response to IGF-I stimulation. The results from these experiments indicate that pretreatment with PTX and Ly294002 partially inhibited the IGF-I-induced degradation of IGF-1R while that with PD980590 had no effect, suggesting significant roles of G-proteins and PI3Ks, but not of MAPKs in the IGF-I-mediated IGF-1R degradation in MDA-MB-231 cells. Notably, even though the activity of G-proteins and PI3Ks are not involved in the process of IGF-1R internalisation, they have a partial effect on the degradation of the receptor.

5.3 Discussion and Conclusion

The studies described in this chapter have provided evidence for coregulation of CXCR4 and IGF-1R expression and function in the highly metastatic MDA-MB-231 cells. While CXCL12 fails to induce downregulation of either CXCR4 or IGF-1R, the two receptors are coregulated by IGF-I. IGF-I induces internalisation of CXCR4 without degradation. Moreover, the level of internalisation of CXCR4 induced by IGF-I is sufficiently high to alter the level of activation of CXCR4 by its ligand CXCL12. On the other hand, downregulation of IGF-1R by IGF-I involves both internalisation and degradation. IGF-1R degradation is mediated through signal transduction involving G-proteins and potentially CXCR4. Moreover, the downregulation process of both receptors is also controlled at the level of downstream activation systems, PI3Ks and MAPKs. Whereas IGF-I-mediated CXCR4 internalisation is dependent on the activation of both PI3Ks and MAPKs, IGF-1R degradation by IGF-I requires only PI3Ks. The coregulation of CXCR4 and IGF-1R observed in breast cancer cells may have a major impact on biological outcomes of activation of these receptors in both physiological and pathological conditions including breast cancer metastasis.

Numerous studies indicate an impact of GPCR/RTK interaction on the signal transduction promoting cellular responses mediated by these receptors. For instance, as pointed out in the previous chapter, a physical association of PDGFRs and S1P₁ receptors leads to the integration of mitogenic signalling in response to their ligands, PDGF and S1P, in human embryonic kidney 293 and airway smooth muscle cells (Alderton *et al.* 2001; Waters *et al.* 2003). Transactivation of EGFR by the ligand for CXCR2, CXCL8 (IL-8), is required for CXCL8-mediated migration of endothelial cells (Schraufstatter *et al.* 2003). IGF-I/IGF-1R

also transactivates CCR5-and CXCR4-mediated signal transduction participating in IGF-I-mediated migration of certain breast cancer cell lines (Akekawatchai *et al.* 2005; Mira *et al.* 2001). It is also apparent that the GPCR/RTK cross-talk plays an important role in downregulation of receptors even though this aspect has not been as extensively investigated as the activation pathways. Amongst the few published studies, it has been demonstrated that overexpression of HER2/ErbB2 enhances CXCR4 expression and inhibits ligand-induced CXCR4 degradation in breast cancer cells (Li *et al.* 2004). Accumulating recent studies also indicate the involvement of β -arrestins and GRKs, major adaptor proteins required for desensitisation of GPCRs, in regulation of growth factor RTKs (Hupfeld and Olefsky 2007).

In the present study, coregulation of CXCR4 and IGF-1R expression and function by IGF-I in MDA-MB-231 cells has been demonstrated. As outlined in section 1.3.2.2, it is well established that the activity of RTKs including IGF-1R can be downregulated by the processes of internalisation and degradation. In accordance with several previous studies (Carelli *et al.* 2006; Chow *et al.* 1998; Girnita *et al.* 2005), the data shown here indicate that exposure of both MDA-MB-231 and MCF-7 cells to IGF-I resulted in the internalisation and degradation of IGF-1R. Due to the evidence for transactivation of CXCR4 by IGF-I in MDA-MB-231 cells (see Chapter 4), it was hypothesised that incubation of the cells with IGF-I may also downregulate CXCR4 expression and activity. As predicted, the results showed that there was co-internalisation of IGF-1R and CXCR4 following prolonged stimulation of the cells with IGF-I. This phenomenon led to a decrease in the number of CXCR4 receptors on cell surface and consequently a reduction of the extent of CXCR4-mediated signal transduction (assessed by the induction of calcium mobilisation induced by CXCL12). Of importance, this co-internalisation of IGF-

1R and CXCR4 was not observed in MCF-7 cells. This is entirely consistent with the finding in the previous chapter that there is no transactivation of CXCR4 by IGF-I in MCF-7 cells due to non-functional CXCR4 (Chapters 3 and 4). Taken together, the data indicate that coregulation of expression and function of the two receptors by IGF-I in MDA-MB-231 cells appears to require transactivation of the CXCR4/G-protein signalling pathway. In addition, there is a degree of selectivity in the system because these observations do not extend to the closely-related GPCR, CCR7. This is probably because of a lack of cross-activation between IGF-1R- and CCR7-mediated signal transduction in MDA-MB-231 cells. However, in the present study, the issue of interaction between these two receptors could not be unequivocally clarified and remains unclear.

In contrast to the extensive data demonstrating the ability of IGF-I to induce downregulation of CXCR4 and IGF-1R, experiments demonstrated that CXCL12 was unable to induce internalisation of either IGF-1R or CXCR4, in MDA-MB-231 cells despite the fact that CXCL12 was a potent inducer for chemotactic response in this cell line and that CXCL12 stimulation induced the internalisation of CXCR4 in the control B300-19/huCXCR4 cells. This is not necessarily surprising with respect to IGF-1R, as the results of the previous chapter indicate that CXCL12 does not transactivate IGF-1R. However, it is unusual that CXCL12 does not induce internalisation of CXCR4. Moreover, despite CXCR4 internalisation mediated by IGF-I in this cell line, degradation of the receptors was not observed. Together, these observations are highly unusual because CXCR4 internalisation and degradation are common features of the chemokine receptor deactivation shown in several cellular systems (Cai *et al.* 2004; Fernandis *et al.* 2002; Marchese and Benovic 2001; Marchese *et al.* 2003; Shen *et al.* 2001). It is possible that, in this cell type, activated CXCR4 may not undergo degradation. Rather, as

demonstrated in a number of studies with respect to several chemokine receptors including CXCR4 (Amara *et al.* 1997; Fan *et al.* 2004; Forster *et al.* 1998; Vines *et al.* 2003), they may be subjected to rapid recycling in order to maintain the number of receptors on the cell surface and the level of receptor activity required for cellular responses. The aberrant downregulation of CXCR4 observed in MDA-MB-231 cells may support a role for chemokine receptors, in particular of CXCR4, in acquisition of metastatic phenotype in breast cancer cells.

Generally, as described in sections 1.3.2.2 and 1.3.3.2, IGF-1R activates a number of signal transduction pathways, requiring tyrosine-phosphorylation of the receptors and the activity of adaptor proteins such as IRS, Shc and Grb10. The activation of CXCR4 results in a dissociation of $G_i\alpha$ and the $G\beta\gamma$ complex, both of which subsequently activate downstream signalling systems, as well as serine/threonine phosphorylation of the receptor at the C-terminal region that is known to be responsible for desensitisation signals. It is apparent that both receptors share major downstream signalling systems including PI3K and MAPK pathways. The data described in chapter 4 indicate that in MDA-MB-231 cells IGF-I is capable of activating both a classical IGF-1R-mediated tyrosine kinase-dependent and an alternative CXCR4/G-protein signalling pathway. Indeed, it appears that IGF-I induces a dissociation of $G_i\alpha$ and $G\beta$ subunits from the CXCR4, in a similar manner to that of CXCL12 (see Chapters 3 and 4). However, it was demonstrated in the present chapter that IGF-I failed to induce calcium mobilisation, an intracellular event downstream of PLC activation, despite the fact that this event was observed following stimulation of the cells with CXCL12. The PLC activation pathway is well established as a common downstream system of the activation of chemokine receptors including CXCR4 and is shown to be involved in chemokine-mediated cell

migration in leukocytes (Hendey and Maxfield 1993; Henschler *et al.* 2003; Thelen 2001). This observation indicates that, despite the ability of IGF-I to activate CXCR4/G-protein dependent signal transduction, the signalling by IGF-I is different from that by CXCL12 in MDA-MB-231 cells at least at the level of PLC activation. This issue remains to be further explored.

It is well documented that, similar to other chemokine receptors, ligand-induced CXCR4 internalisation requires phosphorylation of the C-terminus domain of the activated receptors presumably by GRKs, allowing β -arrestins to bind to the receptor complex and subsequently mediate receptor internalisation (Bunemann and Hosey 1999; Cheng *et al.* 2000; DeFea 2007; Mellado *et al.* 2001; Orsini *et al.* 1999). Most previous studies indicate that chemokine receptor phosphorylation and internalisation is a PTX-insensitive process (Amara *et al.* 1997; Forster *et al.* 1998; Giannini and Boulay 1995; Signoret *et al.* 1998). The process has been shown to be regulated by signalling at C-terminal region of the phosphorylated receptors (Cheng *et al.* 2000; Futahashi *et al.* 2007; Signoret *et al.* 1998). However, receptor phosphorylation-independent internalisation of CXCR4 by CXCL12 has also been reported in rat basophilic leukaemia cells adding to the complexity of this issue (Haribabu *et al.* 1997). The data generated in the present study demonstrate a novel form of chemokine receptor internalisation that is induced by a heterologous ligand, IGF-I. It was shown that prior treatment of MDA-MB-231 cells with PTX partially inhibited IGF-I-mediated CXCR4 internalisation suggesting that internalisation of CXCR4 by IGF-I is modulated by both G_i/α -dependent and-independent mechanisms. Based on those previous studies (Cheng *et al.* 2000; Futahashi *et al.* 2007; Signoret *et al.* 1998), the PTX-

insensitive CXCR4 internalisation induced by IGF-I presumably resulted from the activation of receptor phosphorylation-dependent pathways.

It has previously been shown that IGF-I activates pathways of PI3K and MAPK, which are responsible for cell migration and survival of breast cancer cells (Dunn *et al.* 2001; Povsic *et al.* 2003; Sliva *et al.* 2002). A previous report also indicates the activation of G_i-coupled PI3Ks and MAPKs downstream of CXCL12/CXCR4, which is obligatory for chemotaxis of T lymphocytes. That study demonstrated that CXCL12-induced MAPK (ERK1/2) activation was inhibited by PI3K inhibitors indicating that the activation of MAPKs is downstream of that of PI3Ks (Sotsios *et al.* 1999). As shown in the present study, the inhibition of PI3Ks and MAPKs decreases the level CXCR4 internalisation by IGF-I in MDA-MB-231 cells indicating essential roles of these signalling systems in this process. While there is no direct evidence showing involvement of MAPK activation in GPCR internalisation, a few studies show the contribution of the activity of PI3Ks to GPCR internalisation (Drake *et al.* 2006; Naga Prasad *et al.* 2001). As demonstrated in the studies with respect to β 2-adrenergic receptors, PI3Ks constitutively form the complex with GRKs. The ligation of receptors by ligands leads to translocation of the complex to activated receptors and subsequent recruitment of an adapter AP-2 protein and clathrin, resulting in receptor endocytosis (Naga Prasad *et al.* 2002).

As is the case with other RTKs, IGF-1R undergoes ubiquitination and degradation processes, which are preceded by tyrosine-phosphorylation of the receptors (Bache *et al.* 2004; Carelli *et al.* 2006; Lipkowitz 2003). Several adapter proteins have been identified as regulators for these processes. For example, an adaptor protein Grb10 has been shown to interact with a ubiquitin protein ligase Nedd-4, and this complex could mediate the

targeting of IGF-1R to endocytotic machinery (Vecchione *et al.* 2003). Another adapter protein, MDM2, has also been shown to serve as a ubiquitin ligase in IGF-1R ubiquitination and degradation (Girnita *et al.* 2003). Accumulating recent studies have revealed a role for GPCRs and their associated proteins, GRKs and β -arrestins, in signal transduction and downregulation of RTKs (Hupfeld and Olefsky 2007; Waters *et al.* 2004). A physical association of G-proteins, GRKs or β -arrestins with growth factor RTKs including IGF-1R has been shown (Akekawatchai *et al.* 2005; Alderton *et al.* 2001; Dalle *et al.* 2001), and clathrin-mediated endocytosis of IGF-1R has been shown to require β -arrestin activity (Lin *et al.* 1998). β -arrestins also act as an adapter for MDM2, which is an E3 ubiquitin ligase. This participates in IGF-1R ubiquitination, and depletion of β -arrestin could eliminate degradation of IGF-1R (Girnita *et al.* 2005). In the present study, one aspect of IGF-I-mediated IGF-1R downregulation in MDA-MB-231 cells was investigated, with specific emphasis on involvement of CXCR4/G-protein signalling in this process. The data show that $G_{i\alpha}$, PI3Ks and MAPKs are not necessary for IGF-1R internalisation by IGF-I in MDA-MB-231 cells as shown by the experiment that neither PTX, Ly294002 nor PD980590 had any effect on IGF-I-induced IGF-1R internalisation. Nonetheless, a partial inhibitory effect of PTX on the degradation process of IGF-1R was observed in the cells indicating involvement of GPCR-mediated signal transduction in the degradation process. Due to the evidence for transactivation of CXCR4 by IGF-I as well as co-internalisation of CXCR4 and IGF-1R in MDA-MB-231 cells, it is possible that activation of CXCR4 signalling is partially responsible for IGF-I-mediated IGF-1R degradation. In addition, the data shown here demonstrate that the activation pathway of PI3Ks, but not of MAPKs, is partially involved in the degradation process of IGF-1R. As mentioned earlier, PI3Ks could be activated by both IGF-1R-mediated tyrosine kinase and

CXCR4/G-protein signalling pathways. A previous study indicates that IGF-I-induced PI3K activation is dependent on the activity of the GPCR-associated proteins β -arrestins. In β -arrestin double knockout mouse fibroblast cells, expression of β -arrestin 1 is required for IGF-I-induced activation of PI3Ks and subsequent activation of PKB/Akt, leading to anti-apoptosis. This pathway is independent of the tyrosine kinase activity of IGF-1R (Povsic *et al.* 2003). However, in the case of PI3K activation of IGF-I-mediated IGF-1R degradation in MDA-MB-231 cells, the signalling elements upstream of PI3Ks could not be identified.

The data generated in the present study indicate a potentially important role for CXCR4 and its downstream signalling systems in IGF-1R downregulation by IGF-I in MDA-MB-231 cells. Nonetheless, two main aspects have to be further clarified. Firstly, direct evidence to implicate CXCR4 signalling in IGF-1R degradation is required. To achieve this, either the effect of PTX on IGF-1R degradation in MCF-7 cells, lacking CXCR4 transactivation by IGF-I, or the IGF-I-induced degradation of IGF-1R in siRNA-mediated CXCR4 knockdown MDA-MB-231 cells will need to be examined. Secondly, the roles of GPCR associated proteins, GRKs and β -arrestins, which are well-known as classical signalling proteins required for GPCR deactivation will need to be investigated in IGF-I-mediated CXCR4 and IGF-1R downregulation in MDA-MB-231 cells. Together, these aspects will provide clearer mechanisms for the coregulation of these two receptors in this cell type.

In conclusion, the data generated in this chapter have provided evidence for the coregulation of CXCR4 and IGF-1R by IGF-I in the highly metastatic MDA-MB-231 cells. IGF-I/IGF-1R is capable of regulating expression and function of CXCR4 while

downregulation of IGF-1R by IGF-I is dependent on signal transduction of GPCRs. The current study also reveals mechanisms for downregulation of CXCR4 and IGF-1R, which may have a major impact on an understanding of biological outcomes of IGF-1R and CXCR4 activation which have been shown in a wide range of cellular systems. Importantly, it is apparent that the downregulation of CXCR4 and IGF-1R is not observed in the non-metastatic MCF-7 cells. This is completely consistent with the previous evidence indicating lack of transactivation between CXCR4 and IGF-1R in this cell line. The data presented in this chapter therefore further supports the importance of CXCR4 and IGF-1R cross-talk in breast cancer metastasis.

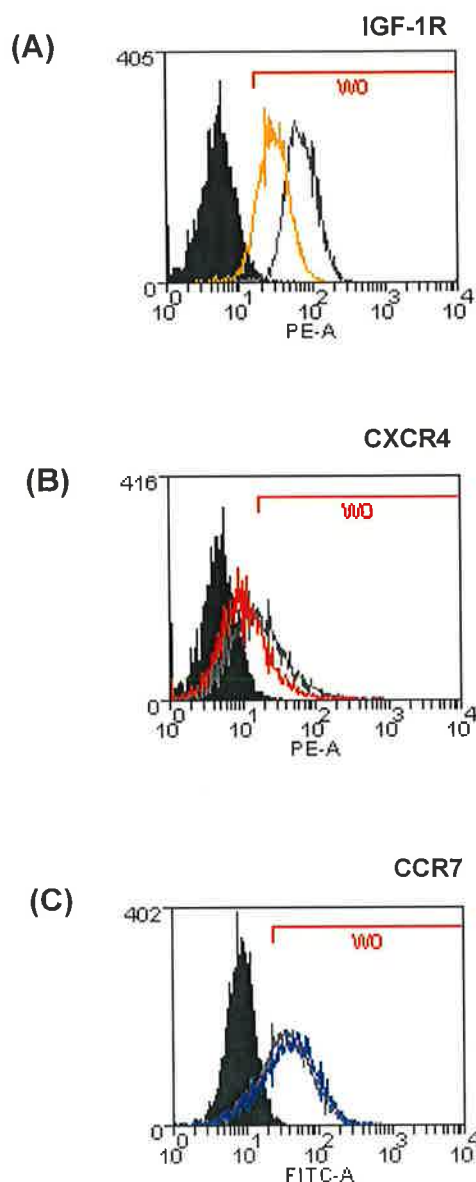


Figure 5.1 Flow cytometric histograms demonstrating effect of IGF-I on surface expression of IGF-1R, CXCR4 and CCR7 on MDA-MB-231 cells

In serum-free condition, cells were left untreated for 24 hours or treated with 10 nM IGF-I for 1, 3, 6 and 24 hours followed by examining the expression of IGF-1R, CXCR4 and CCR7 on surface of the cells by flow cytometry using specific Abs to these receptors (mouse monoclonal anti-IGF-1R (7C2), anti-CXCR4 (12G5) and rat monoclonal anti-CCR7 respectively). The representative histograms demonstrating surface expression of IGF-1R (A), CXCR4 (B) and CCR7 (C) on the untreated or IGF-I-treated cells at 24 hour of treatment are shown. The filled black histograms are isotype control Ab staining. The immunostaining with anti-IGF-1R, -CXCR4 and -CCR7 in untreated cells is depicted as grey blanked histograms whereas that in IGF-I-treated cells is shown as yellow, red and blue blanked histograms for anti-IGF-1R, -CXCR4 and -CCR7, respectively.

