Investigation of PI3Kγ signaling downstream of IGF-1R-CXCR4 transactivation in metastatic MDA-MB-231 breast cancer cells

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Abstract

Breast cancer metastasis is a multi-step process regulated by a number of homeostatic factors. The insulin-like growth factor 1 tyrosine kinase receptor (IGF-1R) and the chemokine G-protein coupled receptor, CXCR4 have been shown to play an important role in breast cancer metastasis. More recently, accumulating evidence suggest that these two distinct receptors may regulate breast cancer cell migration through receptor transactivation. However, the underlying molecular mechanisms by which IGF-1R-CXCR4 transactivation regulates breast cancer cell metastasis remain unclear. Since phosphoinositide 3 kinases (PI3Ks) are known to be key signaling molecules governing cell migration, PI3K signaling downstream of IGF-1R-CXCR4 transactivation was investigated. In the present study the expression of class I PI3K isoforms was investigated in metastatic MDA-MB-231 breast cancer cells compared to that in non-metastatic MCF-7 cells. The data show that high levels of class IB PI3K catalytic subunit, p110γ are restricted to the highly metastatic cell types, correlating with the metastatic potential of the cell lines. Moreover, PI3Kγ is the major PI3K isoform regulating cell migration and activation of Akt downstream of IGF-1R-CXCR4 transactivation in metastatic MDA-MB-231 cells. Finally, several downstream targets that are dependent on PI3Kγ were identified using 2-D Fluorescence Difference Gel Electrophoresis (DIGE) and mass spectrometry analysis, including eukaryotic elongation factor 2 (eEF2), pyruvate kinase isozymes M1/M2 (PKM1/M2) and phosphoglycerate kinase 1 (PGK1) with PI3Kγ being shown to regulate phosphorylation of eEF2. In summary, the data in this study demonstrate a novel role for PI3Kγ in regulating cell migration downstream of IGF-1R-CXCR4 transactivation, potentially by attenuating cell proliferation via inhibition of eEF2 activation. The understanding of molecular mechanisms underlying receptor transactivation, including PI3K signaling transduction pathways in the progression of breast cancer metastasis and invasion may lead to development of more effective diagnostic and therapeutic strategies.

Declaration

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Poster Presentation

Table of Contents

List of Figures

List of Tables

Abbreviations

Ab	antibody
BCA	bicinchoninic acid
bp	base pair
BSA	bovine serum albmin
°C	degrees Celsius
CCL	CC chemokine ligand
CCR	CC chemokine receptor
cDNA	complementary deoxyribonucleic acid
CXCL	CXC chemokine ligand
DIGE	Difference Gel Electrophoresis
DMEM	Dulbecco' s modifeied Eagle' s medium
DMSO	dimethyl sulfoxide
DNA	Deoxynucleic triphosphate
dNTPs	deoxynucleic triphosphates
eEF2	Eukaryotic elongation factor 2
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	estrogen receptor
FCS	fetal calf serum
g	gram
GPCR	G-protein coupled receptor
G protein	GTP-binding protein
GRK	G-protein coupled receptor kinase
GTP	guansine 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 groth factor I or II
IGF-1R	insulin-like groth factor-1 receptor

IGF-2R	insulin-like groth factor-2 receptor
IGFBP	insulin-like groth factor binding protein
IR	insulin receptor
IRS	insulin receptor substrate
JAK	Janus-family tyrosone kinase
kDa	kiloDalton
KIRA	kinase receptor activation assay
l	liter
MS	mass spectrometry
mTOR	mammalian target of rapamycin
m	metre
mA	milliampare
MAPK	mitogen-activated protein kinase
mg	milligram
ml	milliliter
mM	millimolar
mRNA	messenger RNA
µ	micron
µg	microgram
µl	microliter
n	nano
nm	nanometer
nM	nanomolar
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
PKB	protein kinase B
PKC	protein kinase C
PMSF	phenylmethylsulfonylfluoride

PTEN	phosphatase and tensin homolog deleted on chromosome ten
PTX	pertussis toxin
Raf	Ras activated factor
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institue medium
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulphate
Shc	src homology proteins
SOS	son of sevenless
S1P	sphingosine 1-phophate
S1P1	sphingosine 1-phophate receptor
STAT	signal transducer and activator of transcription
TAE	tris acetate EDTA
TBS	tris buffered saline
TEMED	N,N,N'-tetramethyl ethylenediamine
Tween-20	polyoxyethylene sorbitan monolaurate
V	volt
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

Introduction

Chapter 1: Introduction

1.1 Breast cancer

1.1.1 Overview

Worldwide, breast cancer is the most common cancer and the leading cause of cancer-related death among women (Muti *et al.*, 2006). According to an estimate 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). The mortality rates of breast cancer vary geographically. The breast cancer mortality rates in Western society is stable or even declining slightly, possibly due to widespread mammographic screening, more precise diagnosis and improvement in treatment (Veronesi *et al.*, 2005). However, rates in eastern European and Asian countries have been rising recently, thus breast cancer continues to be an important public health issue on a global scale (Veronesi *et al.*, 2005). Most of the deaths from breast cancer arise from invasive and metastatic carcinomas. It has been reported that regardless of the advance of treatment for primary tumours, breast cancer shows a 20-30% recurrence and low overall survival rates of 24-36 months (Sanchez-Munoz *et al.*, 2009).

Clinically, breast cancer is believed to be a highly heterogeneous disease developed through sequential multi-step stages (Kenemans *et al.*, 2004). Breast cancer progresses through atypical duct hyperplasia, low-grade ductal carcinoma *in situ* (DCIS) to fully developed invasive breast cancer characterized by stromal invasion and metastasis to regional lymph nodes or distant organs (Veronesi *et al.*, 2005). Depending on the stage of the disease, different treatments such as surgery, radiotherapy and chemotherapy have been developed. More recently, new strategies targeting the biomolecular markers for breast cancer have been developed and in the case of hormone-and endocrine-based therapies, it has significantly decreased the mortality rates in the past decade (Veronesi *et al.*, 2005). Several other molecular targets and targeted therapeutic drugs have also been investigated

based on the improving understanding of the molecular mechanisms underlying the progression of this cancer.