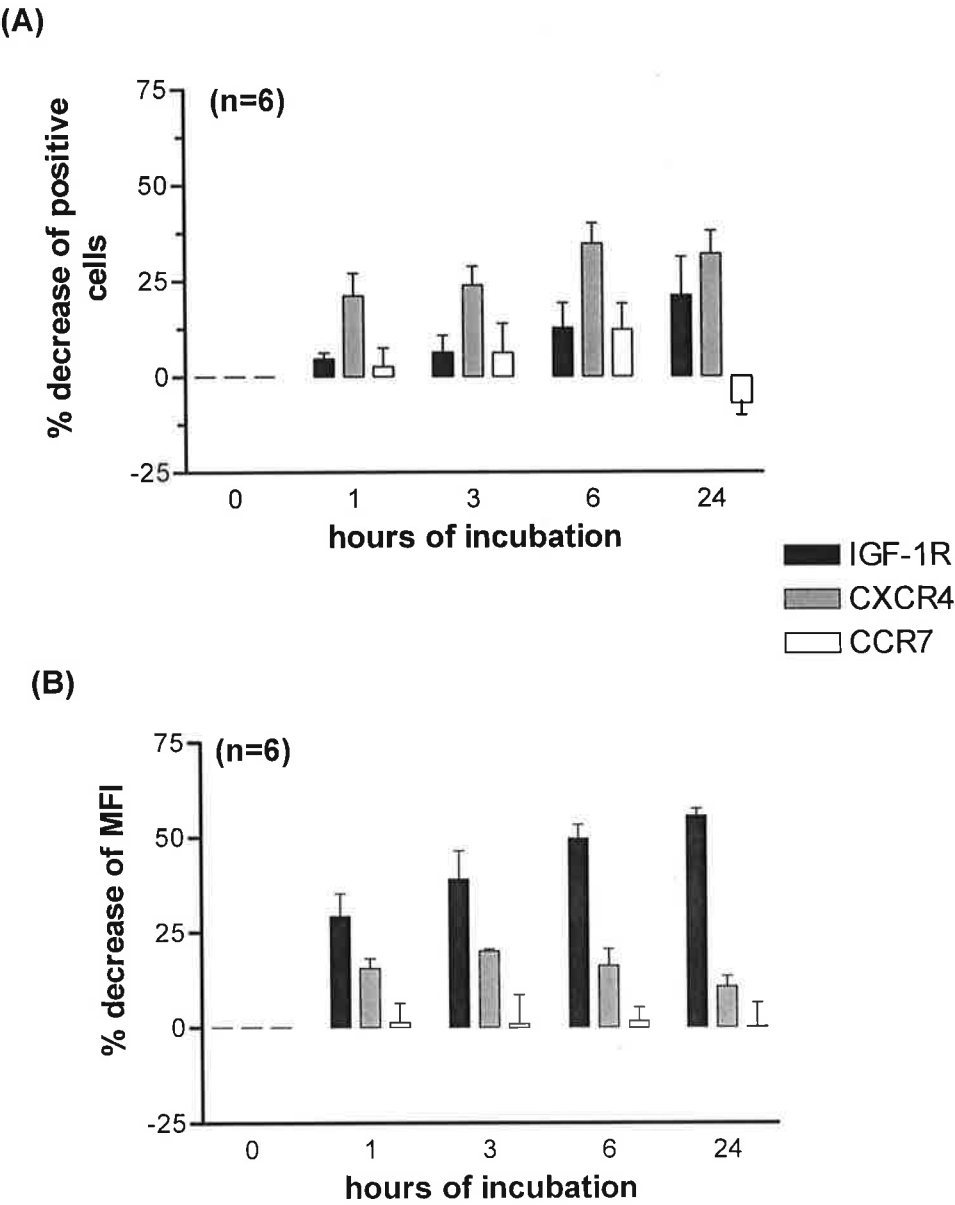


Figure 5.2 Co-internalisation of IGF-1R and CXCR4, but not CCR7, induced by IGF-I in MDA-MB-231 cells

The cells were left untreated for 24 hours or treated with 10 nM IGF-I for 1, 3, 6 and 24 hours in serum-free medium followed by examining the expression of IGF-1R, CXCR4 and CCR7 on surface of the cells by flow cytometry using specific Abs to these receptors (mouse monoclonal anti-IGF-1R (7C2), anti-CXCR4 (12G5) and rat monoclonal anti-CCR7 respectively). Loss of receptors on the cell surface following incubation with IGF-I was analysed and presented as % decrease of positive staining cells (A) and % decrease of mean fluorescent intensity (MFI) (B). Data are mean $\pm$ S.E.M. pooled from 6 separate experiments.

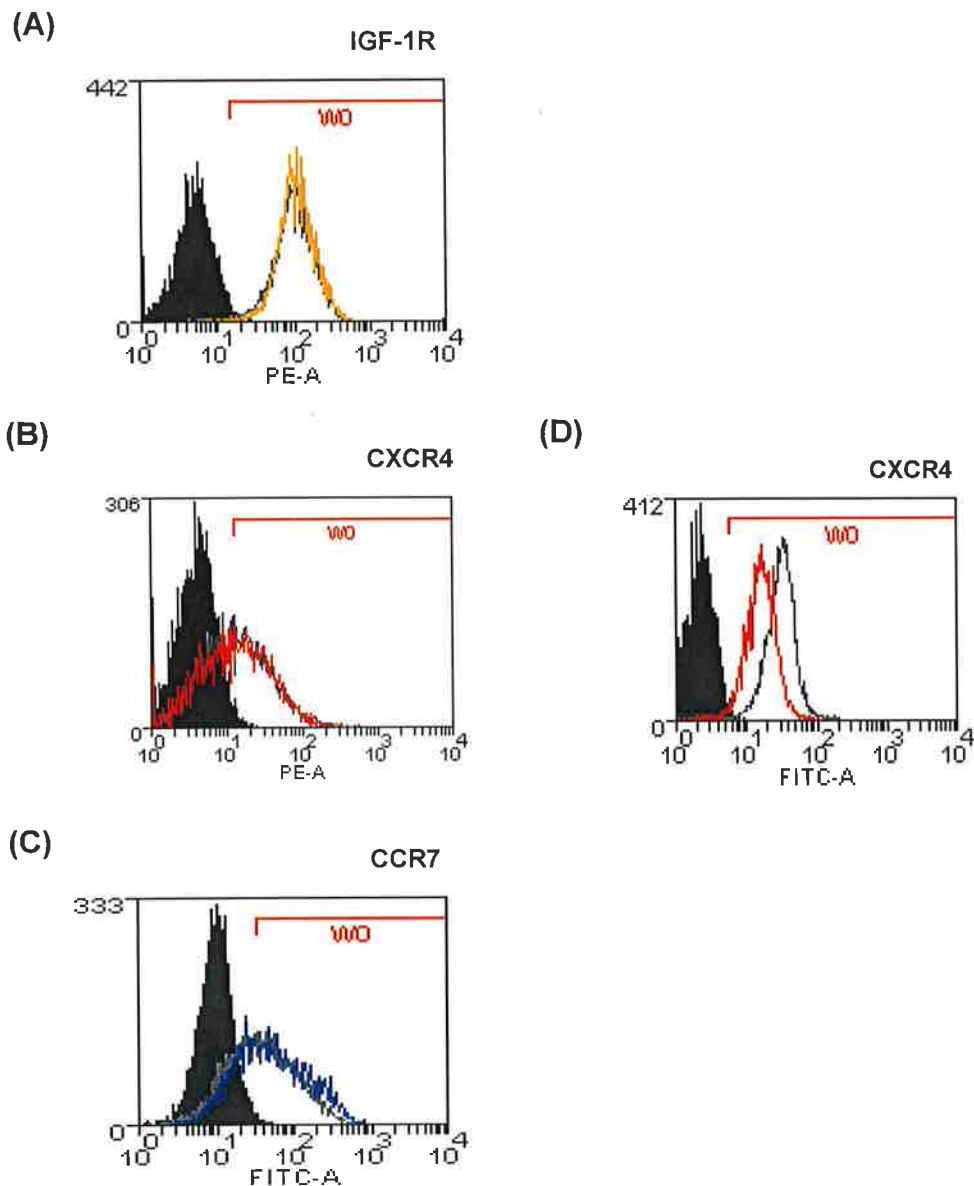


Figure 5.3 Flow cytometric histograms demonstrating the effect of CXCL12 on surface expression of IGF-1R, CXCR4 and CCR7 on MDA-MB-231 cells

In serum-free condition, the cells were left unexposed for 24 hours or exposed to 100 nM CXCL12 for 1, 3, 6 and 24 hours. The expression of IGF-1R, CXCR4 and CCR7 on surface of the cells was examined by flow cytometry using specific Abs to these receptors (mouse monoclonal anti-IGF-1R (7C2), anti-CXCR4 (12G5) and rat monoclonal anti-CCR7 respectively). (A), (B) and (C) are representative histograms showing the expression of IGF-1R, CXCR4 and CCR7, respectively in untreated and CXCL12-treated cells at 24 hour of treatment. (D), the surface expression of CXCR4 on unstimulated and CXCL12-stimulated B300-19/huCXCR4 cells, stably expressing functional CXCR4, at 1 hour of stimulation. The filled black histograms are isotype control Ab staining. The immunostaining with anti-IGF-1R, -CXCR4 and -CCR7 in untreated cells is depicted as grey blanked histograms whereas that in CXCL12-treated cells is shown as yellow, red and blue blanked histograms for anti-IGF-1R, -CXCR4 and -CCR7 respectively.

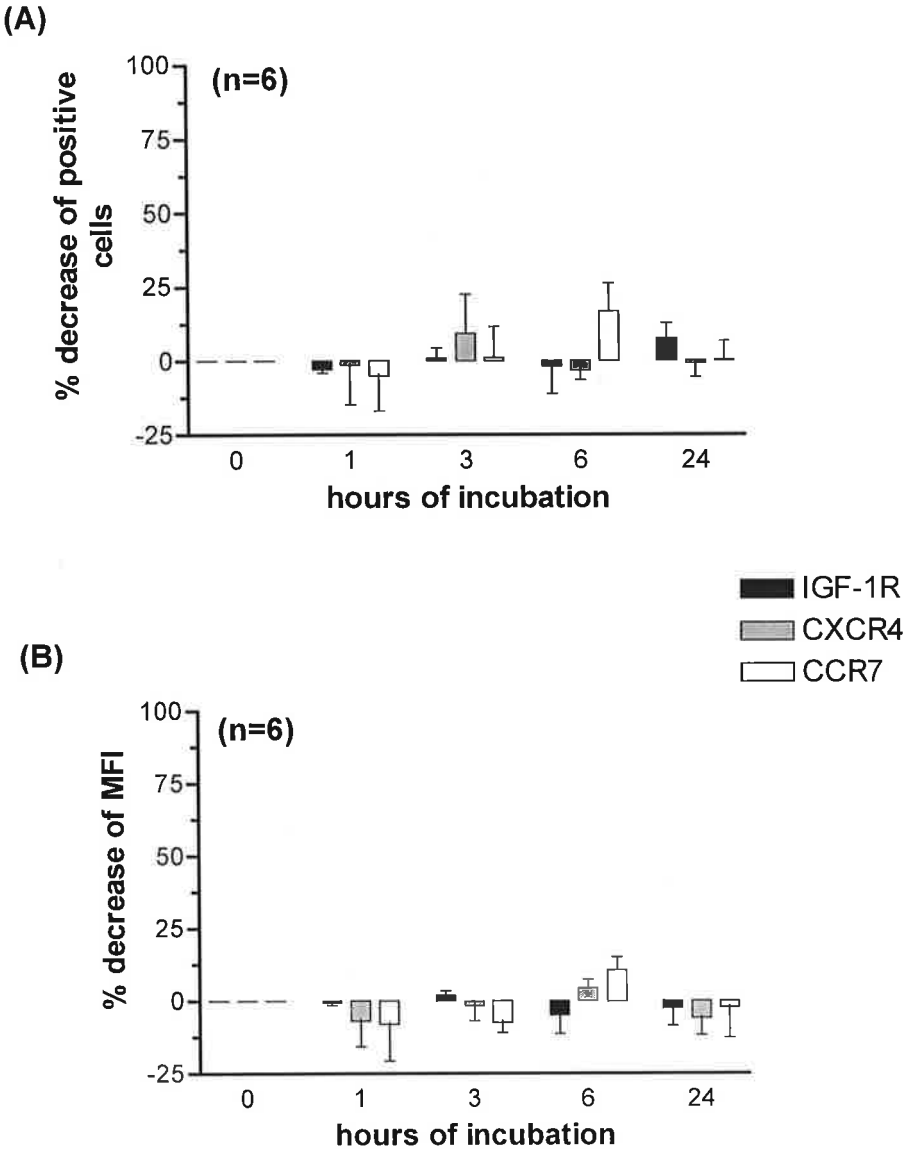


Figure 5.4 Lack of internalisation of IGF-1R, CXCR4 and CCR7 induced by CXCL12 in MDA-MB-231 cells

The cells were left untreated for 24 hours or treated with 100 nM CXCL12 for 1, 3, 6 and 24 hours in serum-free medium. Flow cytometric analysis was performed to measure the expression of surface IGF-1R, CXCR4 and CCR7 on control cells and those treated with CXCL12 using specific Abs to these receptors (mouse monoclonal anti-IGF-1R (7C2), anti-CXCR4 (12G5) and rat monoclonal anti-CCR7 respectively). Reduction of surface receptors was calculated and expressed as % decrease of positive staining cells (A) and % decrease of mean fluorescent intensity (MFI) (B). Data are mean $\pm$ S.E.M. pooled from 6 separate experiments.

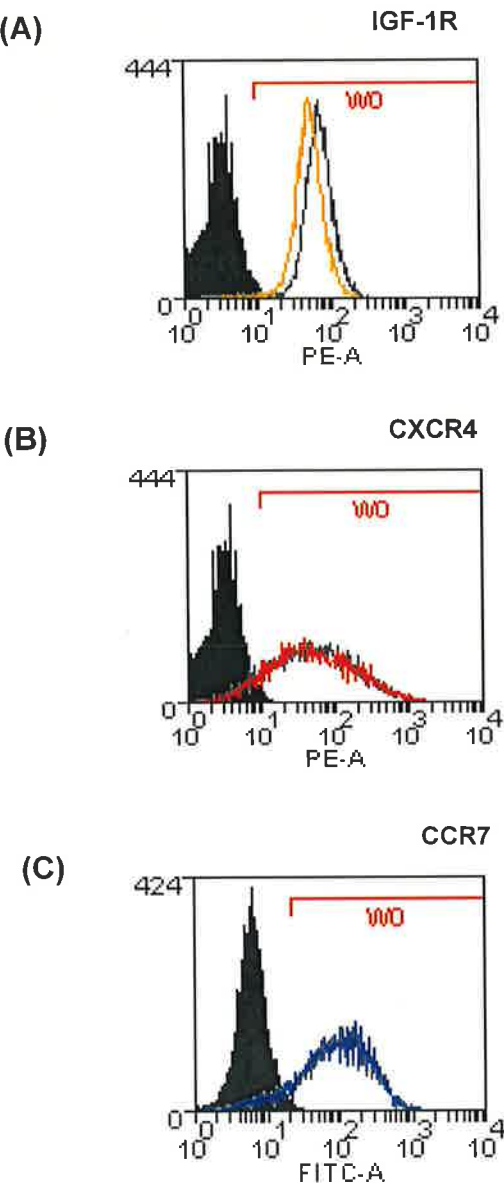


Figure 5.5 Flow cytometric histograms demonstrating the effect of IGF-I on expression of IGF-1R, CXCR4 and CCR7 on MCF-7 cells

The cells were left untreated for 24 hours or treated with 10 nM IGF-I for 1, 3, 6 and 24 hours in serum-free condition. The surface expression of IGF-1R, CXCR4 and CCR7 was examined by flow cytometric analysis using specific Abs to these receptors (mouse monoclonal anti-IGF-1R (7C2), anti-CXCR4 (12G5) and rat monoclonal anti-CCR7 respectively). (A), (B) and (C) are representative histograms of 5 separate experiments showing the expression of IGF-1R, CXCR4 and CCR7 respectively in control cells and those incubated with IGF-I at 24 hour. The filled black histograms are isotype control Ab staining. The immunostaining with anti-IGF-1R, -CXCR4 and -CCR7 in untreated cells is depicted as grey blanked histograms whereas that in IGF-I-treated cells is shown as yellow, red and blue blanked histograms for anti-IGF-1R, -CXCR4 and -CCR7, respectively.