1.1.2 Breast cancer tumourigenesis

1.1.2.1 Inherited factors

Genetic predisposition has been suggested to be one of the factors resulting in breast cancer (Polyak, 2001). Mutations of tumour susceptible genes such as *BRCA1* and *2* are the most commonly identified genetic syndromes identified in breast cancer. These account for approximately 10% of all breast cancer cases (Marcus *et al.*, 1996) and increase the risk of developing breast cancer 40-85% (Armstrong *et al.*, 2000; Robson & Offit, 2007; Singletary, 2003). Evidence suggests that there are several other genes that are associated with breast cancer at a 5% significance level (de Jong *et al.*, 2002), some of which may be associated with breast cancer susceptibility observed in several rare genetic syndromes, such as Cowden Syndrome (Brody & Biesecker, 1998).

1.1.2.2 Environmental factors

The majority of breast cancer cases are non-inherited resulting from multiple environmental effects that contribute to the acquisition of somatic genetic changes. The precise molecular mechanisms underlying sporadic breast cancer tumourigenesis have not been fully understood, even though several genes have been shown to promote breast cancer progression in sporadic cases. These genes include those encoding hormonal receptors such as estrogen and progesterone receptor, proto-oncogene, *HRAS*, DNA repair genes, *XRCC1* and *XRCC3* (Weber & Nathanson, 2000), and a member of epidermal growth factor receptor (EGFR) family, *HER2* (Kakarala & Wicha, 2008).

1.1.2.3 Breast cancer stem cells (BCSCs)

Recently, there is some evidence emerging that both inherited and sporadic breast cancer may develop through dysregulation of stem-cell self-renewal (Brabletz *et al.*, 2005) and these aberrant stem cells are termed as breast cancer stem cells (BCSCs). BCSCs have been identified (Al-Hajj *et al.*, 2003) and isolated (Charafe-Jauffret *et al.*, 2009; Dontu *et al.*, 2003; Ginestier *et al.*, 2007; Liu *et al.*, 2006) *in vitro*. Moreover, it has been

demonstrated that injection of as few as 1000 of these cells is able to generate tumours in immunodeficient mice (Ponti *et al.*, 2005). The detailed molecular mechanism by which BCSCs are regulated, especially a series of oncogenetic pathways has also been investigated in several studies (Foulkes, 2004; Kakarala & Wicha, 2008; Korkaya & Wicha, 2009; Liu *et al.*, 2008a; Shimono *et al.*, 2009; Wellner *et al.*, 2009; Yu *et al.*, 2007), which opens the door to potential new strategies for the therapeutic development (McDermott & Wicha, 2010).

1.1.3 Breast cancer and metastasis

During the cancer-development process, the formation of metastasis has been characterized as a major reason for the failure of current therapeutic methods. Over the last decade significant progress has been made towards a better understanding of metastasis, however our knowledge remains limited and treatments targeting metastasis remain a priority.

Metastasis is a complex process and is composed of sequential and interrelated steps (Chambers *et al.*, 2002; Fidler, 2002). It involves the initial cellular transformation and growth at the primary site, formation of a vascular network called angiogenesis, shedding of tumor cells into blood circulation, named as intravasation, arrest of the cells in the target organs, penetration into surrounding tissue, known as extravasation and establishment of secondary tumours after proliferation (Figure 1.1). The establishment of the secondary tumours almost certainly involves the blood circulatory system. However, the lymphatic system has also been shown to play a significant role (Weigelt *et al.*, 2005). It is believed that tumour cells can enter the lymph nodes through the lymphatic system during the early stages of tumour growth (Chambers *et al.*, 2002).

It has been proposed that metastasis is an inefficient process based on the observation that only a small percentage of cells from primary tumour can establish metastases (Weiss, 1996). The underlying molecular mechanisms have been recently investigated (Cameron *et al.*, 2000; Fidler, 1970; Luzzi *et al.*, 1998; Weiss, 1996), including a concept of migrating cancer stem (MCS) cells (Brabletz *et al.*, 2005). That study, in colorectal cancer, suggests that stationary cancer stem cells embedded in the epithelial tissues can not

disseminate whereas migrating cancer stem cells located predominantly at the tumour-host interface that are derived from stationary cancer stem cells through acquisition of a transient epithelial-mesenchymal transition (EMT) can efficiently form distant metastasis. However, it still requires verification in other types of human tumours than colorectal cancer.

Metastasis also represents a highly organized, non-random and organ-selective process (Nicolson, 1993). The most common places for metastasis in breast cancer are lung, liver, bone marrow and lymph nodes (Muller *et al.*, 2001). This organ preference of cancer metastasis was firstly discussed by Paget, who presented a theory of "seed" and "soil" in 1889 (Fidler, 2002). Recent studies provide further insight, including that cancer cells selectively settle at a site producing sufficient growth factors to facilitate proliferation, adhesion molecules to recruit the circulating cells and chemoattractants to promote tumor cells travelling to particular sites (Liotta, 2001; Liotta & Kohn, 2001; Moore, 2001; Murphy, 2001). As an example, it has been shown that the bone niche provides appropriate homing signaling to tumor cell and tumor cells generate a series of molecules resulting in the modification of the bones to facilitate tumor cells proliferation and survival (Guise, 2010). This complex interplay between the tumor cells and organ microenviroments is also described as "vicious cycle".

1.2 Molecular basis of breast cancer

1.2.1 Overview

A number of homeostatic factors have been implicated in breast cancer biology, including insulin-like growth factors (IGFs) and chemokines. They assist tumour cell proliferation, survival and migration which promote growth and invasion of tumours (Liotta, 2001; Singer *et al.*, 2000).

IGFs and chemokines mediate multiple cell functions through their cognate receptors, which belong to tyrosine kinase receptor (RTK) and G-protein-coupled receptor (GPCR)

families, respectively. Recent studies have shown that the signaling pathways initiated following ligation of RTKs and GPCRs are not necessarily linear in that, they create a complex signaling network involving crosstalk. Such crosstalk between these two kinds of receptors contributes to this complexity and has been claimed to play an important role in regulating multiple cell activities including metastasis.