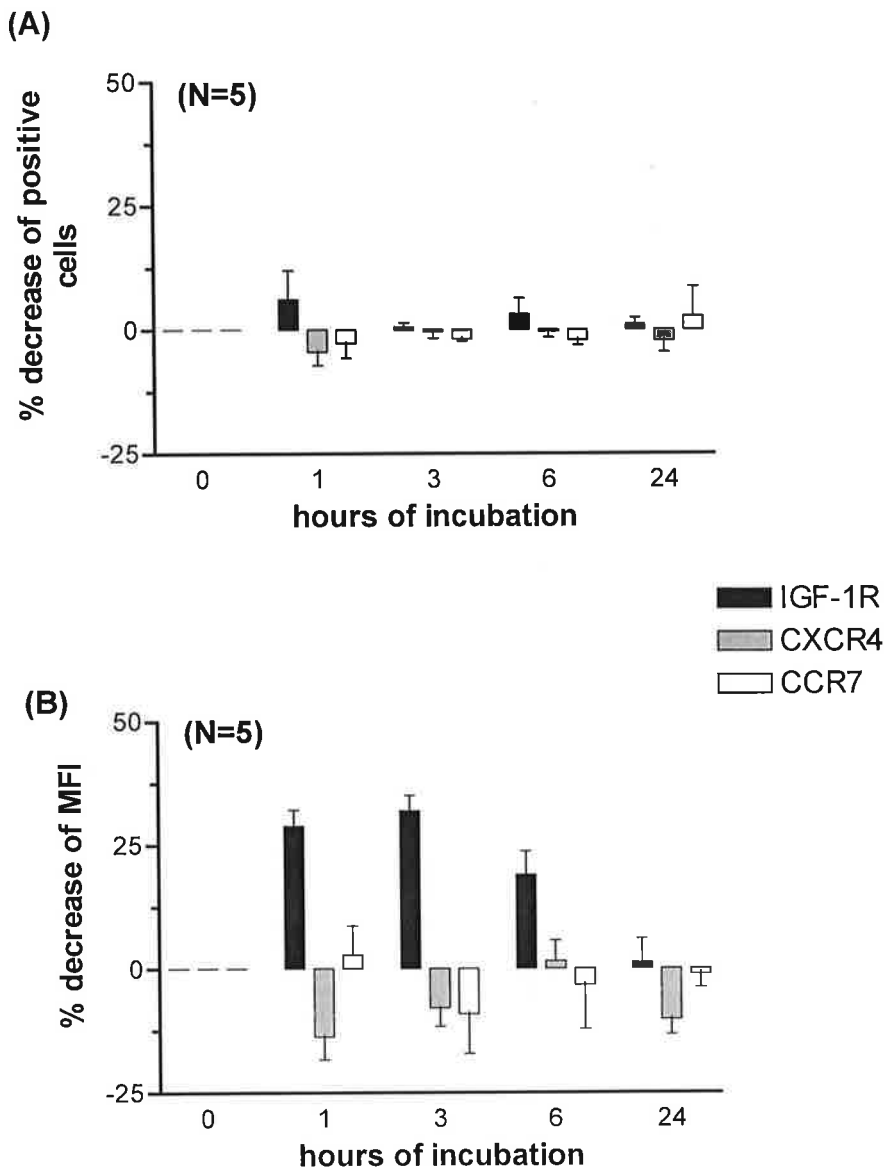


Figure 5.6 Internalisation of IGF-1R, but not CXCR4 and CCR7, induced by IGF-1 in MCF-7 cells

The cells were left untreated for 24 hours or treated with 10 nM IGF-I for 1, 3, 6 and 24 hours in serum-free condition. The surface expression of IGF-1R, CXCR4 and CCR7 on untreated and IGF-I-treated cells was examined by flow cytometric analysis using specific Abs to these receptors (mouse monoclonal anti-IGF-1R (7C2), anti-CXCR4 (12G5) and rat monoclonal anti-CCR7 respectively). Loss of receptors on cell surface following IGF-I stimulation was analysed and presented as % decrease of positive staining cells (A) and % decrease of mean fluorescent intensity (MFI) (B). Data are mean $\pm$ S.E.M. pooled from 5 separate experiments.

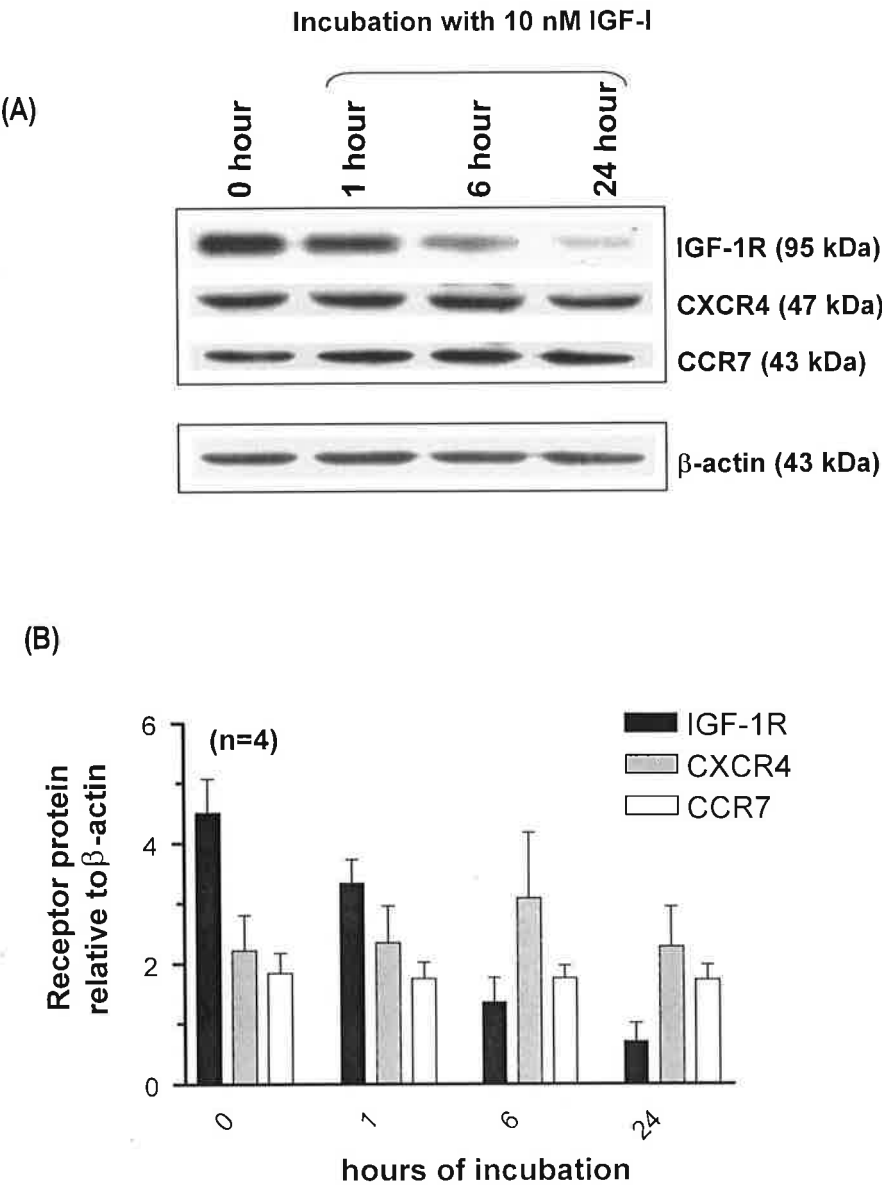


Figure 5.7 Degradation of IGF-1R, but not CXCR4 and CCR7, induced by IGF-I stimulation in MDA-MB-231 cells

The cells were left untreated for 24 hours or incubated with 10 nM IGF-I for 1, 6 or 24 hours in serum-free condition. Cell lysates were prepared and subjected to SDS-PAGE and Western blot analysis for the expression of IGF-1R, CXCR4 and CCR7 using specific Abs. (A), a representative Western blot showing the receptor content detected in the cell lysates. Western blot for β-actin demonstrates the equivalence of total protein loading. (B), the levels of receptor content in the cell lysates were expressed relative to the levels of β-actin protein. Data are mean ± S.E.M. pooled from 4 separate experiments.

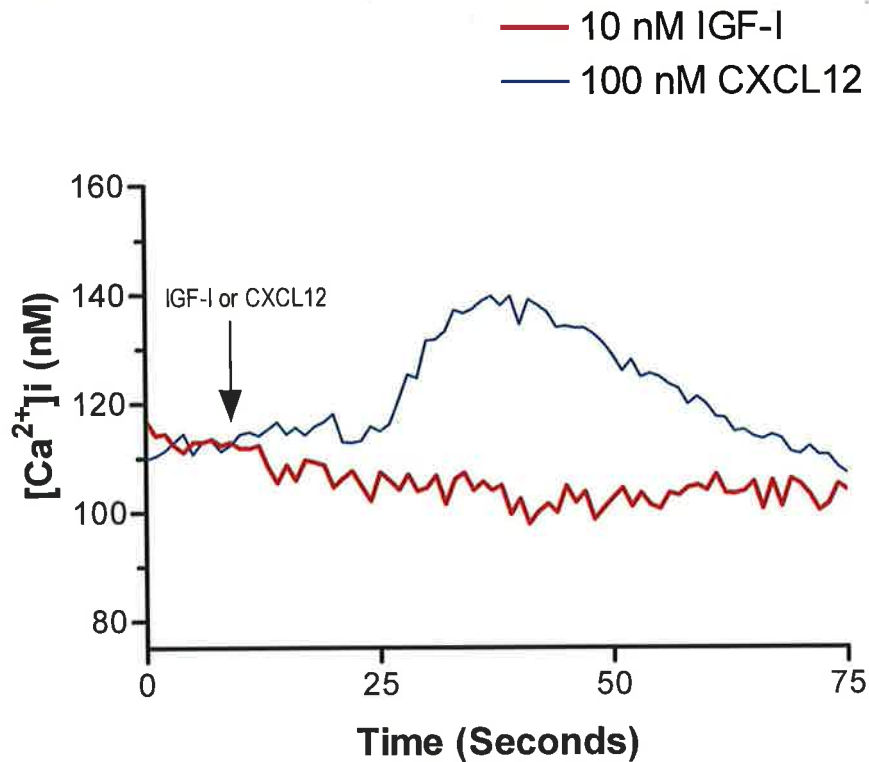


Figure 5.8 *Effect of IGF-I and CXCL12 on intracellular calcium levels in MDA-MB-231 cells*

Cells were cultured in serum-free medium for 2-3 hours before being labelled with Fura-2/AM. The cells were then treated with either 10 nM IGF-I or 100 nM CXCL12, and assayed for intracellular calcium mobilisation by monitoring intracellular calcium-related fluorescence changes on a luminescence spectrometer. The data shown are representative of at least 3 experiments with similar results.

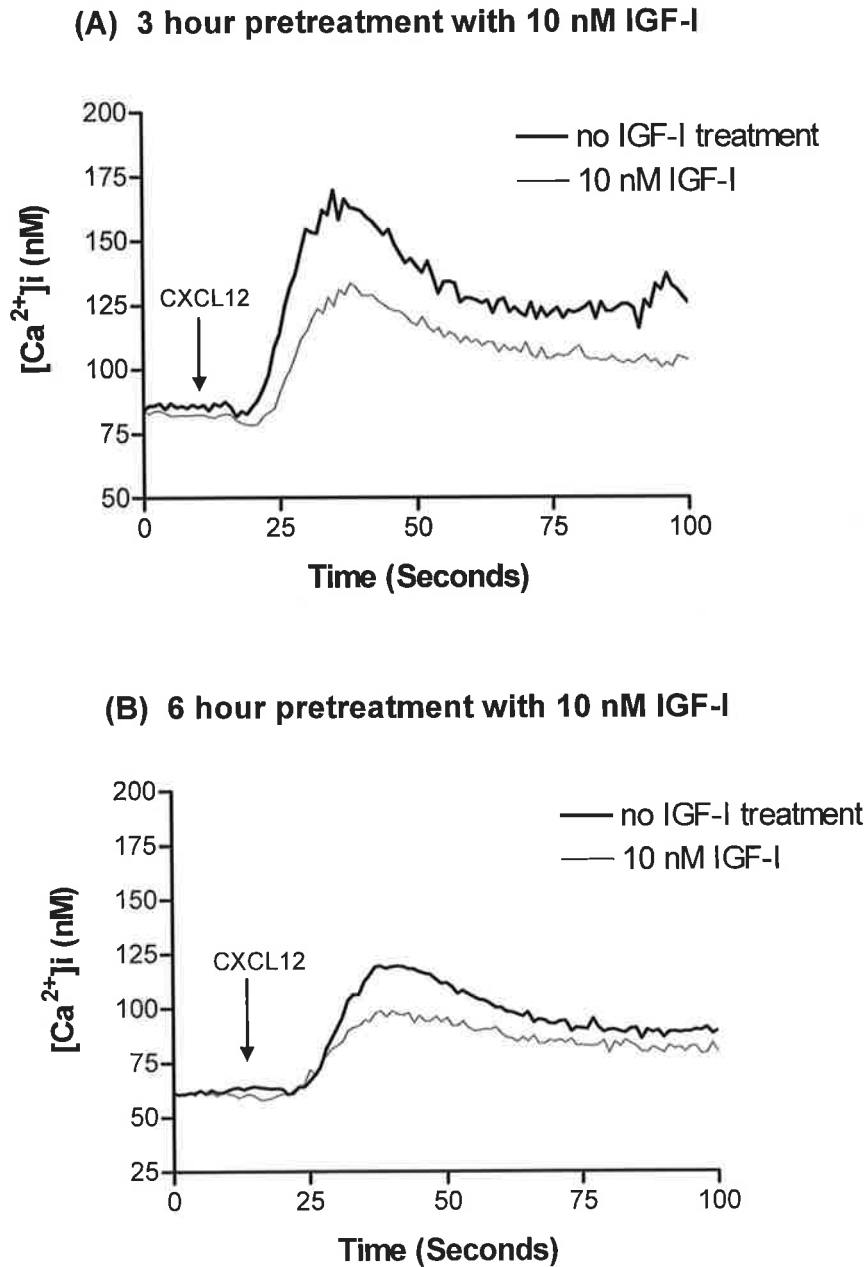


Figure 5.9 Effect of IGF-I treatment on CXCL12-mediated calcium influx in MDA-MB-231 cells

MDA-MB-231 cells were left untreated or treated with 10 nM IGF-I for 3 and 6 hours in serum-free condition. The cells were then incubated with Fura-2/AM and subsequently evaluated for intracellular calcium mobilisation in response to 100 nM CXCL12. The data shown are representative results showing CXCL12-mediated calcium mobilisation in the untreated or IGF-I treated cells at (A) 3 and (B) 6 hour.

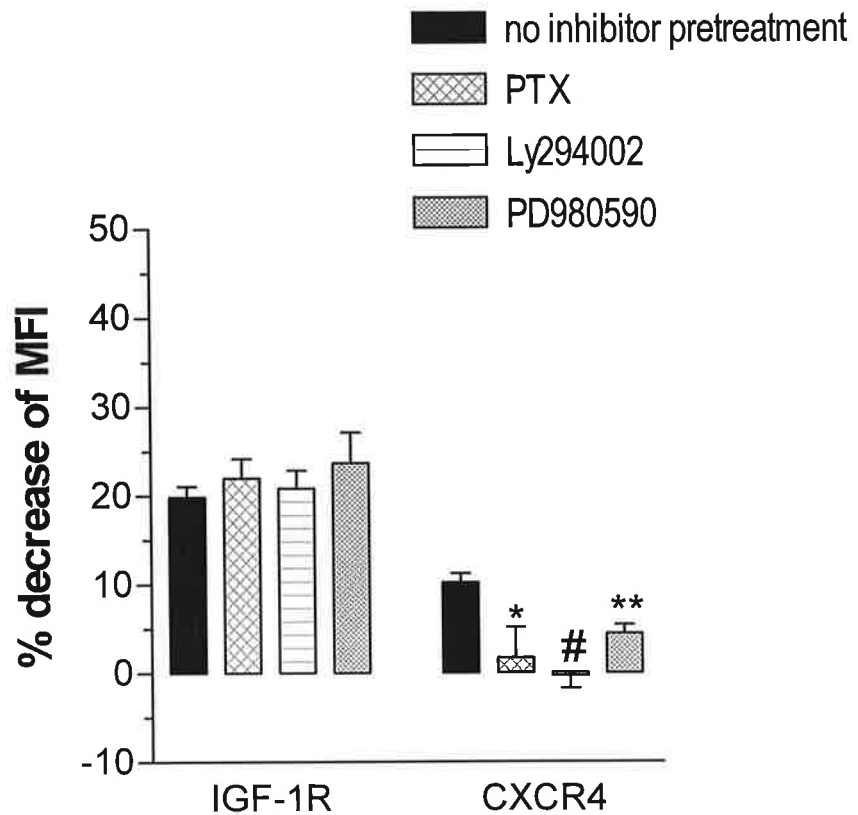


Figure 5.10 *Effect of inhibition of G-proteins, PI3Ks and MAPKs on the internalisation of IGF-1R and CXCR4 in MDA-MB-231 cells in response to IGF-I*

The cells were left untreated or pretreated with PTX (100 ng/ml), Ly294002 (100 μ M) and PD980590 (20 μ M), inhibitors for G-proteins, PI3Ks and MAPKs, respectively for 2-3 hours prior to stimulation or not, with 10 nM IGF-I for 24 hours. Surface expression of IGF-1R and CXCR4 in untreated and IGF-I-treated cells was examined by flow cytometry using specific Abs to IGF-1R and CXCR4 (monoclonal anti-IGF-1R (7C2) and anti-CXCR4 (12G5) respectively). Loss of the receptors on the cell surface was determined and calculated as % decrease of mean fluorescent intensity (MFI). Data are expressed as mean $\pm$ S.E.M. from at least 4 separate experiments. Asterisks indicate values statistically different from control values (student's unpaired t test) at *, $p < 0.05$; **, $p < 0.005$; #, $p < 0.0001$.