This section focuses on the established roles of IGF, especially IGF-I, and chemokines and their receptors, in particular CXCL12/CXCR4 in cancer and the molecular mechanisms by which these molecules influence cancer biological processes.

1.2.2 The IGF system and breast cancer

1.2.2.1 The IGF system

The IGF system is composed of three peptide hormones: IGF-I, IGF-II and insulin, four cell surface receptors: IGF receptor type 1 (IGF-1R), IGF receptor type II (IGF-2R)/mannose 6-phosphate receptor (M6P-R), insulin receptor (IR) and hybrid of IGF/insulin receptor, six high-affinity binding proteins (IGFBPs 1-6) and IGFBP protease (Adams *et al.*, 2000; Froesch *et al.*, 1963; Lelbach *et al.*, 2005; Pavelic *et al.*, 2007; Pollak, 2008; Sachdev & Yee, 2001; Tao *et al.*, 2007) (Figure 1.2). Most circulating IGFs are generated by the liver and stimulated by both hormonal and nutritional factors. They are also expressed in an autocrine or paracrine manner in numerous target tissues, such as breast tissue (Sachdev & Yee, 2001), indicating their role as both hormones and tissue growth factors (Pollak, 2008; Tao *et al.*, 2007). IGF-I is more important in postnatal growth and development while IGF-II is required for embryogenesis. Mice bearing a homozygous deletion of the IGF-I gene are born with 60% decrease in weight compared with wild type mice and the majority of the mice die soon after birth due to hypodevelopment of the lung and diaphragm (Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993). IGF-II is predominantly expressed during prenatal growth and development. Heterozygous IGF-II gene knockout in the mice results in a 60% smaller size compared with wild type counterparts (DeChiara *et al.*, 1990). The physiological activities of IGFs

are mediated by their association with the IGFBPs. IGFBPs are a structurally related superfamily of secreted proteins which can bind to IGFs with higher affinity than their interactions with the IGF-1R (Clemmons, 1998; Rosenfeld *et al.*, 1999). Apart from mediating IGF-independent biological effects (Jones & Clemmons, 1995), they regulate activities of IGFs in several ways. For instance, they transport IGFs from the circulation to peripheral tissues and maintain a reservoir of IGFs in the circulation (Rosenfeld *et al.*, 1999). Moreover, following cleavage by specific serine proteases, IGFBP proteases, or binding to extracellular matrix (ECM), IGFBPs have a reduced affinity for IGF-I and IGF-II, thereby increasing IGF signaling by releasing IGFs (Denley *et al.*, 2005; Jones & Clemmons, 1995; Sachdev & Yee, 2001). The IGF system regulates various cellular responses including cell proliferation, survival, and migration through interaction with these components as well as downstream signaling transduction pathways. The ligands IGF-I, IGF-II, and insulin bind to the various members of the insulin and IGF-receptor family with different binding efficiency (Gauguin *et al.*, 2008; Pavelic *et al.*, 2007). However, it is apparent that the biological effects of IGF-I are mediated mainly through IGF-1R.

IGF-1R is a heterotetramer tyrosine kinase receptor that shares at least 60% homology with the insulin receptor (Fujita-Yamaguchi *et al.*, 1986; Steele-Perkins *et al.*, 1988). The mature cell membrane-bound IGF-1R consists of two 130- to 135-kDa α–chains and two 90- to 95-kDa β–chains, with several α-α and α-β disulfide bridges (Massague & Czech, 1982) (Figure1.3). The α subunits, which are entirely extracellular, form the ligand-binding domain that binds one ligand molecule whereas the β subunits containing a single transmembrane domain and an intracellular segment, with tyrosine kinase activity form multiple binding sites for signaling substrates (Adams *et al.*, 2000). IGF-1R is expressed in all cell types except hepatocytes and T lymphocytes (Sachdev & Yee, 2001). IGF-1R knockout mice are 45% smaller in size at birth compared to their littlemates and generally die after birth due to respiratory failure (Liu *et al.*, 1993).

IGF-2R is a monomeric transmembrane protein that lacks intrinsic signaling activity and acts as a negative regulator of IGF activity. IGF-2R regulates sequestration, endocytosis, and degradation of IGF-II (Kornfeld, 1992; Scott & Firth, 2004), thereby reducing the half-life of IGF-II and decreasing the interaction between IGF-II and IGF-2R (Moschos & Mantzoros, 2002). IGF-2R knockout mice show increased serum and tissue levels of IGF-II, associated with an approximately 40% increase in size and generalized organomegaly (Lau *et al.*, 1994; Wang *et al.*, 1994) whereas mice exposed to increased IGF-2R dosage show decreased body and organ size (Wutz *et al.*, 2001; Zaina & Squire, 1998).

1.2.2.2 Expression of IGF components in breast cancer

Accumulating evidence has revealed an association of the expression of IGF-1R with breast cancer with overexpression of IGF-1R in breast cancer cells being observed in several studies (Koda *et al.*, 2003; Papa *et al.*, 1993; Resnik *et al.*, 1998). Moreover, transgenic overexpression of IGF-1R can induce mammary tumour formation (Jones *et al.*, 2007). More recently, expression of the activated IGF-1R has also been shown to be related to a poor survival in a variety of invasive breast cancer subtypes (Law *et al.*, 2008). The mechanisms by which IGF-1R is overexpressed in breast cancer cells are incompletely understood although several studies indicate that oncogenes and/or anti-oncogenes such as *BRCA1* and *p53* mutations may be involved (Hudelist *et al.*, 2007; Maor *et al.*, 2007; Ohlsson *et al.*, 1998).

The expression of IGFs has also been implicated in breast cancer, acting mainly through endocrine and paracrine ways. It has been shown that the circulating level of IGF-I is higher in breast cancer patients than those in normal controls. In addition, a high level of circulating IGF-I and low level of IGFBP-3 expression have been reported to be associated with an increased risk of breast cancer (Decensi *et al.*, 2003; Furstenberger & Senn, 2002; Krajcik *et al.*, 2002; Toropainen *et al.*, 1995). Local production of IGFs in breast cancer cells has also been observed in several studies. It has been shown that IGF-I (Yee *et al.*, 1989) and IGF-II (Giani *et al.*, 1996) are expressed in the stromal cells, suggesting a paracrine role in breast cancer cells. Moreover, it has also been shown that malignant

breast epithelial cells can induce the expression of IGF-II in the stroma *in vitro* (Singer *et al.*, 1995). Recently, it has been reported that human IGF-II levels also remain high post-natally, and there is evidence for IGF-II-1R autocrine loops in some cancers, including oral cancer cells, that promote cell proliferation (Brady *et al*., 2007). Taken together, these studies imply multiple mechanisms of action of IGFs in cancer.