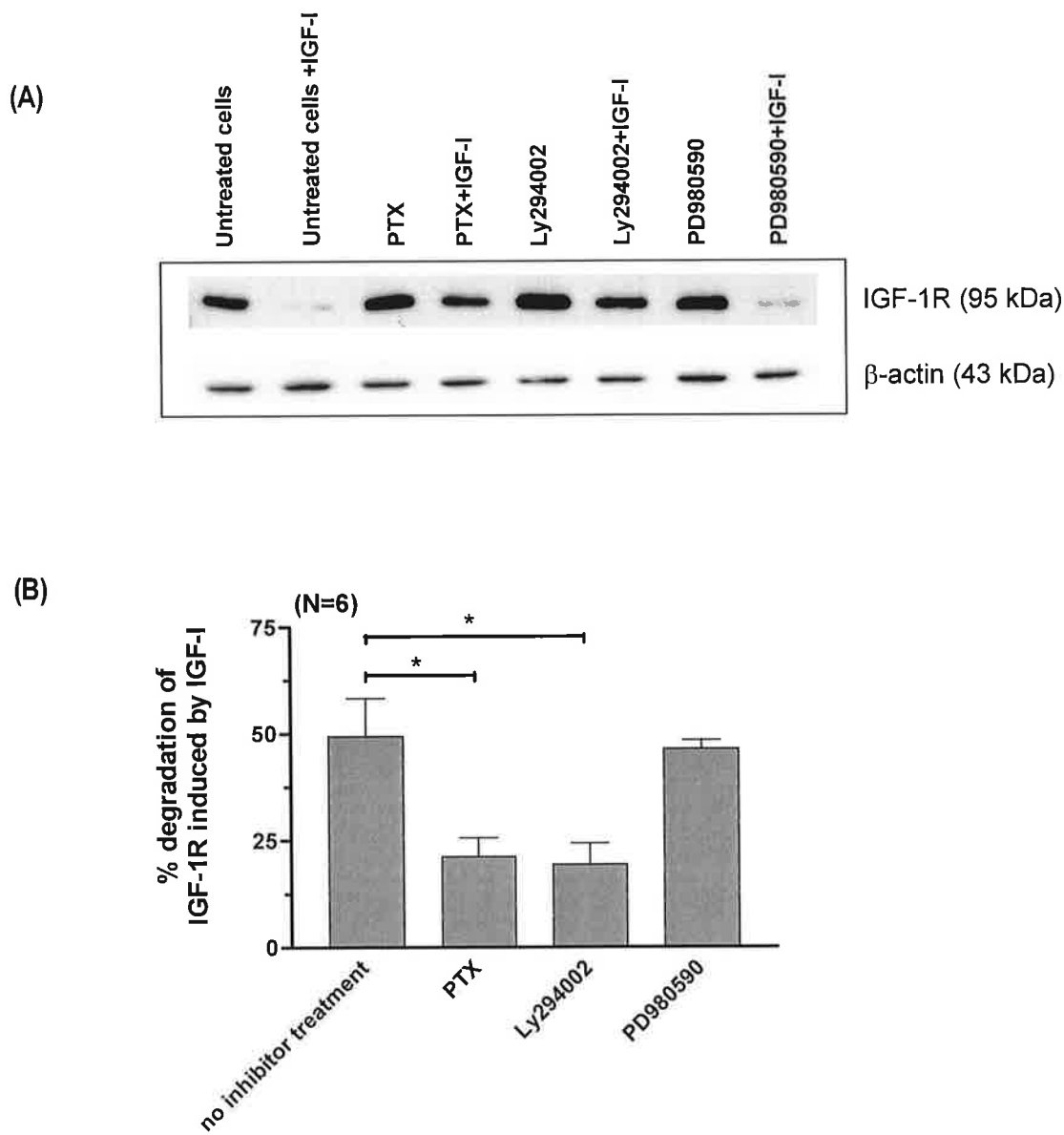


Figure 5.11 Effect of inhibition of G-proteins, PI3Ks and MAPKs on the degradation of IGF-1R in MDA-MB-231 cells induced by IGF-I

The cells were left untreated or pretreated with PTX (100 ng/ml), Ly294002 (100 μ M) and PD980590 (20 μ M), inhibitors for G-proteins, PI3Ks and MAPKs, respectively for 2-3 hours prior to stimulation or not, with 10 nM IGF-I for 24 hours. Cell lysates were prepared and subjected to SDS-PAGE and Western blot analysis for degradation of IGF-1R. (A), a representative Western blot demonstrating the effect of the inhibitors on IGF-I-mediated IGF-1R degradation. (B), semiquantitative data demonstrating the effect of the inhibitors on the IGF-I-induced IGF-1R degradation in cell lysates. Results are expressed as mean $\pm$ S.E.M. pooled from 6 separate experiments. * ($p < 0.05$) indicates statistically significant effect of inhibitors on the IGF-1R degradation (student's unpaired t test).

CHAPTER 6

General Discussion and Conclusion

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6.1 Introduction

Advances in cancer research have clearly implicated homeostatic factors in cancer progression. Previous studies have indicated significant roles of IGF-1R in the progression of metastatic and invasive breast carcinoma (Bartucci *et al.* 2001; Dunn *et al.* 1998; Sachdev *et al.* 2004). More recently, CXCR4 and to a lesser extent CCR7, have also been implicated in the process of breast cancer metastasis and invasion (Chen *et al.* 2003; Liang *et al.* 2005; Muller *et al.* 2001). A number of molecular studies have demonstrated that the binding to these receptors by their corresponding ligands, IGF-I, CXCL12 and, CCL19 and CCL21, respectively initiates signal transduction pathways leading to cell migration, which is a mandatory cellular activity for the acquisition of the metastatic and invasive potential of cancer cells. In breast cancer epithelial cells, migrational signal transduction induced by IGF-1R has been partially defined whereas those by CXCR4 and CCR7 are virtually undocumented, even though the chemotactic signalling pathways mediated by these receptors have been well established in leukocytes (Bartucci *et al.* 2001; Mellado *et al.* 2001; Thelen 2001). In addition, based on accumulating recent studies, there is the potential for cross-talk between the signal transduction pathways activated by growth factor RTKs and GPCRs which may significantly affect various biological and physiological responses induced by these receptors (Gavi *et al.* 2006; Waters *et al.* 2004). Accordingly, an investigation into migrational signal transduction induced by IGF-1R and

chemokine receptors, particularly an interaction between the two receptor systems in breast cancer epithelial cells, is definitely required for an understanding in molecular terms of metastasis and invasion of breast cancer.

The studies described in this thesis have begun to document the signal transduction pathways activated by IGF-1R and chemokine receptors, CXCR4 and CCR7, that are involved in migration of breast cancer epithelial cells. In particular, for the first time, evidence for cross-talk between IGF-1R and CXCR4 in the highly metastatic MDA-MB-231 cell line has been demonstrated. The studies have also provided mechanisms by which the interaction between these two receptors affects activation of cell migration as well as deactivation of the receptors. Importantly, the cross-talk appears to play a potentially important role in establishing the metastatic and invasive ability of breast cancer cells.

6.2 Cross-talk between GPCR and RTK signal transduction: evidence for an interaction between CXCR4 and IGF-1R in human breast cancer epithelial cells

A growing body of evidence has indicated the existence of cross-talk between signal transduction pathways activated by different types of receptors, particularly between that of GPCRs and RTKs. As outlined in section 1.3.4, several forms of interaction between GPCRs and RTKs have been reported and the molecular basis of such interactions is being investigated to a greater extent. It is apparent that the cross-talk may occur at multiple levels in signal transduction pathways, including at the levels of receptor-to-receptor interactions and at crucial downstream signalling systems such as PI3K, Akt/protein kinase B and MAPK (Pyne *et al.* 2003; Vazquez-Prado *et al.* 2003). Based on

accumulating evidence emerging at the time that the current study was conducted, several models had been proposed to explain how these receptors integrate their signal transduction, which is subsequently translated into cellular responses.

Cross-talk between GPCRs and RTKs can require the activity of a second ligand produced by a cell in activating the second receptor, called a sequential or autocrine mechanism (see Figure 6.1A). This is primarily found in the well established transactivation of EGFR by GPCR ligands. Several GPCR ligands including lysophosphatidic acid (LPA), endothelin-1 (ET-1) and thrombin have been shown to induce rapid tyrosine phosphorylation of EGFR and recruitment of the adapter proteins Shc and Grb1, leading to p42/p44 MAPK activation in different cellular systems (Daub *et al.* 1997; Daub *et al.* 1996; Gschwind *et al.* 2002). Typically, the GPCR agonist-mediated activation of p42/p44 MAPK requires tyrosine kinase activity of EGFR. Specific inhibition of EGFR function by either a dominant-negative EGFR mutant or treatment of cells with inhibitors specific to EGFRs, suppresses p42/p44 MAPK activation induced by these GPCR ligands (Daub *et al.* 1997; Daub *et al.* 1996). This cross-activation appears to depend on the presence of heparin-binding EGF (HB-EGF), which is primarily synthesised as a proligand of EGF (proHB-EGF) and expressed on the cell membrane. Upon GPCR-ligand interaction, proteolytic cleavage of proHB-EGFs, probably by the activity of a metalloprotease enzyme, results in release of HB-EGFs that subsequently bind to and transduce the autocatalytic tyrosine kinase activity of EGFR, leading to the activation of MAPKs (Gschwind *et al.* 2002; Prenzel *et al.* 1999; Yan *et al.* 2002). This type of cross-talk has also been demonstrated in reverse transactivation of GPCRs by growth factor receptors. A previous report has demonstrated that IGF-I-mediated migration in breast cancer cells requires the production and release of one of the ligands for CCR5, CCL5, which is

induced by stimulation of the cells with IGF-I. This study shows that blockade of CCL5 activity by a neutralising Ab to CCL5 inhibits cell migration in response to IGF-I (Mira *et al.* 2001). PDGF/PDGFR has been shown to stimulate cell motility in a S1P₁ receptor-dependent manner in human embryonic kidney 293 cells. This is based on the observation that PDGF can activate the synthesis and release of S1P, which in turn binds to and activates the S1P₁ receptor to induce Rac, PKB, and subsequent cell motility (Hobson *et al.* 2001).

Another theoretical model for GPCR/RTK transactivation is based on a physical formation of a receptor complex (see Figure 6.1B). This has been proposed recently for the cross-talk between one of the receptors for S1P, S1P₁, and PDGFR in the induction of mitogenic signal transduction (Pyne *et al.* 2003). Co-immunoprecipitation studies have revealed that, in human embryonic kidney 293 cells, recombinant PDGFR is tethered to endogenous S1P₁, providing a platform on which PDGFR and S1P₁ can interact, resulting in more efficient p42/p44 MAPK activation (Alderton *et al.* 2001). In airway smooth muscle cells, the functionally active complex of endogenous PDGFR and S1P₁ is also observed and it is demonstrated that S1P and/or PDGF can induce the formation of endocytic vesicles containing the two receptors, which has been suggested to be essential for further activating MAPK pathways (Waters *et al.* 2003). Due to a close interaction between these receptors, it is predicted that PDGF stimulation is dependent on the S1P₁ receptor, whereas S1P stimulation requires the PDGFR. G_iα and Gβγ subunits are primarily recruited to the receptor complex with S1P₁ and are released from the complex upon the stimulation with S1P. PDGF activates the intrinsic kinase activity of PDGFR which apparently tyrosine-phosphorylates G_iα subunits. These molecules further induce downstream signalling mediating endocytic “pinching off” of vesicles. This leads to the

activation of $G_i\alpha$ -dependent p42/p44 MAPK pathways and subsequent DNA synthesis (Alderton *et al.* 2001; Waters *et al.* 2003). In contrast to the sequential loop model previously reported (Hobson *et al.* 2001), these studies rule out a role for second ligands, either S1P or PDGF, in the activation of p42/p44 MAPK through $S1P_1$ and PDGFR respectively, and propose the GPCR/RTK signalling complex as a major mechanism for transactivation between $S1P_1$ and PDGFR. Nevertheless, both the sequential and receptor signalling complex models could mechanistically explain reverse transactivation of PDGFR by S1P observed in vascular smooth muscle cells. It appears that S1P ligands are able to induce G_i -dependent tyrosine-phosphorylation of PDGFR, leading to the activation of downstream signalling molecules, an adapter protein Shc and a p85 of class IA PI3K, which are required for mitogenic responses (Tanimoto *et al.* 2004).

The formation of signalling complex of GPCR/RTK is also potentially required for transactivation between several other GPCRs and RTKs. Nerve growth factor (NGF) activates a $G_i\alpha$ -dependent p42/p44 MAPK pathway. This seems to rely on the interaction between a receptor for NGF, Trk, and the LPA_1 receptor and its associated proteins, GRKs and β -arrestins (Pyne *et al.* 2003). It has been demonstrated that GRKs are constitutively associated with the Trk receptor and the stimulation with NGF promotes binding of β -arrestins to the GRK/TrK receptor complex, resulting in clathrin-mediated endocytic signalling to p42/p44 MAPK (Rakhit *et al.* 2001). Insulin and IGF-I are well-known to stimulate sequestration of β_2 -adrenergic receptors, attenuating β -catecholamine action in a similar way that the β_2 -adrenergic receptor agonist, isoproterenol, attenuates catecholamine action (Gavi *et al.* 2006; Karoor *et al.* 1995). Several studies have shown that β_2 -adrenergic receptors can be phosphorylated by insulin and IGF-I at particular

residues in the C-terminus domain and a second intracellular loop of the receptors respectively, providing docking sites similar to IRS-1 and IRS-2 on the β_2 -adrenergic receptors that allow SH2-and SH3-domain containing proteins, including Grb2, dynamin, Src and Shc, to bind to the receptors. This results in the activation of Sos/Ras/Raf pathways to MAPK (Gavi *et al.* 2006; Gavi *et al.* 2005; Karoor *et al.* 1995; Karoor and Malbon 1996; Malbon and Karoor 1998)(see Figure 6.1C). Despite there being no direct evidence for the physical formation of the receptor complex in certain cells, *in vitro* reconstitution and phosphorylation assays using recombinant insulin receptors and β_2 -adrenergic receptors have demonstrated that β_2 -adrenergic receptors are a substrate for insulin receptor tyrosine kinases, suggesting the requirement of a direct interaction between the two receptor proteins in this cross-activation (Baltensperger *et al.* 1996).

GPCR and RTK signalling also converge at multiple levels of downstream signalling systems including MAPK, PI3K and Akt/ protein kinase B (PKB). In an emerging body of evidence, the cross-talk at MAPK activation is the most well established. MAPK activation by growth factors is enhanced in the presence of GPCRs such as the case of PDGFR and S1P₁ (Alderton *et al.* 2001; Waters *et al.* 2004; Waters *et al.* 2003). Conversely, GPCRs have been shown to activate MAPK independently or through transactivation of RTKs as found in the activation of EGFR by many GPCR ligands (Daub *et al.* 1997; Daub *et al.* 1996; Gschwind *et al.* 2002). Several other non-receptor kinases including PI3K and PKB have also been shown to be key molecules for RTK/GPCR cross-talk. For example, with respect to the interaction between insulin and β_2 -adrenergic receptors (see Figure 6.1C), the cross-talk occurs not only at the level of receptor-receptor interaction but also at the level of activation of PI3K and Akt/PKB. Insulin is well-known to induce the activation of PI3K and its downstream

serine/threonine kinase Akt/PKB. Inhibition of PI3K activation by a PI3K inhibitor, Ly294002, and dominant-negative expression of Akt/PKB block the sequestration of β_2 -adrenergic receptors induced by insulin. The β_2 -adrenergic receptor mutant, lacking phosphorylation sites for Akt/PKB, also does not undergo internalisation in response to insulin (Doronin *et al.* 2002).

The studies described in this thesis have provided evidence for a novel form of cross-talk between CXCR4 and IGF-1R signal transduction in the highly metastatic breast cancer MDA-MB-231 cells. These studies were conducted in comparison with the non-metastatic MCF-7 cells. Based on the data generated in the present study, a hypothetical model for interaction between these two receptors has been proposed (see Figure 6.2). In MDA-MB-231 cells, the cross-talk between CXCR4 and IGF-1R occurs at the level of receptor-to-receptor interaction and appears to be unidirectional. While CXCL12 does not induce activation of IGF-1R, IGF-I is able to transactivate CXCR4/G-protein signal transduction in this cell type. It is apparent that this cross-talk is not mediated through a sequential or autocrine mechanism as supported by the fact that production and release of the ligand for CXCR4, CXCL12, are not required for such transactivation. Rather, similar to the model for PDGFR/S1P₁ receptor interaction shown in Figure 6.1 (B) (Pyne *et al.* 2003), the cross-talk between signal transduction of CXCR4 and IGF-1R depends on the physical formation of a complex containing IGF-1R, CXCR4 and G-proteins, G_i α_2 and G β (see Figure 6.2 A (1) and (2)). This receptor complex appears to be constitutively formed and probably provides a sufficiently close proximity of these proteins to allow IGF-I/IGF-1R to transactivate CXCR4 through release of G_i α and G β . These activation pathways apparently work together with a classical tyrosine kinase-dependent pathway downstream of IGF-I/IGF-1R to drive a chemotactic response of the cells. In MCF-7 cells, the physical

association of IGF-1R and CXCR4/G-proteins is also found at the resting state (see Figure 6.2 B (1) and (2)). Nonetheless, IGF-I is unable to transactivate CXCR4/G-protein activation pathways due to non-functional CXCR4 expressed in this cell line (Akekawatchai *et al.* 2005). The defect of CXCR4 signalling in MCF-7 cells is found at the level of G-protein activation. Thus, chemotaxis of MCF-7 cells by IGF-I is presumably mediated only through IGF-1R tyrosine kinase-dependent pathways. The model for CXCR4/IGF-1R interaction demonstrated in the present study clearly shows how IGF-1R and CXCR4 coordinately activate signal transduction, leading to a migrational response of the highly metastatic MDA-MB-231 cells (Akekawatchai *et al.* 2005).

The interaction between CXCR4 and IGF-1R signalling is also observed with respect to deactivation of the two receptors in MDA-MB-231 cells as shown in Figure 6.2 A (3). The two receptors are co-internalised following prolonged exposure of MDA-MB-231 cells to IGF-I, leading to a reduction in ability of CXCR4 to induce signal transduction. However, only IGF-1R subsequently undergoes the process of degradation while CXCR4 is potentially subjected to recycling. The capability of growth factors to downregulate GPCRs is also found in the case of β_2 -adrenergic receptor internalisation by insulin or IGF-I, although this seems to play an important role in the activation of MAPK (Gavi *et al.* 2006; Karoor *et al.* 1995). Clearly, the coregulation of CXCR4 and IGF-1R by IGF-I observed in the present study is dependent on the induction of CXCR4/G-protein activation pathways because of the fact that the co-internalisation of these receptors is not observed in MCF-7 cells, which lack transactivation of CXCR4 by IGF-I (see Figure 6.2 B (3)). Importantly, it was observed that CXCR4 was resistant to CXCL12-induced internalization in MDA-MB-231 cells. Taken together, these data suggest that the cross-

talk between IGF-1R and CXCR4 plays an important role in removing CXCR4 from the surface of metastatic breast cancer cells.

The downregulation process of CXCR4 and IGF-1R by IGF-I found in MDA-MB-231 cells appears to be very complicated and controlled at multiple levels of signalling downstream of the two receptors. Previous studies have shown that IGF-1R forms a complex with several signalling molecules known to be downstream of CXCR4, including G-proteins, GRKs and β -arrestins (Akekawatchai *et al.* 2005; Alderton *et al.* 2001; Cheng *et al.* 2000; Dalle *et al.* 2001; Hupfeld and Olefsky 2007; Orsini *et al.* 1999). To the best of our knowledge, no direct interaction site for these molecules on IGF-1R has been identified. Together with the evidence shown in the present study that the presence of CXCR4 is obligatory for induction of $G_i\alpha$ dependent chemotaxis by IGF-I in MDA-MB-231 cells and that CXCR4 and IGF-1R can be co-precipitated, it is likely that a physical interaction between IGF-1R and CXCR4 allows these CXCR4-associated proteins to participate in the signalling receptor complex. Therefore, upon stimulation with IGF-I, these signalling molecules are involved in activation-induced downregulation of both CXCR4 and IGF-1R. As shown in this model (see Figure 6.2 A (3)), it is apparent that CXCR4 is internalised partially via the $G_i\alpha$ activation pathway. Importantly, IGF-1R also undergoes a degradation process requiring the $G_i\alpha$ pathway, although the receptors do not utilise this pathway at the internalisation step. GRKs and β -arrestins, which are also well-known as key molecules for downregulating CXCR4, have also been shown to play an important role in RTK signalling (Cheng *et al.* 2000; Hupfeld and Olefsky 2007; Mellado *et al.* 2001; Orsini *et al.* 1999). IGF-1R internalisation and degradation are regulated by the activity of β -arrestins (Girnita *et al.* 2005; Lin *et al.* 1998). Thus, it is possible that

upon stimulation of MDA-MB-231 cells with IGF-I, GRKs and β -arrestins are also recruited to the receptor complex with CXCR4 and this mediates downregulation of both CXCR4 and IGF-1R. However, this aspect of receptor downregulation involving the activity of these molecules remains unclear and still requires further investigation.

The downregulation process of CXCR4 and IGF-1R by IGF-I observed in MDA-MB-231 cells is also modulated by PI3K and MAPK (see Figure 6.2 A (3)). Whereas PI3K and MAPK are required for CXCR4 internalisation, none of these pathways is involved in internalisation of IGF-1R. However, PI3K is essential for the degradation process of IGF-1R. These observations support the notion that the two receptors share downstream signalling systems that regulate their deactivation process and the data collected in this thesis indicate the involvement of the cross-talk between CXCR4 and IGF-1R in the downregulation process.

In contrast to the coregulation of surface expression of CXCR4 and IGF-1R found in MDA-MB-231 cells, IGF-1R is not coregulated with CXCR4 in response to IGF-I in MCF-7 cells (see Figure 6.2 B (3)). Indeed, it appears that only IGF-1R is internalised and degraded following exposure of the cells to IGF-I. This observation is consistent with the results shown in the previous chapters indicating that CXCR4 is non-functional in MCF-7 cells. Without the activation of CXCR4/G-protein pathways, mechanisms for downregulation of IGF-1R in MCF-7 cells are likely to be different from those in MDA-MB-231 cells and may be dependent only on the activation pathways initiated by tyrosine kinase activity of IGF-1R. In this hypothetical model, many aspects in downregulation of IGF-1R in MCF-7 cells remain to be clarified including whether IGF-1R uses $G_i\alpha$, PI3K and MAPK and whether there is contribution of GRKs and β -arrestins in the processes of

internalisation and degradation of IGF-1R. These issues will provide a clearer understanding in downregulation of CXCR4 and IGF-1R in these breast cancer cells and will be the subject of future experiments.

In the past decade, several lines of evidence have been increasingly supported that signal transduction initiated by receptors on the cell surface in response to extracellular stimuli is influenced by formation of membrane microdomains, enriched in cholesterol and glycosphingolipids, termed lipid rafts. Lipid rafts containing a set of membrane signalling proteins have been considered as a platform that mediates intracellular signal transduction (Pike 2004; Simons and Toomre 2000). Currently, a number of GPCRs and RTKs have been shown to be associated with lipid rafts and this is obligatory for ligand-receptor binding and receptor signalling (Pike 2003). While the disruption of lipid raft structure by cholesterol depletion inhibits ligand binding to the receptors and impairs the ability of receptors to mediate signalling, replenishment of cell membrane with cholesterol effectively restores the receptor ability to transduce signals (Nguyen and Taub 2002; Remacle-Bonnet *et al.* 2005). It is hypothesised that rafts contain a distinctive protein and lipid composition that is important to accomplish specific signalling functions. It is also possible that establishment of lipid rafts may be responsible for transactivation among different types of receptors especially between GPCRs and RTKs. This is the case for transactivation of PDGFR and EGFR by the GPCR ligand, S1P. It appears that the transactivation is abolished by depletion of cholesterol, suggesting requirement of the cholesterol-rich microdomains for this cross-activation (Tanimoto *et al.* 2004). Both CXCR4 and IGF-1R are raft-associated proteins and their ability to mediate signalling is also supported by lipid raft formation (Nguyen and Taub 2002; Remacle-Bonnet *et al.* 2005). As discussed in the present study, the cross-talk between CXCR4 and IGF-1R

signalling observed in MDA-MB-231 cells is based on the formation of a signalling complex containing IGF-1R, CXCR4 and CXCR4-associated proteins. The complex allows IGF-I to transduce signal transduction downstream of both IGF-1R and CXCR4 and regulate the expression and function of the two receptors. While mechanisms by which these proteins are recruited to form the complex are largely unclear, it is likely that lipid rafts support the establishment of this GPCR/RTK signalling complex.

At present, cross-talk between GPCRs and RTKs in various cellular systems is increasingly being established. Despite numerous studies supporting the existence of the cross-talk and its biological outcomes, further studies are still required, particularly an investigation into a possible contribution of the cross-talk to physiological functions mediated by these receptors in different biological settings. Nonetheless, this requires more solid knowledge of the mechanisms involved in the establishment of GPCR/RTK transactivation, which will lead to the approaches to disrupt or modify the transactivation of GPCRs and RTKs in a given cell type.

6.3 Implications of cross-talk between signal transduction of CXCR4 and IGF-1R in breast cancer metastasis

CXCL12/CXCR4 and IGF-I/IGF-1R are well-known as mediators for cellular activities such as proliferation, migration and anti-apoptosis. These ligand-receptor pairs are expressed in most cell types and tissues, and contribute to a variety of physiological and pathological conditions. IGF-I/IGF-1R expression is of importance in growth and development of the fetus. A homozygous deletion of either IGF-I or IGF-1R gene in mice leads to decreased birth weight and the majority of mice die shortly after birth because of hypodevelopment of organs, particularly in the respiratory systems (Liu *et al.* 1993).

CXCL12/CXCR4 plays a fundamental role in fetal development and naïve leukocyte trafficking. Mice in which CXCL12 or CXCR4 has been knocked out die *in utero* and are defective in many physiological systems including vascular development, haematopoiesis and cardiogenesis (Nagasawa *et al.* 1996; Tachibana *et al.* 1998; Zou *et al.* 1998). Certainly, these ligand-receptor systems share similarities in their physiological functions and this in itself suggests a potential contribution of the cross-talk between their signalling to these physiological outcomes.

IGF-1R and CXCR4 have also been implicated in the development of various pathological conditions including cancer. In particular, as outlined in the introduction to this thesis, both receptors contribute to metastasis and invasion of breast cancer (Bartucci *et al.* 2001; Dunn *et al.* 1998; Muller *et al.* 2001; Sachdev *et al.* 2004). In the present study, important aspects of expression and function of these receptors, as well as the cross-talk between their signal transduction pathways, have been investigated in the highly metastatic MDA-MB-231 in comparison with non-metastatic MCF-7 cells. As demonstrated, the two cell lines exhibit differential characteristics in terms of expression and function of these receptors. While MDA-MB-231 cells expressed low levels of IGF-1R expression and function, the cells exhibit functionally active CXCR4/G-proteins. Moreover, in this cell type, IGF-I is able to transactivate CXCR4/G-protein signal transduction and modulates expression and function of CXCR4. MCF-7 cells show a high level of functional IGF-1R but express a non-functional phenotype of CXCR4 leading to a lack of the cross-talk between IGF-1R and CXCR4 in the cells. Despite the essential role of IGF-1R in induction of migrational responses observed in different breast cancer cell lines, low levels of expression of IGF-1R and its major downstream signalling molecule IRS are associated with more metastatic and invasive phenotypes of breast carcinoma (Bartucci *et*

al. 2001; Pennisi *et al.* 2002; Sepp-Lorenzino *et al.* 1994; Surmacz 2000). A recent study examining CXCR4 expression and function in a panel of metastatic and non-metastatic breast cancer cell lines found that, while all cell lines examined expressed high levels of CXCR4, the receptor was only functional on the metastatic cell lines. This suggests that the presence of CXCR4 in cell lines and tissues may not correlate with metastasis and invasion in breast cancer. Rather, the study shows that this pathological process may require functionally active CXCR4/G-proteins embedded in given breast cancer cells (Holland *et al.* 2006). Importantly, the present study has also revealed that the CXCR4/G-protein signalling apparatus in the highly metastatic MDA-MB-231 cells can also be utilised by IGF-I/IGF-1R. The data present in this thesis also indicate a link between IGF-1R internalisation and degradation, and active CXCR4 in metastatic cells. Therefore, the lower level of IGF-1R which correlates with metastatic ability may be related to the ability of IGF-1R to interact with functional CXCR4 on metastatic cells. While the phenotypes involving expression and function of IGF-1R and CXCR4 observed only in the highly metastatic breast cancer cell line are possibly important for breast cancer cells in establishing a metastatic phenotype, extensive studies in a variety of breast cancer cell lines and tissues are required to support this aspect of involvement of these receptors in development of breast cancer metastasis.

6.4 Concluding remarks and future studies

The studies described in this thesis have provided further evidence that both IGF-1R and chemokine receptors, CXCR4 and CCR7, are important for the migration of human breast cancer epithelial cells, which is obligatory for establishing the metastatic and invasive ability in breast cancer. In particular, the studies have provided evidence for a novel cross-

talk between IGF-1R and CXCR4 in highly metastatic breast cancer cells. This cross-talk appears to be essential for optimal induction of migrational response and for the coregulation of CXCR4 and IGF-1R expression and function in this cell type in response to IGF-I. Of interest, it is likely that there is a degree of selectivity in establishing this RTK/GPCR cross-talk. The present study demonstrated that surface expression of the closely-related GPCR, CCR7, was not regulated by IGF-I/IGF-1R and this potentially results from a lack of interaction between IGF-1R and CCR7 in this cell type. Nonetheless, the issue of IGF-1R and CCR7 interaction is unequivocally clarified in the present study and should be the subject for further studies.

IGF-1R and CXCR4 are important mediators of a number of biological outcomes. The molecular basis of the observed cross-talk is therefore potentially of significance in understanding the roles of these receptors in various physiological and pathological conditions including cancer metastasis. While the present study has documented the interaction between IGF-1R- and CXCR4-mediated signal transduction in the highly metastatic MDA-MB-231 cells and its potential contribution to an acquisition of metastatic and invasive phenotypes of breast cancer, several relevant aspects remain to be further clarified. For example, it would be important to extensively investigate for similar cross-talk in a variety of cell lines and tissues from breast cancer, other types of cancer and under normal circumstances. Different factors potentially involved in establishing the CXCR4/IGF-1R signalling complex must be examined in order to elucidate the precise mechanism of the cross-talk. It is also important to conduct a more detailed analysis of signal transduction pathways downstream of CXCR4 and IGF-1R complex following the stimulation of IGF-I. Finally, the role of this cross-talk in the development of breast cancer metastasis and invasion would need to be studied in *in vivo* models once specific

approaches to inhibit the cross-talk are available. Accordingly, these will provide a clearer understanding of the roles of these receptors in progression of breast cancer metastasis and invasion which may lead to development of more effective diagnostics and therapy for cancer.

Figure 6.1 *Three hypothetical models for cross-talk between GPCRs and RTKs*

(A), Cross-talk between EGFR and a GPCR represents a sequential or autocrine loop model. EGFR can be transactivated by many GPCR ligands such as lysophosphatidic acid (LPA), endothelin-1 (ET-1) and thrombin, requiring the activity of metalloproteinase in cleavage of the proligand, HB-EGF and subsequent release of free EGF to bind to, and activate EGFR. The activated EGFR becomes autophosphorylated which results in activation of a series of signalling molecules, Grb2/Sos/Ras/Raf, leading to MAPK activation. (B), A model for GPCR/RTK cross-talk based on the formation of a GPCR/RTK signalling complex that allows these receptors to exploit one another's downstream signalling pathways (Modified from (Pyne *et al.* 2003)). This has been proposed for cross-talk between PDGFR and the S1P₁ receptor. The Gβγ complex and G_iα are released by the stimulation of S1P₁ with S1P. Due to a close interaction between these receptors, PDGF activates the intrinsic tyrosine-kinase activity of PDGFR that subsequently tyrosine-phosphorylates G_iα. G_iα and the Gβγ complex together with other signalling molecules including GRKs/β-arrestins and the complex of PI3K/Grb1/dynamin subsequently promote the endocytic “pinching off” of the PDGFR/S1P₁ complex, leading to MAPK activation and mitogenesis (Pyne *et al.* 2003). (C), Cross-talk at the level of downstream signalling systems shown in the interaction between IR and the β₂-adrenergic receptor (modified from (Gavi *et al.* 2006)). In response to insulin, β₂-adrenergic receptors can be phosphorylated by either the intrinsic kinase of IR or its downstream non-receptor kinases, PI3K and Akt/PKB, leading to a sequestration of β₂-adrenergic receptors and therefore a reduction of β-catecholamine activity.

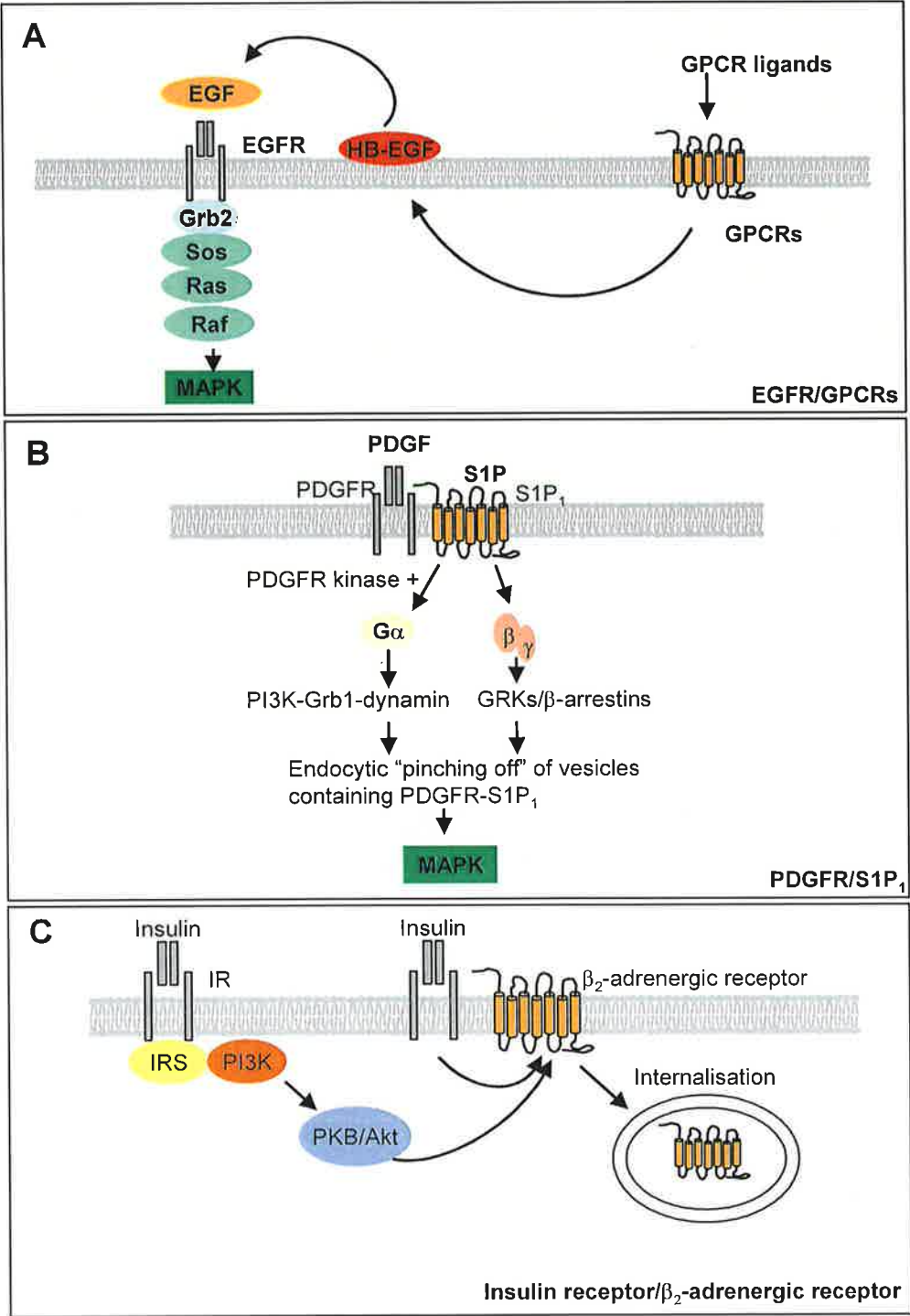
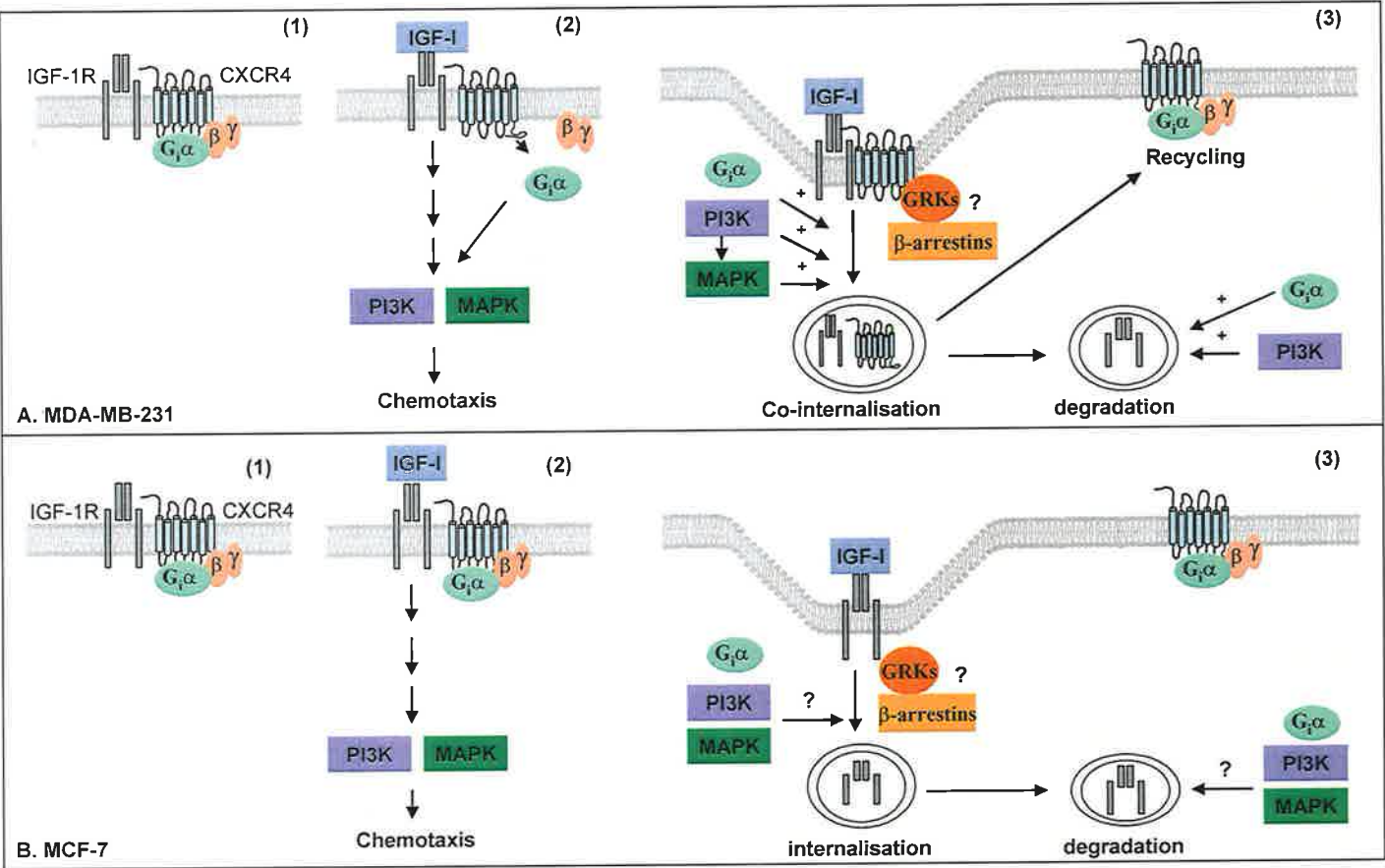