1.2.2.3 The IGF system and breast cancer transformation, growth and survival

A number of studies demonstrate that dysregulation of the IGF system is associated with breast cancer transformation (Baserga *et al.*, 2003). For instance, it has been shown that various tumourigenic agents fail to induce a transformed phenotype in mouse fibroblast cells from IGF-1R knockout mice (R^-) (Burgaud *et al.*, 1995). Similarly, blockade of either expression or function of IGF-1R by anti-sense-IGF-1R RNA or using neutralizing anti-IGF-1R antibodies or dominant-negative mutants, respectively, leads to reduced transforming potential in various cell types, including breast cancer cells (Burgaud *et al.*, 1995; Surmacz, 2000). On the other hand, overexpression of IGF-1R in mice induces mammary tumor formation which is associated with increased IGF downstream signaling (Jones *et al.*, 2007). IGF-1R has also been shown to be essential in cellular transformation mediated by multiple oncogenes, such as Ras and c-Src527 (Baserga *et al.*, 1997; Gatzka *et al.*, 2000; Valentinis *et al.*, 1997). The transforming effect of IGF-I in breast cancers has also been reported in several studies (Hadsell *et al.*, 1996; Neuenschwander *et al.*, 1996; Pollak *et al.*, 2001). One of them shows that targeted expression of IGF-I and des (1-3) hIGF-I (which has reduced affinity to IGFBPs) to the mammary gland results in a delay in involution and is associated with mammary adenocarcinomas in mice undergoing multiple lactations (Neuenschwander *et al.*, 1996). Additionally, decreased circulating IGF-I levels resulting from the expression of a GH antagonist in transgenic mice showed a reduced incidence of mammary tumours after exposure to the carcinogen DMBA (Pollak *et al.*, 2001).

IGF-I and IGF-1R also promote breast cancer cell proliferation, especially of estrogen receptor positive (ER^+) breast cancer cells through interactions with ER (Gross & Yee, 2003; Sachdev & Yee, 2001). Estrogen and IGF-I are potent mitogens for most breast

cancer cell lines, and there is considerable interaction between ER and IGF-1R, although their signaling pathways contrast. Addition of IGF-I and estradiol (E2) induces a synergistic effect in ER^+ MCF-7 cell growth whereas this effect is not observed in MCF-7-derived cells with decreased IGF-1R expression (Dupont *et al.*, 2000). In line with this, blocking of ER by an antagonist, tamoxifen leads to a decreased proliferation in response to IGF-I (Lee *et al.*, 1997; Sachdev & Yee, 2001). This synergistic effect results from estrogen-induced upregulation of IGF components, including IGF-1R (Lee *et al.*, 1999), which subsequently leads to enhanced IGF signaling as well as activation of some cell cycle molecules, such as cyclin D1 and cyclin E (Dupont & Le Roith, 2001). Another major action of IGF-I/IGF-1R in breast cancer cells is to inhibit apoptosis and promote survival (Yanochko & Eckhart, 2006). Downregulation of IGF-1R using different techniques, such as anti-sense to the IGF-1R, antisense oligodeoxynucleotides or dominant-negative mutants leads to massive apoptosis of a wide range of cancer cells, including melanoma, prostate cancer and glioblastoma (Kulik *et al.*, 1997; Reiss *et al.*, 1998; Resnicoff *et al.*, 1994a; Resnicoff *et al.*, 1994b). The effects of IGF-1R on cell apoptosis result from the activation of IGF-1R signaling upon the ligand binding (Hermanto *et al.*, 2000; Kulik *et al.*, 1997; Novosyadlyy *et al.*, 2008). IGF-1R signal transduction is discussed in more detail in section 1.2.2.5.

1.2.2.4 The IGF system and metastasis and invasion

1.2.2.4.1 Expression and metastasis

Increasing evidence support the notion that the IGF system plays an important role in metastasis and invasion of various types of cancers (Furukawa *et al.*, 2005; Kornprat *et al.*, 2006; Long *et al.*, 1995; Surmacz, 2000). It has been shown that elevated circulating levels of IGF-I and IGFBP-3 are associated with an increase risk of breast cancer recurrence (Decensi *et al.*, 2003). Although several reports have demonstrated that an elevated level of IGF-1R is associated with cancer invasion and metastasis (Brodt *et al.*, 2000; Dunn *et al.*, 1998; Long *et al.*, 1995; Long *et al.*, 1998; Sachdev *et al.*, 2004), a decreased level of IGF-1R expression has also been observed in some types of advanced cancer (Nakamura *et al.*, 2004; Sarfstein *et al.*, 2006; Schnarr *et al.*, 2000). With regards

to breast cancer cells, high levels of IGF-1R are observed in less aggressive phenotypes (Guvakova & Surmacz, 1997; Lee *et al.*, 1999) while low levels of IGF-1R are found in the more aggressive breast cancer cell lines (Bartucci *et al.*, 2001; Sepp-Lorenzino *et al.*, 1994). This is also supported by another study which showed that reduced levels of IGF-1R lead to a more metastatic phenotype with up to a three-fold increase in migration (Pennisi *et al.*, 2002). While no convincing correlation between IGF-1R levels and prognosis has been established, the activation status of IGF-1R may be a more promising readout for cancer progression (Sachdev, 2008). A recent study shows that the IGF-I signature is associated with more aggressive breast cancers (Creighton *et al.*, 2008). In that study, an IGF-I signature pattern comprising 800 genes whose expression was altered after IGF-I treatment has been identified in MCF-7 cells and this signature was found in 80% of ER^- cancers with a poor prognosis.