Figure 6.2 *A hypothetical model for CXCR4 and IGF-1R cross-talk in the highly metastatic MDA-MB-231 and non-metastatic MCF-7 cells*

The figure depicts signal transduction induced by IGF-I stimulation in MDA-MB-231 (A) and MCF-7 (B) cells in the resting state (1), activation state (2) and deactivation state (3). The constitutive formation of a signalling complex containing IGF-1R, CXCR4/G-proteins, $G_i\alpha$ and $G\beta\gamma$, is found in both MDA-MB-231 and MCF-7 cells (A and B (1)). In MDA-MB-231 cells (A), IGF-I can transactivate CXCR4/G-protein signalling pathways that work coordinately with tyrosine kinase-dependent pathways of IGF-1R to promote a chemotactic response of the cells (A (2)). With prolonged exposure to IGF-I, CXCR4 and IGF-1R are co-internalised, however only IGF-1R undergoes degradation whereas CXCR4 is potentially recycled back to the cell surface. These processes are $G_i\alpha$ dependent and regulated by several signalling systems downstream of both receptors including the pathways of PI3K and MAPK. In MCF-7 cells (B), in contrast, there is no transactivation of CXCR4/G-protein signal transduction by IGF-I due to non-functional CXCR4 expressed in this cell type. Chemotaxis of MCF-7 cells induced by IGF-I may be dependent solely on tyrosine kinase-dependent pathways of IGF-1R (B (2)). This lack of CXCR4/G-protein signalling in response to IGF-I means that IGF-I is unable to downregulate CXCR4 in this cell line (B (3)). Mechanisms for the downregulation of IGF-1R by IGF-I in these cells remain to be clarified in more detail.



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APPENDIX

Transactivation of CXCR4 by the Insulin-like Growth Factor-1 Receptor (IGF-1R) in Human MDA-MB-231 Breast Cancer Epithelial Cells*

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In the multimolecular environment in tissues and organs, cross-talk between growth factor and G protein-coupled receptors is likely to play an important role in both normal and pathological responses. In this report, we demonstrate transactivation of the chemokine receptor CXCR4 by the growth factor insulin-like growth factor (IGF)-1 is required for IGF-1-induced cell migration in metastatic MDA-MB-231 cells. The induction of chemotaxis in MDA-MB-231 cells by IGF-1 was inhibited by pretreatment of the cells with pertussis toxin (PTX) and by RNAi-mediated knockdown of CXCR4. Transactivation of the CXCR4 pathway by IGF-1 occurred independently of CXCL12, the chemokine ligand of CXCR4. Neither CXCR4 knockdown nor PTX had any effect on the ability of IGF-1 to activate IGF-1R, suggesting that CXCR4 and G proteins are activated subsequent to, or independently of, phosphorylation of IGF-1R by IGF-1. Coprecipitation studies revealed the presence of a constitutive complex containing IGF-1R, CXCR4, and the G protein subunits, $G_{i\alpha_2}$ and $G_{i\beta}$, and stimulation of MDA-MB-231 cells with IGF-1 led to the release of $G_{i\alpha_2}$ and $G_{i\beta}$ from CXCR4. Based on our findings, we propose that CXCR4 constitutively forms a complex with IGF-1R in MDA-MB-231 cells, and that this interaction allows IGF-1 to activate migrational signaling pathways through CXCR4, $G_{i\alpha_2}$ and $G_{i\beta}$.

The G protein-coupled receptor (GPCR)² CXCR4 is the receptor for the chemokine CXCL12. Both molecules are essential for life, with genetic deletion in mice of either CXCR4 or CXCL12 resulting in a lethal phenotype (1–3). Activation of CXCR4 by CXCL12 has been implicated in the homeostasis and activation of the immune system, and influences a range of other biological systems under both normal and pathological conditions (4–6). These include angiogenesis (7–9), cell survival (10, 11), and more recently, tumor growth and metastasis (12–14). Indeed, it has recently been shown that CXCR4 is expressed in breast cancer tissues and cell lines, and that CXCL12 is expressed in several target organs of breast cancer metastasis (13). Additionally, treatment of mice with neutralizing Abs against CXCR4 inhibits metastasis in a mouse model of breast cancer, as does RNAi-mediated knock-

down of CXCR4 on orthotopically transplanted breast carcinoma cells (12, 13). These data point to an important role for CXCR4 in cancer.

The cellular signal transduction pathways induced by CXCL12 have been well characterized in leukocytes. Interaction of CXCL12 with CXCR4 leads to the release of the G protein subunits $G_{i\alpha}$ and $G_{i\beta}$ from intracellular domains of CXCR4. These subunits then bind and activate downstream enzyme systems including phospholipase C, which leads to a transient increase in the level of intracellular Ca^{2+} , and phosphoinositide 3-kinase (PI3K), which results in activation of AKT and subsequently, cell migration (15–17). In contrast, the role of CXCR4, including characterization of signal transduction mechanisms in cell types other than leukocytes is less well established despite the fact that CXCR4 is expressed in most tissues and organs.

Cross-talk between GPCRs and growth factor receptor-tyrosine kinase (RTKs) induced signaling pathways has become increasingly well documented in different cellular systems. For example, EGFR is tyrosine-phosphorylated in response to CCL11, a ligand for the GPCR CCR3, leading to MAP kinase activation and IL-8 production in bronchial epithelial cells (18). In rat aortic vascular smooth muscle cells, both PDGFR and EGFR are phosphorylated by sphingosine 1-phosphate (S1P), a lipid mediator that is a ligand for the S1PR family of GPCRs, leading to activation of effectors downstream of PDGFR and EGFR including Shc, and the p85 regulatory subunit of the class IA PI3K (19). In contrast, examples of transactivation of GPCRs by RTKs are less abundant, although recently it has been shown that IGF-1 stimulated phosphorylation of CCR5 in MCF-7 cells. Chemotaxis induced by IGF-1 was inhibited by a neutralizing anti-CCL5 antibody, which indicates that transactivation of CCR5 by IGF-1 is indirect, requiring production of a CCR5 ligand (20).

Because investigating interactions between different receptor classes is essential for our understanding of the mechanisms by which cells process multiple signaling inputs, we have examined potential cross-talk in the signal transduction pathways induced following ligation of CXCR4 and IGF-1R. Our data demonstrate the existence of a physical association between IGF-1R, CXCR4, and the G protein subunits, $G_{i\alpha}$ and $G_{i\beta}$ in the breast cancer epithelial cell lines, MDA-MB-231. This interaction drives a unidirectional transactivation of CXCR4 and G proteins by IGF-1 leading to cell migration in MDA-MB-231 cells, which is independent of the CXCR4 chemokine ligand, CXCL12. These data indicate the existence of a novel form of transactivation between these two important receptors.

MATERIALS AND METHODS

Cell Lines and Cell Culture Conditions—Breast cancer cell lines, MCF-7 and MDA-MB-231, were obtained from the American Type Culture Collection. P6 cells (BALB/c3T3 cells overexpressing human IGF-1R) were kindly provided by Professor R. Baserga (Philadelphia, PA). MCF-7 and P6 cells were cultured in Dulbecco's modified Eagle's

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² The abbreviations used are: GPCR, G protein-coupled receptor; Abs, antibodies; PI3K, phosphoinositide 3-kinase; MAP, mitogen-activated protein; S1P, sphingosine 1-phosphate; PTX, pertussis toxin; BSA, bovine serum albumin; KIR, kinase receptor activation assay; IL, interleukin; RTK, receptor-tyrosine kinase; IGF, insulin-like growth factor; EGF, epidermal growth factor receptor; PDGF, platelet-derived growth factor.

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medium supplemented with 10% fetal bovine serum whereas MDA-MB-231 cells were in RPMI 1640 with 10% fetal bovine serum, at 37 °C in a 5% CO₂ atmosphere.

Reagents—A hybridoma supernatant containing anti-IGF-1R (7C2 clone) was produced in the Monoclonal Antibody Facility in the School of Molecular & Biomedical Science, The University of Adelaide as described.³ A monoclonal anti-IGF-1R 24-31 (21) was a gift from Dr. Leah Cosgrove (CSIRO, Human Nutrition, Adelaide, South Australia). Monoclonal anti-human CXCR4 antibodies (clone 12G5) were purchased from R&D systems (Minneapolis, MN), and polyclonal CXCR4 antibodies were purchased from Chemicon International Inc. Monoclonal anti-IGF-1R antibodies (clone 2C8), antibodies to G_iα₂ (T-19) and Gβ (M-14) and monoclonal control antibodies IgG (anti-hemagglutinin clone F-7) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescein isothiocyanate-conjugated anti-CXCR4 was from R&D Systems whereas PE-conjugated anti-mouse IgG and horseradish peroxidase-labeled donkey anti-rabbit IgG were purchased from Rockland (Gilbertsville, PA). DELFIA Eu-labeling kit reagents composed of europium-labeled anti-phosphotyrosine PY20 Abs and DELFIA enhancement solution were purchased from PerkinElmer Life Sciences. IGF-1 was obtained from GroPep Pty Ltd (Adelaide, South Australia). CXCL12 was kindly provided by Professor Ian Clark-Lewis (UBC, Vancouver). Pertussis toxin (PTX) was purchased from Sapphire Bioscience, NSW, Australia.

Retroviral-mediated RNAi Knockdown of CXCR4—The shRNA 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 5'-gatctGGTGGTCTATGTTGGCGTCTGttcaagaGACAGACGC-CAACATAGACCACCTtttta-3' and 5'-agcttaaaaaGGTGGTCTATGTTGGCGTC TGtctcttgaacagacgccaacatagaccacca-3' were inserted to produce CXCR4 shRNA-expressing construct. The 21-nucleotide CXCR4 target sites at position 470–490 of human CXCR4 cDNA are indicated in capitals in the oligonucleotide sequences. Previously described oligonucleotides containing specific target sequences for *Renilla* luciferase were used to produce the expression vector for the negative control (22).

To produce retroviral supernatants, 293T packaging cells were transfected with 10 μg of specific or control expression vectors, 8 μg of pVPack-VSV-G, 8 μg of pVPack-GP (Stratagene), and 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 fetal 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. MDA-MB-231 cells were plated in a 60-mm tissue culture dish at ~40% confluency, 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 picked and expanded for further analysis.

Immunofluorescent Staining and Flow Cytometric Analysis—Cells were trypsinized and suspended to 5 × 10⁶ cells/ml in staining buffer

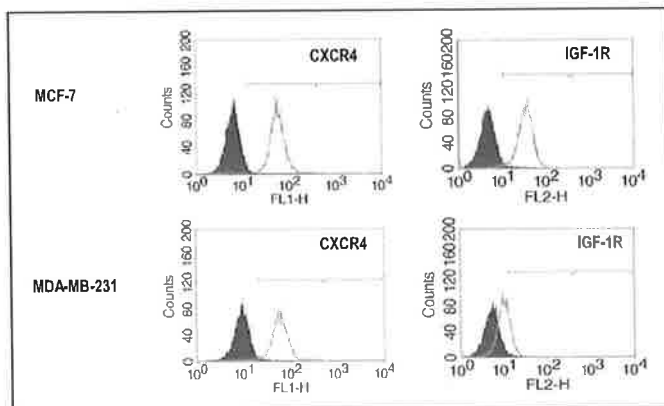


FIGURE 1. Expression of CXCR4 and IGF-1R on MCF-7 and MDA-MB-231 cell lines. The levels of CXCR4 and IGF-1R were assessed by flow cytometry using specific Abs as described under "Material and Methods." This histogram of CXCR4 and IGF-1R expression on MCF-7 and MDA-MB-231 cells is representative of three others performed with similar results. The filled and blank histograms are the isotype control and anti-CXCR4 or anti-IGF-1R staining, respectively.

(phosphate-buffered saline containing 1% BSA and 0.04% sodium azide). After the cells were fixed with 3.7% paraformaldehyde (BDH Laboratory Supplies, Poole, UK) in PBS at room temperature for 10 min, Fc receptors were blocked with purified human IgG (Sigma) (10 μg per 10⁶ of cells) at room temperature for 30 min. The blocked cell suspension (50 μl) was aliquoted to each round bottom tube and incubated each with tested or isotype control Abs at 4 °C for 30 min. For IGF-1R detection, the cells were labeled with hybridoma supernatants and washed with staining buffer, followed by staining with PE-conjugated anti-mouse detection Abs. For CXCR4 detection, the cells were stained with fluorescein isothiocyanate-conjugated anti-CXCR4. The labeled cells were washed with staining buffer followed by phosphate-buffered saline and then detected on a FACscan (BD Australia).

Chemotaxis Assay—Chemotaxis was measured in a Modified Boyden chamber using polycarbonate filters (8 μm for MDA-MB-231 cells and 12 μm for MCF-7 cells, Neuroprobe, Gaithersburg, MD) coated with 25 μg/ml collagen type I (Sigma) in 10 mM acetic acid. Cell suspensions in serum-free medium (RPMI 1640) containing 0.5% BSA were preincubated with calcein-AM (1 μg/ml of final concentration, Molecular Probes (Eugene, OR) for 30 min before being loaded in the upper chamber (5 × 10⁴ viable cells/well) whereas the lower chamber contained various concentrations of CXCL12 and/or IGF-1. After the chamber was incubated at 37 °C (4 h for MDA-MB-231 cells and 6 h for MCF-7 cells), membranes were taken out, and cells on the upper surface were removed. The transmigrated cells on the lower surface were measured by their fluorescent intensity using Molecular Imager[®] Fx (Bio-Rad) and expressed as a migration index, representing the fluorescent signals of stimulated cells compared with those of non-stimulated cells.

Kinase Receptor Activation Assay (KIRA)—The KIRA assay was performed with modifications to a previously described protocol (23–25). Cells (2.5 × 10⁵ cells/well) were cultured in 24-well flat bottom culture plates overnight then placed in serum-free medium (RPMI 1640 with 0.5% BSA) for 4 h before being incubated with various concentrations of CXCL12 or IGF-1. After 10 min of stimulation, cell lysates were prepared by addition of lysis buffer (20 mM HEPES, 150 mM NaCl, 1.5 MgCl₂, 1 mM EGTA, 10% glycerol, and 1% Triton X-100) containing 2 mM Na₃VO₄, 10 mM NaF, and protease inhibitor solution (Sigma) and then transferred to 96-well white polystyrene plates (Greiner Bioone, Germany) which were precoated with anti-IGF-1R antibodies (mAb 24-31) diluted in 50 mM NaHCO₃/Na₂CO₃, pH 9.6 (0.25 μg/well), and blocked with 0.5% BSA in TBST. After overnight incubation, the plates

³ M. Keyhanfar, B. Forbe, L. Cosgrove, G. Booker and J. Wallace, manuscript in preparation.