1.2.2.4.2 Molecular mechanism of IGF signaling in metastasis

Despite the conflicting results of the levels of IGF-1R expression in metastatic breast cancer cells, a number of studies demonstrate that IGF-1R is clearly associated with breast cancer metastasis and invasion. Blocking of IGF-1R by anti-IGF-1R inhibits chemotaxis induced by IGF-I in MCF-7 and MDA-MB-231 cells (Doerr & Jones, 1996). Similarly, a dominant-negative mutant of IGF-1R inhibits invasion and metastasis of breast cancer cells (Byron *et al.*, 2006; Dunn *et al.*, 1998).

Accumulating studies suggest that IGF-I signaling regulates every step of metastasis, such as invasion, angiogenesis, survival and extravasation. It has been shown that IGF-I regulates cell invasion through matrix metalloproteinases (MMPs), a family of collagenases identified as the major molecules involved in ECM degradation and tumour invasion. Activation of IGF signaling leads to upregulation of MMP-2, therefore enhancing cell migration and invasion (Long *et al.*, 1998). Another member of the MMPs, MMP-9 is also regulated by IGF-I signaling as shown in a study demonstrating that IGF-I significantly enhances cell-surface-associated MMP-9 activity and induces migration of MCF-7 cells (Mira *et al.*, 1999). The IGF-I system can also influence the urokinase plasminogen activator receptor (uPAR) system, which is another central mediator of

tumour cell migration and invasion, to promote tumour invasion. It has been shown that IGF-I can increase the expression of uPA, therefore leading to enhanced cell migration, which can be inhibited by blocking IGF-1R with a dominant-negative inhibitor (Dunn *et al.*, 2000; Dunn *et al.*, 2001).

IGF-I has also been implicated in mediating angiogenesis through hypoxia-inducible factor 1α (Hif-1α) (Fukuda *et al.*, 2002) and vascular endothelial growth factor (VEGF) (Clarke *et al.*, 2001; Miele *et al.*, 2000). Several studies have demonstrated that IGF-I can increase Hif-1α (Fukuda *et al.*, 2002) and VEGF (Carroll & Ashcroft, 2006; Fukuda *et al.*, 2002; Stoeltzing *et al.*, 2003) expression in various types of cancer cells. Moreover, inhibition of IGF-I with a dominant-negative construct leads to inhibition of angiogenesis (Reinmuth *et al.*, 2002a; Reinmuth *et al.*, 2002b; Stoeltzing *et al.*, 2003). IGF-I signaling has also been implicated in lymphangiogenesis to facilitate metastasis (Achen *et al.*, 2005; Bjorndahl *et al.*, 2005; Tang *et al.*, 2003).

Furthermore, there is evidence that IGF-I signaling plays a key role in regulating cancer cell survival and colonization at distant sites. IGF-I has been shown to enhance survival in an anchorage-independent manner (Baserga *et al.*, 2003; Valentinis *et al.*, 1999), which is of great importance for metastasis. In addition, inhibition of IGF-I with neutralizing antibodies results in a decreased number of circulating tumour cells and these cells are more susceptible to loss of adhesion-induced cell death, or anoikis (Sachdev *et al.*, 2010).

1.2.2.5 Signal transduction by the IGF-1R

IGFs exert various biological effects through their multiple receptors. However, most of the information on IGF-triggered signaling transduction reviewed here has come from studies of the IGF-1R system, as outlined in Figure 1.4. Two major signal transduction pathways triggered by IGF-1R are the phosphatidylinositol 3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. Upon ligand binding, a conformation change induces activation of the intrinsic tyrosine kinase of the IGF-1R, resulting in autophosphorylation of tyrosines on the intracellular portion of the β subunits, leading to the recruitment of several docking proteins to the phosphorylated site in the cytoplasmic

domain, including insulin receptor substrates (IRSs) 1-4 and sarcoma (SRC) homology 2 domain-containing (Shc) protein (Butler *et al.*, 1998; Samani & Brodt, 2001). IRS-1 is known to be the immediate downstream target of the IGF-1R tyrosine kinase, which binds to the receptor through a phosphotyrosine-binding (PTB) domain (Craparo *et al.*, 1995; Surmacz, 2000). Once phosphorylated, IRS-1 acts as a scaffold for binding downstream targets of the IGF-1R, such as p85 subunit of PI3Ks and growth factor receptor-bound-protein 2 (Grb2) (Delahaye *et al.*, 1998; Giorgetti *et al.*, 1993; Sun *et al.*, 1993). PI3Ks is recruited to the plasma membrane and phosphorylated by the IRS and Shc, leading to activation of several downstream substrates, including 3-phosphoinositide-dependent protein kinase (PDK1) and protein kinase B/Akt. Activation of Akt triggers multiple downstream effecters, such as mammalian target of rapamycin (mTOR) and bcl-associated death promoter (BAD) to regulate apoptosis, cell proliferation and metabolism (Kooijman *et al.*, 1995; Paz-Ares *et al.*, 2009; Petley *et al.*, 1999; Shaw & Cantley, 2006). The detailed signaling downstream of PI3K is further discussed in section 1.3. In parallel to PI3K-driven signaling, recruitment of Grb2/SOS by IRS-1 or Shc results in the activation of Ras/Raf-1/MAPK pathway and downstream nuclear factors, mediating cell growth, survival and differentiation (Grey *et al.*, 2003; Hermanto *et al.*, 2000; Lopez-Calderero *et al.*, 2010; Song *et al.*, 2004). In some cell types, the IGF-1R can also directly activate some intracellular molecules, such as the Janus kinase (JAK)-1 and -2 that are involved in cytokine-regulated signaling, which may in turn phosphorylate IRS-1 (Gual *et al.*, 1998).

1.2.3 Chemokines and breast cancer

1.2.3.1 Chemokines and chemokine receptors

Chemokines are a super family of small secreted peptides that were initially characterized as mediators of leukocyte trafficking during inflammation (Dowsland *et al.*, 2003; Thelen, 2001). However, they are also known to be involved in other biological activities such as homeostasis, cell proliferation, haematopoiesis, viral/cell interaction, angiogenesis, neovascularisation and cancer metastasis (Baggiolini & Loetscher, 2000; Balkwill, 1998; Belperio *et al.*, 2000; Chen *et al.*, 2006; Gale & McColl, 1999; Holland *et al.*, 2006; Hwang *et al.*, 2005; Tanaka *et al.*, 2005). Chemokines are approximately 8-14 kDa in

weight and generally are composed of 70-80 amino acids in length. Chemokines contain at least four conserved cysteines which form the disulfide bonds essential for their distinctive structure. Most chemokines have two important regions, an exposed loop in the backbone between the second and the third cysteines, believed to be the binding region of the receptors, and a variable region at the NH_2-terminus prior to the first cysteine, shown to play a key role in the specificity of the receptor binding and downstream signalling triggered by chemokines (Baggiolini *et al.*, 1997; Clark-Lewis *et al.*, 1995; Olson & Ley, 2002).

Chemokines are defined independently of their function, based on their amino acid composition, especially on the presence of a conserved tetra-cysteine motif (Allen *et al.*, 2007; Horuk, 2001; Olson & Ley, 2002; Rajagopalan & Rajarathnam, 2006; Rossi & Zlotnik, 2000; Zlotnik *et al.*, 2006) (Figure 1.5). Two major subclasses of chemokines are classified as CXC and CC, according to relative position of the first two consensus cysteines. An amino acid is positioned between the first and second cysteines in the CXC group while in CC chemokines, these cysteines are next to each other. Three homologous molecules are also regarded as chemokines, including CX_3CL1 with three intervening amino acids between the first cysteines, XCL1 and XCL2 without two out of four canonical cysteines (Baggiolini *et al.*, 1997; Gale & McColl, 1999; Mellado *et al.*, 2001b; Olson & Ley, 2002).

Chemokine receptors belong to the seven transmembrane G protein-coupled receptor family (Allen *et al.*, 2007; Horuk, 2001; Rajagopalan & Rajarathnam, 2006). Most of the chemokine receptors contain approximately 350 amino acids and have a molecular weight of around 40-50. These receptors comprise two main parts functionally (Figure 1.6). Three extracellular loops with NH_2-terminus act as a binding site for chemokine ligands whereas three intracellular loops with COOH-terminus are associated with intracellular signal transduction. A conserved 10-amino acid sequence in the second intracellular loop in most of the receptors is critical for heterotrimeric G-protein coupling and cysteine

residues in each of the extrcellular loops help to maintain their three-dimensional structure by forming disulfide bridges. Chemokine receptors comprise four major families, CCR CXCR, CR and CX_3CR based on their counterparts of the ligands, CC, CXC, C and CX_3C, respectively (Baggiolini *et al.*, 1997; Horuk, 2001; Mellado *et al.*, 2001b; Olson & Ley, 2002). The chemokine receptors also include at least 4 atypical receptors that bind ligands with a high affinity but do not elicit typical signalling transduction, such as D6, Duffy antigen receptor for chemokine receptor (DARC), ChemoCentryx chemokine receptor (CCX-CKR) and CXCR7 (Boldajipour *et al.*, 2008; Comerford *et al.*, 2006; Comerford *et al.*, 2010; Gosling *et al.*, 2000; Nibbs *et al.*, 1997; Peiper *et al.*, 1995). There is a high degree of redundancy in the chemokine family as multiple chemokines bind to the same receptor (Rossi & Zlotnik, 2000). Generally, the CC receptors are more promiscuous than CXC receptors: some chemokines bind to multiple receptors and some receptors in turn bind to multiple chemokines. However, certain chemokines interact with single receptors and some receptors bind only one chemokines (Ali & Lazennec, 2007).

Chemokines are expressed by a wide range of cell types including leukocytes, platelets, fibroblasts, endothelial and tumour cells (Balkwill, 1998) and therefore are involved in multiple biological processes. According to the function and pattern of expression, chemokines can also be classified into two main subclasses, inflammatory (inducible) and homeostastatic (constitutive) chemokines. However, a new group that share characteristics of both types depending on the pathological and physiological signals has also been elicited, such as CCL20 (MIP-3α) and CXCL9 (Mig) (Dwinell *et al.*, 2001; Kohler *et al.*, 2003). The majority of chemokines are inflammatory chemokines. They are produced by cells of many different tissues in response to proinflammatory cytokines, such as tumour necrosis factor (TNF) and the interferons (IFNs) and are responsible for recruiting particular effector cells to inflammatory sites (Olson & Ley, 2002; Ono *et al.*, 2003). For instance, increased levels of CCL2, CCL3 and CCL5 were shown in patients with rheumatoid arthristis (Charo & Ransohoff, 2006), indicative of their role as mediators of inflammation. On the other hand, homeostatic chemokines are found to be expressed constitutively in certain cells and tissues and are believed to be involved in embryonic

development and maintenance of homeostatic immunosurveillance (Muller *et al.*, 2002; Rossi & Zlotnik, 2000). As an example, CXCL12 (SDF-1) is expressed in a wide range of cells and tissues and plays an important role in B-cell lymphopoiesis, bone marrow myelopoiesis and thymocyte homing (Baggiolini, 1998).

1.2.3.2 Expression of chemokines and chemokine receptors in breast cancer

Expression of a number of chemokines and chemokine receptors has been studied in breast cancer cells and tissues. Of note, the levels of CXCL8, CCL2 and CCL4 are found to be increased in cancer tissues compared to normal tissues (Bieche *et al.*, 2007; Chavey *et al.*, 2007; Greene *et al.*, 1997) and CXCL12 and CXCL5 are present at higher levels at metastatic sites (Kang *et al.*, 2005b; Kang *et al.*, 2005c; Muller *et al.*, 2001; Niwa *et al.*, 2001). Chemokine expression seems to be correlated with a poor prognosis (Chavey *et al.*, 2007; Kang *et al.*, 2005b; Kang *et al.*, 2005c; Niwa *et al.*, 2001). Some chemokine receptors are also found to be overexpressed in various types of breast cancer cells including CXCR4 and CCR7, which bind to CXCL12 or CCL19/CCL21, respectively. Studies using immunohistochemical staining techniques revealed that CXCR4 is expressed at a high level in between 5-73% of all breast cancers whereas the normal breast epithelial cells do not express this receptor (Cabioglu *et al.*, 2005; Kato *et al.*, 2003; Muller *et al.*, 2001). Expression of CXCR4 is positively correlated to lymph node and bone metastasis (Cabioglu *et al.*, 2005; Kang *et al.*, 2005b; Kato *et al.*, 2003). Similar to CXCR4, CCR7 has been shown to be upregulated in breast cancer (Andre *et al.*, 2006; Cabioglu *et al.*, 2005) and is used as a predictor for metastasis (Zlotnik, 2004). Expression of other chemokine receptors such as CX_3CR1, CCR6, DARC and CXCR3 have also been found in breast cancer and are associated with cancer metastasis in different sites (Andre *et al.*, 2006; Goldberg-Bittman *et al.*, 2004; Ou *et al.*, 2006).