TABLE ONE
Flow cytometric analysis of CXCR4 and IGF-1R expression on MCF-7 and MDA-MB-231 cells

Cell line	% Cells positive	Geometric mean
CXCR4 expression		
B300 ^a	49.63 ± 23.24 ^b	38.03 ± 6.59
MCF-7	94.74 ± 4.01	65.28 ± 17.08
MDA-MB-231	95.71 ± 1.10	77.32 ± 18.77
IGF-1R expression		
P6 ^c	98.05 ± 1.38	154.72 ± 35.96
huIRB ^{+d}	1.41 ± 0.36	16.11 ± 4.15
MCF-7	98.85 ± 0.69	26.75 ± 7.12
MDA-MB-231	22.65 ± 9.34	13.10 ± 3.47

^a B300, positive control cell line for huCXCR4.^b Data are pooled from three separate experiments and presented as mean ± S.D.^c P6, positive control cell line for huIGF-1R.^d huIRB⁺, positive control cell line for human insulin receptor B.

were washed with TBST, and the activated receptor complex formed was detected by incubating with europium-labeled anti-phosphotyrosine PY20 (10 ng/well) for 2 h at room temperature. After washing with distilled water, the plates were added with DELFIA enhancement solution (100 μ l/well). Time-resolved fluorescence was then measured using 340-nm excitation and 610-nm emission filters on a BMG Lab Technologies PolarstarTM Fluorometer.

Immunoprecipitations and Western Blot Analyses—Cells were lysed at 4 °C for 20 min in Triton-lysis buffer (20 mM HEPES, 150 mM NaCl, 1.5 MgCl₂, 1 mM EGTA, 10% glycerol, and 1% Triton X-100) containing 2 mM Na₃VO₄, 50 mM NaF, 10 mM phenylmethylsulfonyl fluoride, and protease inhibitor (1:100, Sigma-Aldrich). 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 was determined using the BCA assay (Pierce). For immunoprecipitation, the lysates (1 mg of total proteins) were incubated with 1 μ g of either anti-IGF-1R 2C8,

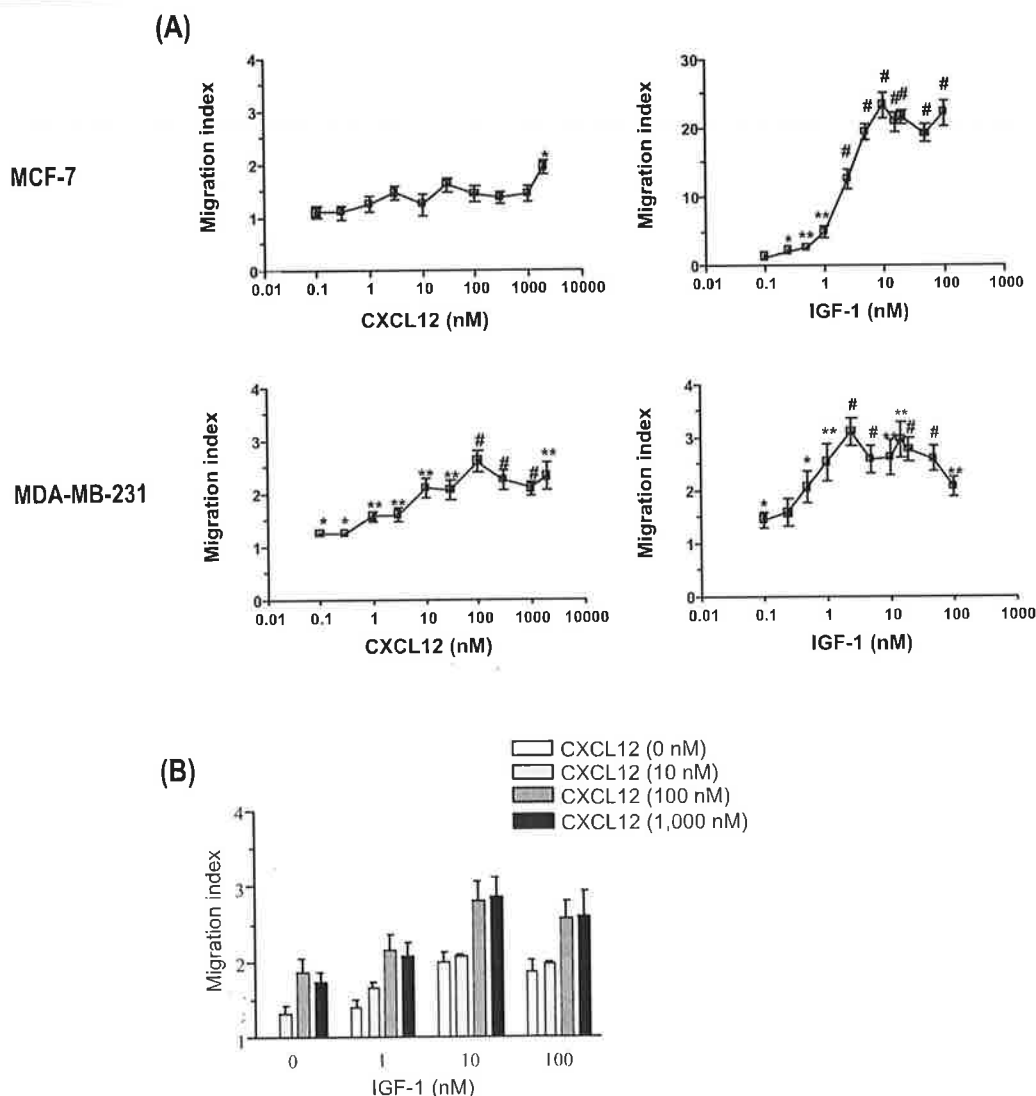


FIGURE 2. The effect of CXCL12 and IGF-1 on chemotaxis of MCF-7 and MDA-MB-231 cell lines. The ability of the cells to migrate in response to CXCL12 and IGF-1 was tested using a modified Boyden chamber as described under "Materials and Methods." The migration index represents the fluorescent signals of stimulated cells compared with those of non-stimulated cells. *A*, the response of MCF-7 and MDA-MB-231 cells to CXCL12 or IGF-1. *B*, the response of MDA-MB-231 cells to combinations of CXCL12 and IGF-1. All panels are expressed mean ± S.E. of migration index from at least three separate experiments each performed in triplicate. Asterisks indicate statistically significantly different from control values (Student's unpaired *t* test) at *, *p* < 0.05; **, *p* < 0.005; #, *p* < 0.0001.

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anti-human CXCR4 12G5, or IgG control antibodies, monoclonal anti-HA, at 4 °C overnight. Immunocomplexes were precipitated with protein G-coated microbeads at 4 °C for 1 h and purified on magnetic microcolumns (Miltenyi Biotec). The bound proteins were eluted from the column in preheated sample buffer (50 mM Tris-HCl pH 6.8, 50 mM dithiothreitol, 1% SDS, 0.005% bromophenol blue, and 10% glycerol). For whole lysate sample preparation, the lysates (50 μ g of total proteins/well) were denatured by boiling for 5 min in sample buffer. The immunoprecipitates and whole lysates were then subjected to 15% SDS-PAGE, transferred to nitrocellulose membrane (HybondTM P, Amersham Biosciences), and analyzed by Western blotting. The transferred membranes were blocked with 1% casein (Roche Applied Science) and incubated with primary Abs (1:1000 of polyclonal anti-CXCR4, 1:500 of anti-G α and -G β) followed by horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:1000). Membranes were visualized by enhanced chemiluminescence (Amersham Biosciences).

RESULTS

Expression of CXCR4 and IGF-1R on the MCF-7 and MDA-MB-231 Breast Cancer Cell Lines—Two breast cancer cell lines, the non-metastatic MCF-7 and metastatic MDA-MB-231, were characterized in terms of the expression and function of CXCR4 and IGF-1R. Flow cytometric analysis showed expression of both CXCR4 and IGF-1R on both cell types (Fig. 1 and TABLE ONE). MCF-7 cells expressed both receptors at high levels (94.74 $\pm$ 4.01% of positive cells with a geometric mean of 65.28 $\pm$ 17.08% for CXCR4 and 98.85 $\pm$ 0.69% of positive cells with a geometric mean of 26.75 $\pm$ 7.12% for IGF-1R) whereas MDA-MB-231 cells showed a high level of CXCR4 (95.71 $\pm$ 1.10% positive cells with a geometric mean of 77.32 $\pm$ 18.77%) and a lower level of IGF-1R expression (22.65 $\pm$ 9.34% of positive cells with a geometric mean of 13.10 $\pm$ 3.47%). Western blot analysis also confirmed the expression of CXCR4 and IGF-1R in both cell lines (data not shown).

Chemotactic Response of MCF-7 and MDA-MB-231 Cells to CXCL12 and IGF-1—The function of CXCR4 and IGF-1R on MCF-7 and MDA-MB-231 cells was examined by testing the migrational response of the cells to respective ligands, CXCL12 and IGF-1, using a modified Boyden chamber chemotaxis assay. Interestingly, even though CXCR4 expression was similar on MDA-MB-231 and MCF-7 cells, only the former responded to CXCL12 (Fig. 2A). In contrast, both cell lines migrated in response to IGF-1. However, in keeping with the lower level of IGF-1R expression, IGF-1-induced chemotaxis of MDA-MB-231 cells was lower than that observed in MCF-7 cells. Additional experimentation was conducted in MDA-MB-231 cells to determine the effect of combined stimulation with CXCL12 and IGF-1. The results of these experiments indicated an additive effect of those ligands on chemotaxis of MDA-MB-231 cells (Fig. 2B).

CXCL12 Does Not Transactivate IGF-1R on MDA-MB-231 Cells—To investigate potential cross-talk between CXCR4 and IGF-1R-induced signal transduction pathways, we initially determined whether there is cross-activation of IGF-1R by CXCL12 on MDA-MB-231 cells. Because activation of IGF-1R by IGF-1 leads to the rapid formation of a tyrosine-phosphorylated receptor complex, a KIRA assay was performed to compare the levels of IGF-1R activation induced by CXCL12 and IGF-1. Preliminary experiments indicated that in P6 (positive control), MCF-7 and MDA-MB-231 cells, maximal levels of activated IGF-1R complex formed after stimulation with 10 nM IGF-1 at 10 min (data not shown). Therefore, in subsequent experiments, the cells were stimulated with various concentrations of IGF-1 and CXCL12 for 10 min. The results of these experiments indicate that IGF-1 dose-dependently induced the

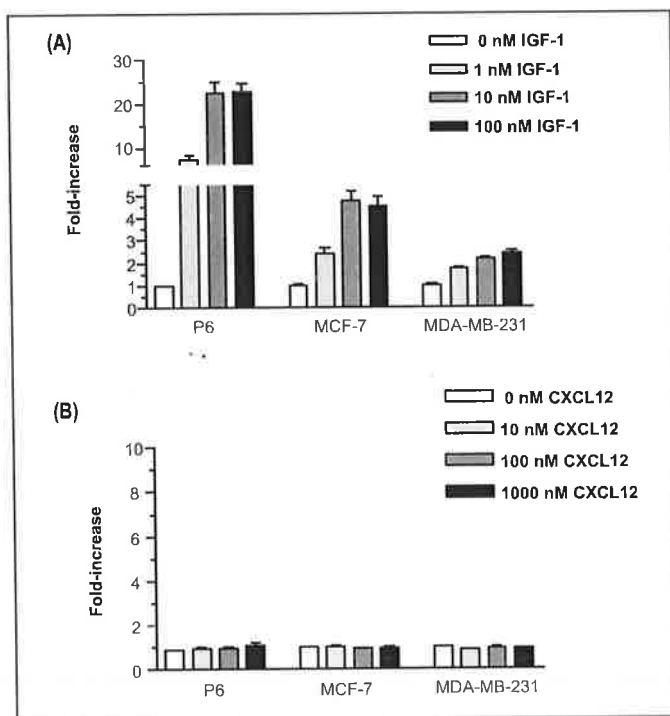


FIGURE 3. IGF-1 but not CXCL12 induces activation of IGF-1R in MCF-7 and MDA-MB-231 cells. A KIRA assay was performed to measure the level of tyrosine-phosphorylated IGF-1R complex formed after incubation with CXCL12 or IGF-1. Fold-increase represents the level of receptor complex formed in stimulated compared with non-stimulated cells. A and B show the level of IGF-1R activation induced by different doses of IGF-1 and CXCL12, respectively. The cell line P6 that overexpresses human IGF-1R was used as a positive control. Data are presented as the mean $\pm$ S.E. from at least three independent experiments each performed in triplicate.

activation of IGF-1R in all three cell lines (Fig. 3A) whereas CXCL12 failed to do so at any of the concentrations tested (Fig. 3B).

Pertussis Toxin Inhibits CXCL12- and IGF-1-induced Chemotaxis but Does Not Affect the Activation of IGF-1R Induced by IGF-1 in MDA-MB-231 Cells—To investigate the involvement of G α in IGF-1-induced chemotaxis of MDA-MB-231 cells, the cells were treated with various concentrations of PTX, a specific inhibitor of G α subunits. The cells were then tested for their chemotactic response to various concentrations of both CXCL12 and IGF-1. As shown in Fig. 4, A and B, PTX at a concentration of 10 ng/ml completely blocked the response to CXCL12 and partially inhibited that to IGF-1 in MDA-MB-231 cells. Similar levels of inhibition were observed when the cells were pretreated with 100 and 1,000 ng/ml PTX (data not shown). These data indicate a contribution of G α to IGF-1-induced chemotaxis of MDA-MB-231 cells. Pretreatment of MCF-7 cells with PTX had no effect on IGF-1-induced chemotaxis at any of the three doses tested (Fig. 4C).

To test the possibility that blocking G α with PTX inhibits the activation of IGF-1R by IGF-1, the lysates of cells untreated or treated with PTX were assayed for the level of tyrosine-phosphorylated IGF-1R complex formed in response to IGF-1 using the KIRA assay. Two different doses of PTX (10 and 100 ng/ml) failed to alter the level of IGF-1R activation in either MDA-MB-231 or MCF-7 cells (Fig. 5, A and B) indicating that G α is not involved in IGF-1-induced formation of the activated IGF-1R complex.

RNAi of CXCR4 Inhibits Both CXCL12- and IGF-1-induced Chemotaxis but Has No Effect on the Activation of IGF-1R in MDA-MB-231 Cells—The involvement of CXCR4 in IGF-1-induced chemotaxis of MDA-MB-231 cells was examined using CXCR4-deficient cells. MDA-MB-231 cells were infected with a retrovirus expressing either RNAi to

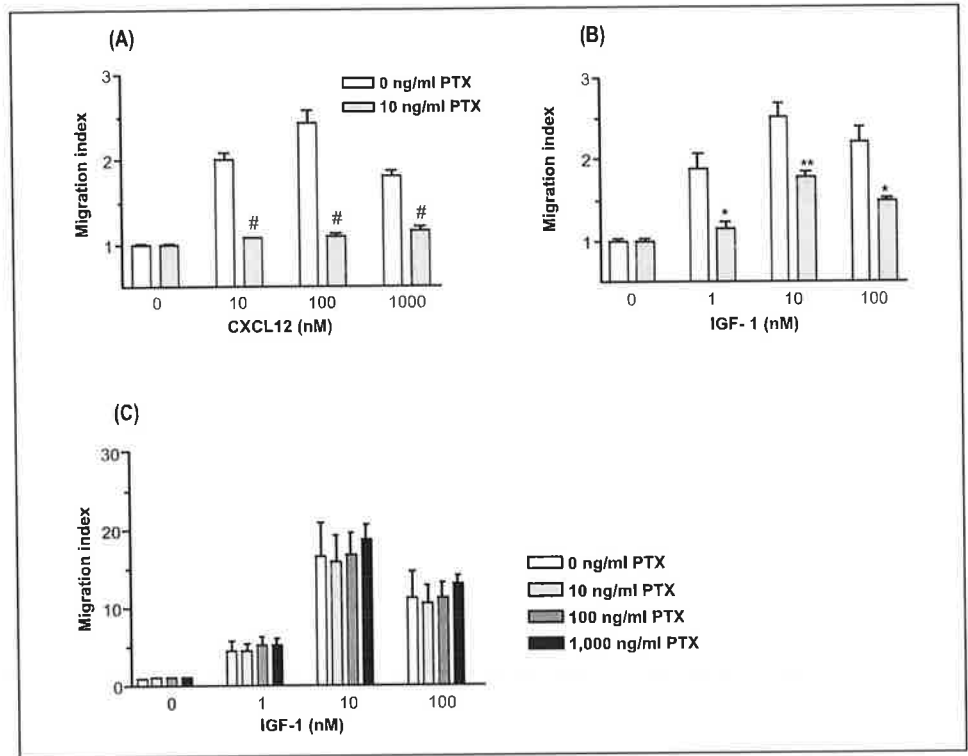


FIGURE 4. Pertussis toxin inhibits CXCL12- and IGF-1-mediated chemotactic responses of MDA-MB-231 cells but does not affect the IGF-1-induced response in MCF-7. The cells were pretreated with various concentrations of PTX prior to testing their chemotactic ability using the Modified Boyden chamber assay. PTX completely blocked the response mediated by CXCL12 in MDA-MB-231 (A); partially inhibited that by IGF-1 in MDA-MB-231 cells (B); had no effect on the IGF-1-induced response in MCF-7 cells (C). The data are represented as the mean $\pm$ S.E. of migration index; $n = 3$ (A and B) and $n = 5$ (C) each performed in triplicate. Asterisks indicate statistically significantly different from control values (Student's unpaired *t* test) at *, $p < 0.05$; **, $p < 0.005$; #, $p < 0.0001$.

knockdown CXCR4 or a retrovirus expressing specific target sequences for *Renilla* luciferase as a negative control. Individual clones were isolated and characterized for CXCR4 surface expression by flow cytometry and CXCR4 function was determined by assessing calcium mobilization and chemotaxis in response to CXCL12. Compared with wild-type MDA-MB-231 cells and the negative control clone, RNAi clones 11, 21, and 27 demonstrated a significant reduction of surface CXCR4 expression (Fig. 6A, shown only for clone 11) and of calcium mobilization in response to CXCL12 (data not shown). The surface expression of IGF-1R was not affected by RNAi CXCR4 knockdown in any of the clones (Fig. 6A shown only for clone 11). Compared with wild-type cells and the negative control clone, RNAi clones 11, 21, and 27 displayed a significant reduction in chemotaxis in response to CXCL12 (Fig. 6B) and IGF-1 (Fig. 6C). In contrast, knockdown of CXCR4 did not have any effect on IGF-1-induced IGF-1R activation as determined in the KIRA assay (Fig. 6D).