Chemokines and chemokine receptor expression is regulated by different mechanisms. For instance, estrogens downregulate CCL2 and CCL3 expression in murine mammary gland tissue (Fanti *et al.*, 2003) while they upregulate CXCL12 in human epithelial cells (Lengi *et al.*, 2007; Tsutsumi *et al.*, 2011). Transfection of HER2 results in up-regulation of

CXCR4 in breast cancer cells (Li *et al.*, 2004). Other molecules such as Hif-1 and Hif-2, transcription factor NF-κB have also been implicated in regulating expression of chemokines and their receptors (Cabioglu *et al.*, 2005; Gupta *et al.*, 2007; Li *et al.*, 2004; Luker & Luker, 2006; Shim *et al.*, 2006).

1.2.3.3 Chemokines and tumour transformation, growth and survival

A number of chemokines and their receptors have been implicated in the process of cancer progression, including transformation, survival and growth (Arya *et al.*, 2003; Vicari & Caux, 2002). Overexpression of CXCL2 and CXCL3, ligands for CXCR2 increases melanocyte tumourigenecity *in vitro* and *in vivo* (Owen *et al.*, 1997). A point mutation of CXCR2 leads to constitutive signaling of the receptor and cellular transformation (Bais *et al.*, 1998; Burger *et al.*, 1999). Apart from their role in transformation, chemokines and chemokine receptors are also involved in tumour cell growth and survival in various types of cancers (Barbero *et al.*, 2003; Burns *et al.*, 2006; Nagpal *et al.*, 2006; Pan *et al.*, 2004; Sun *et al.*, 2003; Zhou *et al.*, 2002b). For instance, CXCL8 has been shown as an important autocrine factor to promote malignant melanoma cell proliferation and this effect is inhibited by blocking CXCL8 using neutralizing monoclonal antibodies (Schadendorf *et al.*, 1993). Additionally, some chemokines may support tumour growth through their anti-apoptosis effect. As an example, inhibition of CXCR4 with the antagonist AMD3100, reduces cell proliferation and increases apoptosis of human brain cancer cells (Rubin *et al.*, 2003) and small cell lung cancer cells (Hartmann *et al.*, 2005).

1.2.3.4 Chemokines and angiogenesis

Angiogenesis is proposed to be one of the pre-metastatic effects in cancer (Karnoub & Weinberg, 2006) and is regulated by multiple factors, including chemokines and their receptors. A subset of chemokines such as CXCL1, CXCL5 and CXCL8 containing an ELR (Glu-Leu-Arg) sequence have been shown to possess pro-angiogenic activity (Weidner, 1996; Weidner & Folkman, 1996). The release of CCL2, CCL4 or CCL5 in breast cancer cells leads to production of MMP9, which cleaves and therefore mobilizes vascular endothelial growth factor (VEGF) (Belotti *et al.*, 2003; Pollard, 2004; Robinson *et al.*, 2002). VEGF induces the secretion of CXCL8, which in turn promotes endothelial cell

branching (Heidemann *et al.*, 2003). In contrast, chemokines without the ELR sequence are angiostatic, such as CXCL9 and CXCL10 (Weidner, 1996; Weidner & Folkman, 1996). They not only inhibit the neovascularisation effects of the angiogenic chemokines, but they also inhibit more classical angiogenic factors including VEGF (Baggiolini *et al.*, 1997; Murphy *et al.*, 2000; Rossi & Zlotnik, 2000). Thus, the balance operating between ELR^- and ELR^+ chemokines greatly influences the course of angiogenesis (Karnoub & Weinberg, 2006).

CXCR4 is one of the chemokine receptors that have been shown to directly regulate angiogenesis (Folkman, 2002). Engineered mice lacking CXCR4 or CXCL12 have a deficiency in the formation of blood vessels in the gastrointestinal tract, suggesting their essential role in embryonic vascularization. Recent studies show that CXCR4 mediates normal microvascular angiogenesis in the colon and also stimulates mature endothelial cells to migrate and form capillary-like structures (Salvucci *et al.*, 2002). This pro-angiogenic effect of CXCR4 is believed to act through upregulation of VEGF, which in turn increases expression of CXCR4 to form a positive feedback loop (Bachelder *et al.*, 2002; Kijowski *et al.*, 2001). Moreover, a recent study shows that CXCR4 may exert its angiogenesis effect through regulation of phosphoglycerate kinase 1 (PGK1). In that study, it was shown that PGK1, a glycolytic enzyme is involved in producing angiostatin by cleaving extracellular plasminogen to release the angiostatic clamp, and that a high level of CXCR4/CXCL12 signaling can downregulate PGK1, therefore promoting angiogenesis. This process is proposed to be important for the survival of the metastasized colony of tumour cells and also for metastasis from metastatic site (Wang *et al.*, 2007).

Other chemokine receptors have also been implicated in angiogenesis, with CXCR1 and CXCR2 being angiogenic while CXCR3 appears to be angiostatic (Baggiolini *et al.*, 1997; Murphy *et al.*, 2000; Rossi & Zlotnik, 2000).