IGF-1R Is Physically Associated with CXCR4 and G Protein Subunits in Both MDA-MB-231 and MCF-7 Cells, but IGF-1 Activates the CXCR4 Signaling Pathway through G Proteins Only in MDA-MB-231 Cells—The nature of the interaction between IGF-1R, CXCR4, and G proteins in MCF-7 and MDA-MB-231 cells was investigated. Immunoprecipitations were performed on cell lysates using an anti-IGF-1R mAb, anti-human CXCR4, or control IgG, followed by Western blots for either CXCR4, $G_i\alpha_2$, or $G\beta$. The immunoprecipitations with control IgG failed to coprecipitate CXCR4, $G_i\alpha_2$, and $G\beta$, despite the fact that these latter three proteins were readily detectable in whole cell lysates subjected directly to Western blot (Fig. 7A). In contrast, immunoprecipitation of IGF-1R and CXCR4 in both MCF-7 and MDA-MB-231 cells led to coprecipitation of all three proteins, indicating the existence of a constitutive complex between IGF-1R, CXCR4, $G_i\alpha_2$, and $G\beta$. Of note, the levels of $G_i\alpha_2$ and $G\beta$ in immunoprecipitates of the two receptors consistently appeared to be higher in MDA-MB-231 cells than in MCF-7 cells.

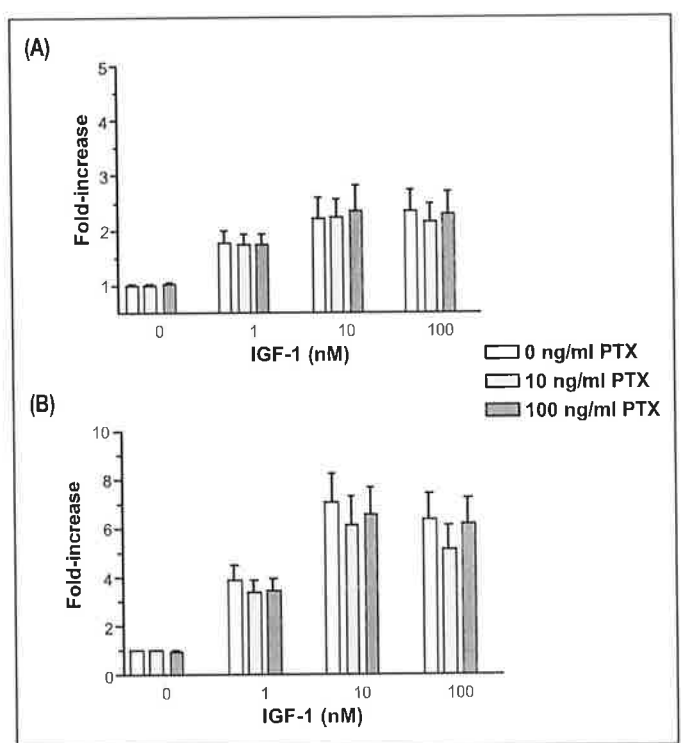
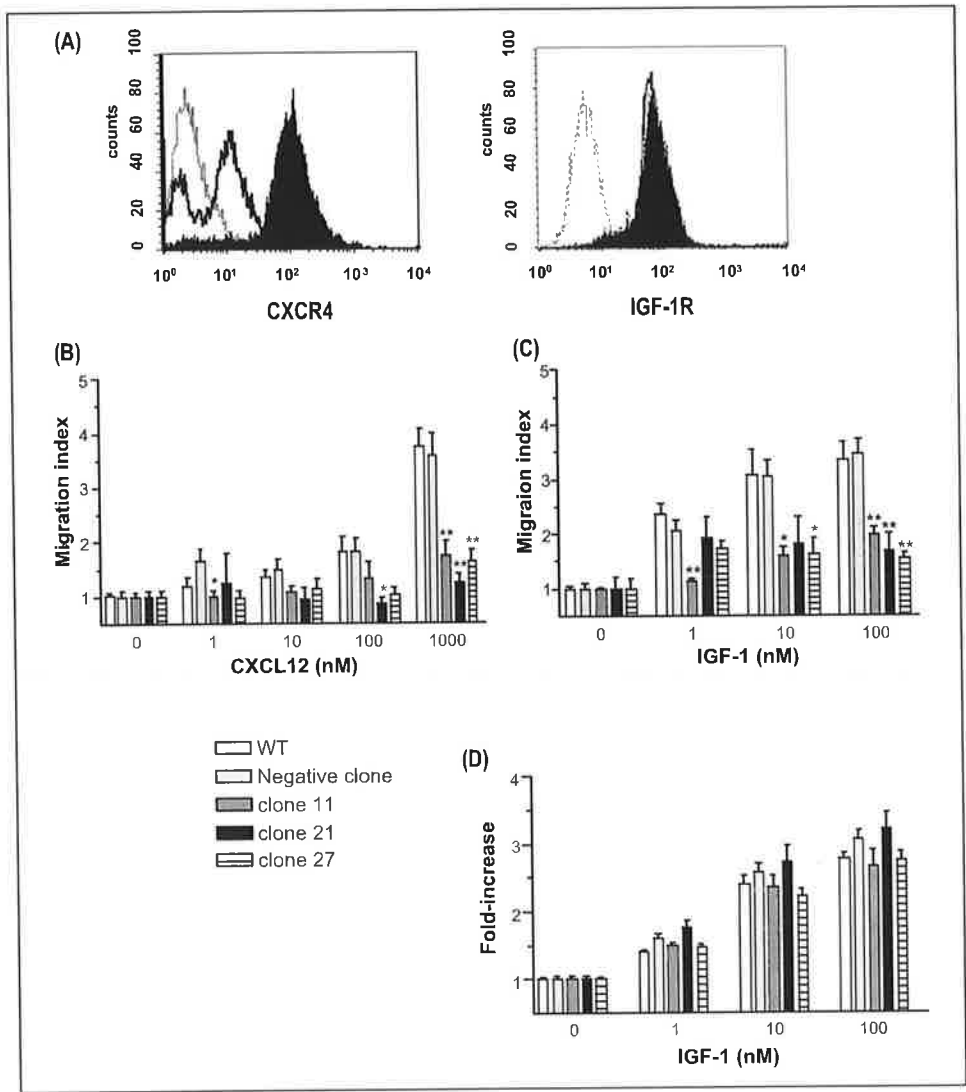


FIGURE 5. Pretreatment with pertussis toxin has no effect on IGF-1-induced IGF-1R activation. MDA-MB-231 and MCF-7 cells were pretreated with various concentrations of PTX and the level of activated IGF-1R complex induced by incubation with the indicated concentrations of IGF-1 for 10 min was determined using a KIRA assay as described under "Materials and Methods." MDA-MB-231 (A) and MCF-7 (B) cells were used. The levels of activated IGF-1R complex are expressed as fold-increase in relation to the level observed in unstimulated cells. The data are represented as the mean $\pm$ S.E. of $n = 5$ independent experiments each performed in triplicate.

Transactivation of CXCR4 by IGF-1

FIGURE 6. Retroviral shRNA specifically inhibits CXCR4 surface expression and CXCL12- and IGF-1-induced migration of MDA-MB-231 breast cancer cells. Cells were infected with retroviruses producing shRNAs, individual clones were selected in puromycin-containing media and analyzed by fluorescence-activated cell sorting (FACS) for the surface expression of CXCR4 and IGF-1R (solid line) together with the wild-type untreated cells (filled histogram). Isotype-matched IgGs were used as a negative control (dotted line). A, histogram showing reduction in the surface levels of CXCR4 and no change in the levels of IGF-1R for the MDA-MB-231 clone 11. Clones 11, 21, and 27 were assessed their ability to migrate in response to CXCL12 (B) and IGF-1 (C) compared with the negative clone and wild-type cells. D, the level of activated IGF-1R complex formed in RNAi CXCR4 knockdown clones compared with the negative clone and wild type. Data are shown as mean $\pm$ S.E. of migration index from 2–5 separate experiments each performed in quadruplicate. Asterisks indicate statistically significantly different from control values (Student's unpaired t test) at *, $p < 0.05$ or **, $p < 0.005$.



The ability of IGF-1 to transactivate CXCR4 was investigated by examining the effect of stimulation with IGF-1 on the level of association of $G_i\alpha_2$ and $G\beta$ with CXCR4 (Fig. 7B). The results of these experiments showed that stimulation of MCF-7 cells with IGF-1 failed to release either $G_i\alpha_2$ or $G\beta$ from the CXCR4/IGF-1R complex, whereas, in contrast, both $G_i\alpha_2$ and $G\beta$ were released from the complex in MDA-MB-231 cells.

DISCUSSION

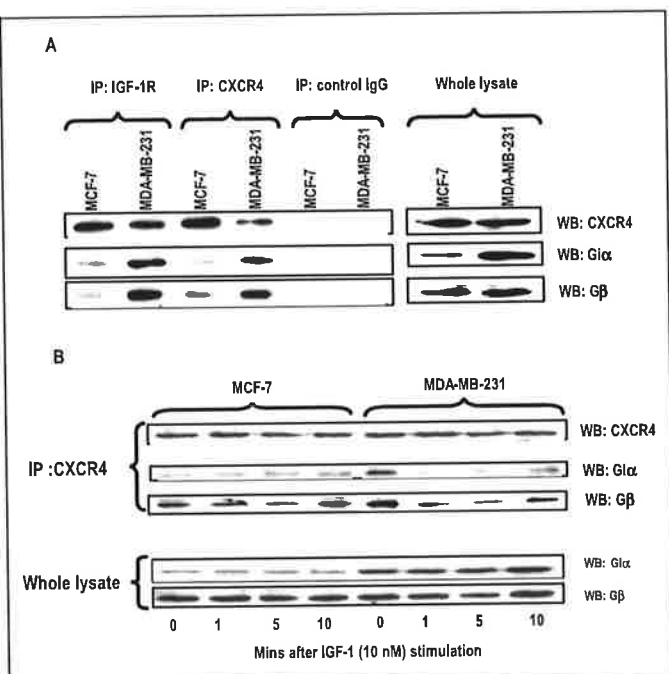
Here we present evidence of cross-talk between CXCR4 and IGF-1R in the human epithelial breast cancer cell line, MDA-MB-231. The basis of this cross-talk appears to depend on a physical association between CXCR4 and IGF-1R. It is unidirectional, involving activation of G protein subunits by IGF-1 that is dependent on the presence of a functional pool of CXCR4, but independent of the CXCR4 ligand CXCL12. These observations potentially have major implications for our understanding of the intracellular signaling of these two important receptors in both normal and pathological situations.

In this study, two breast cancer cell lines, the non-metastatic MCF-7 and the highly metastatic MDA-MB-231 were characterized in terms of expression and function of CXCR4 and IGF-1R. MCF-7 cells exhibited a high level of IGF-1R expression and a strong chemotactic response to

IGF-1 whereas MDA-MB-231 cells expressed a lower level of the receptor and a lower response to IGF-1. The lower level of IGF-1R expression in the metastatic MDA-MB-231 cells compared with the non-metastatic MCF-7 cells correlates well with the results of a recent study demonstrating that reduced expression of IGF-1R in MCF-7 cells leads to a more metastatic phenotype in those cells (26).

Although the two cell lines expressed high levels of CXCR4, only MDA-MB-231 cells responded functionally to CXCL12, indicating uncoupling of receptor expression and function in the MCF-7 cells. This phenomenon has been observed previously with respect to CXCR4 in the human hepatoma cell line HepG2 (27), and other chemokine receptors in a range of cell types (28, 29), although the molecular basis for this non-functional phenotype, at least with respect to cell migration, was not defined in those studies. However, the results of our studies suggest at least two mechanisms: differences in the level of expression of $G_i\alpha_2$ and $G\beta$ in MB-MDA-231 cells and MCF-7 cells, which results in different levels of association of $G_i\alpha_2$ with CXCR4 in those cells, and the failure of $G_i\alpha_2$ and/or $G\beta$ to uncouple from CXCR4 upon activation of the receptor (data not shown).

Three forms of cross-talk between GPCR and RTK systems have been demonstrated in different cellular systems. First, RTKs can be transactivated by GPCRs. For example, EGF-R is phosphorylated in response to



We found that formation of the tyrosine-phosphorylated IGF-1R complex is independent of CXCR4 and G proteins. This suggests the signal transduction pathway leading to activation of CXCR4 bifurcates either before or following tyrosine phosphorylation of IGF-1R. Of note, the inhibition of IGF-1-induced chemotaxis by PTX and knockdown of CXCR4 was only partial even though CXCL12-induced migration of MDA-MB-231 cells was completely inhibited. The partial inhibition of IGF-1-induced cell migration indicates CXCR4- and G protein-independent induction of chemotaxis through IGF-1R. Cell migration in eukaryotic cells is known to depend on activation of Class IA and IB PI3Ks (31, 32). In general, Class IA PI3Ks link RTKs to cell migration, whereas Class IB PI3Ks mediate cell migration in response to ligation of GPCRs (33, 34). IGF-1 is known to induce activation of PI3K and this is dependent on tyrosine phosphorylation. In contrast, ligation of CXCR4 activates Class IB PI3K, and this is inhibited by PTX. Therefore, the residual cell migration observed in response to IGF-1 in PTX-treated cells is likely because of tyrosine phosphorylation-dependent Class IA PI3K activation. A physical interaction between IGF-1R and $G_{i\alpha}$ and G_{β} has previously been demonstrated (35, 36). Moreover, in those studies, PTX was shown to inhibit IGF-1-induced activation of MAPK in neuronal cells (36), and IGF-1-induced mitogenesis of HIRcB cells and 3T3L1 adipocytes (35). In contrast to the conclusion from that study that IGF-1R functions as a G protein-coupled receptor (35), our findings suggest that the association of the G protein subunits with IGF-1R is indirect, and requires the presence of CXCR4. Certainly, our data indicate that the presence of functional CXCR4 is required for G protein-dependent cell migration in response to IGF-1.

Interestingly, CXCR4 and IGF-1R could be coprecipitated in both MCF-7 and MDA-MB-231 cells, indicating that the lack of involvement of CXCR4 in IGF-1-induced chemotaxis of MCF-7 cells was not because of a lack of association of CXCR4 and IGF-1R in those cells. Rather, our data indicate that the cross-talk in MDA-MB-231 cells is mediated at the level of G protein activity; both $G_{i\alpha_2}$ and $G\beta$ are associated with the complex in both cell lines; however, activation of the complex, as determined by release of $G_{i\alpha_2}$ and $G\beta$ from CXCR4, only occurs in MDA-MB-231 cells. This is consistent with our observation that MCF-7 cells do not respond to CXCL12, at least in terms of the migratory response.

In summary, our data provide evidence of a novel transactivation between RTK and GPCR signal transduction pathways. We have observed the coprecipitation of IGF-1R, CXCR4, and the G protein subunits, $G_i\alpha_2$ and $G\beta$, indicating a constitutive physical association between these molecules. Based on our data, we propose that this IGF-1R/CXCR4 complex allows CXCR4 and G proteins to act partially in IGF-1-induced chemotaxis of MDA-MB-231 cells probably through activation of class IB PI3K activity. Our data also demonstrate that CXCR4 and G proteins operate independently of the activation of IGF-1R because neither PTX pretreatment nor CXCR4 knockdown affected the levels of tyrosine-phosphorylated IGF-1R complex formed

CCL11, a ligand for the GPCR CCR3, leading to the MAP kinase activation and IL-8 production in bronchial epithelial cells (18). This appears to depend on activation of CCR3 by CCL11. Second, GPCRs can be transactivated by RTKs. For example, it has been shown that IGF-1 stimulates phosphorylation of CCR5 in MCF-7 cells. Chemotaxis induced by IGF-1 was inhibited by a neutralizing anti-CCL5 antibody. Transactivation of CCR5 by IGF-1 was therefore indirect, requiring the activity of the ligand (CCL5) for the second receptor (20). Finally, bidirectional transactivation between the same two receptor systems has also been observed: PDGFR is phosphorylated by S1P leading to activation of downstream effectors including Shc, and the p85 regulatory subunit of the class IA PI3K (19), and PDGF has been demonstrated to transactivate the S1P receptor (30).

In MDA-MB-231 cells, transactivation of IGF-1R following activation of CXCR4 was not observed: incubation of the cells with CXCL12 failed to stimulate the formation of the tyrosine-phosphorylated IGF-1R complex, one of the earliest detectable events that occurs following ligation of IGF-1R by IGF-1 (23–25). Moreover, neither pretreatment of the cells with pertussis toxin nor RNAi-mediated knockdown of CXCR4 inhibited IGF-1-induced formation of the activated IGF-1R complex. Taken together, these data indicate that neither G proteins nor CXCR4 are involved in formation of the phosphorylated IGF-1R signaling complex. In contrast, our data demonstrate transactivation of CXCR4 by IGF-1. Blocking $G_i\alpha$ by pretreatment of the cells with PTX, (which completely inhibits the chemotactic response to CXCL12), partially blocked the chemotactic response of the cells to IGF-1. In addition, RNAi-mediated knockdown of CXCR4 significantly inhibited IGF-1-induced chemotaxis of MDA-MB-231 cells. Together, these data indicate that IGF-1-induced chemotaxis is dependent on functional expres-

Transactivation of CXCR4 by IGF-1

after IGF-1 stimulation. The fact that this pathway does not appear to be active in the non-metastatic MCF-7 cells suggests that CXCR4/IGF-1R receptor integration may play an important role in cancer metastasis. In addition, both IGF-1/IGF-1R and CXCL12/CXCR4 are essential for life (3, 37–39) raising the possibility that transactivation between IGF-1R and CXCR4 may be involved in development. Further experimentation comparing IGF-1R signaling complexes in both MCF-7 and MDA-MB-231 cells may provide further insights.

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Typographical errors:

Page IV, third paragraph: Mehrnaz Keyhanfar

Page 23, line 3 from bottom: (Figure 1.7)

Page 29, line 4: antibodies appear

Page 53, line 2: ECL, definition is "Enhanced Chemi-Luminiscence"

Page 53, 2nd paragraph, line 6: The oligonucleotides.

Page 72, line 4: The previous studies