1.2.3.5 Chemokines and metastasis and invasion

1.2.3.5.1 Expression of chemokines and chemokine receptors and metastasis

In addition to a role in transformation, growth, survival and angiogenesis, increasing data show that chemokines and chemokine receptors are involved in homing of tumour cells to the sentinel lymph nodes, metastasis to specific organs and metastasis of metastatic lesions. It has been shown that multiple CXC chemokines, such as CXCL8, CXCL1-3, CXCL5 and CXCL6 are highly expressed at metastases, suggesting they may account for the higher aggressiveness of breast cancers (Bieche *et al.*, 2007). In line with this, elevated expression of CXCL8 leads to an increased invasiveness of breast cancer cells into matrigel and blockade of CXCL8 by neutralizing antibody specifically inhibits CXCL8-induced invasion (Lin *et al.*, 2004). It is also the case for CXCL12, a ligand for CXCR4. Knockout of CXCL12 leads to a decreased ability of invasion and migration in breast cancer cells (Kang *et al.*, 2005a). CC chemokines such as CCL1-5, CCL10 and CCL7, have also been implicated in metastasis (Allinen *et al.*, 2004; Kang *et al.*, 2005a; Mira *et al.*, 2001; Youngs *et al.*, 1997). Neutralizing antibodies to CCL2 prevent the formation of lung metastasis in mice bearing CCL2-expressing MDA-MB-231 cells in a human breast cancer xenograft model (Salcedo *et al.*, 2000). Chemokine receptors have also been reported to be involved in metastasis. Blocking of CXCR4 expression by anti-CXCR4 or siRNA decreases breast cancer cell invasion *in vitro* (Chen *et al.*, 2003; Lapteva *et al.*, 2005) and inhibits lung metastasis in SCID mice (Gutzkow *et al.*, 2003; Hatse *et al.*, 2002; Liang *et al.*, 2005; Muller *et al.*, 2001; Tamamura *et al.*, 2003). Overexpression of CCR7, the receptor for CCL19 and CCL21 induces significant lymph node metastasis in mice and this effect is blocked by neutralizing anti-CCL21 antibodies (Wiley *et al.*, 2001). The involvement of CXCR4 and CCR7 in metastasis has also been reported in other malignancies, such as non-small cell lung cancer (Phillips *et al.*, 2003), colorectal cancer (Gunther *et al.*, 2005), prostate (Balkwill, 2004), melanoma (Balkwill, 2004; Wiley *et al.*, 2001), pancreatic (Balkwill, 2004), and ovarian cancers (Balkwill, 2004).

1.2.3.5.2 Molecular mechanisms of chemokine signaling in metastasis

A molecular mechanism of chemokine-mediated metastasis for breast cancer cells was first established by Muller and colleagues (Muller *et al.*, 2001). CXCR4 and CCR7 are highly expressed in human breast cancer cell lines, malignant breast tumours and metastases while their corresponding ligands, CXCL12 and CCL21, respectively, are present at a peak level in all the target organs for breast cancer metastasis, such as lung, liver, bone marrow and lymph nodes. *In vitro* data show that CXCR4 or CCR7 mediate chemotactic and invasive response through actin polymerization and pseudopodia formation.

Subsequent studies showed that various effector molecules are essential for CXCR4 to promote breast cancer cell metastasis. CXCR4 can activate components of FAK, related adhesion focal tyrosine kinase (RAFTK/Pyk2), cytoskeleton protein, Crk and paxillin, tyrosine phosphatase SHP2 as well as increase the association between SHP2 and PI3K. Inhibition of PI3Ks and RAFTK/Pyk2 and phosphatase tyrosine leads to a significant reduction of chemotaxis and chemoinvasion (Fernandis *et al.*, 2004). Moreover, CXCR4 promotes adhesion to components of ECM, including collagen and fibronectin through activation of integrins (Hartmann *et al.*, 2005). CXCR4 also induce secretion of MMP2 and MMP9 resulting in degradation of ECM molecules (Kang *et al.*, 2005a).

1.2.3.6 Signaling transduction by chemokine receptors

Chemokine receptors regulate a variety of cellular processes as discussed before through initiating multiple signaling pathways upon ligand binding. Some of the key signaling pathways have been identified, including heterotrimeric G-protein-dependent and heterotrimeric G-protein-independent signaling (Figure 1.7).

Chemokines are the first members of the cytokine family that are characterized by their ability to bind heterotrimeric G proteins (Ali & Lazennec, 2007). The heterotrimeric G protein is composed of Gα, Gβ and Gγ subunits, and Gα is further grouped into $G\alpha_i$, $G\alpha_s$, $G\alpha_q$ and $G\alpha_{12}$ according to sequence similarity. Upon ligand binding, a conformational change of the receptor leads to the activation of the G protein by facilitating the exchange of bound GDP with GTP. The activated G protein subsequently dissociates from the receptor as active G_α and $G_{\beta\gamma}$, binding to GTP to trigger downstream signaling cascades.

Different Gα subunits mediate GPCR signaling in unique routes/pathways (Goldsmith & Dhanasekaran, 2007; Mellado *et al.*, 2001a). Chemokine receptor signaling is primarily $G\alpha_i$-mediated, which can be inhibited by pertusis toxin (PTX) (Goldsmith & Dhanasekaran, 2007; Mellado *et al.*, 2001a), although recent studies show that chemokine receptor signaling may also be mediated through other Gα (Rubin, 2009). Activated $G\alpha_i$ can activate PI3K, resulting in the phosphorylation of several focal adhesion molecules, such as FAK and Crk, as well as Janus kinase, JAK2 and JAK3, to regulate chemotaxis (Wang *et al.*, 2000; Zhang *et al.*, 2001). The detailed signaling downstream of PI3K is further discussed in section 1.3. MAPK has also been shown to be activated by $G\alpha_i$ during the chemotaxis process (Bendall *et al.*, 2005; Mellado *et al.*, 2001a). More recently, it has become clear that the $G_{\beta\gamma}$ subunit also plays an important role in chemokine receptor signal transduction. For instance, the $G_{\beta\gamma}$ subunit can initiate phopholipase C (PLC) activation and formation of the inositol trisphophate (IP_3) and diacylglycerol (DAG), resulting in mobilization of Ca^{2+} from intracellular pools (Kiselyov *et al.*, 2003; Mellado *et al.*, 2001a). Additionally, $G_{\beta\gamma}$ regulates the activation of PI3Kγ, the beta-adrenergic receptor kinase (Neer, 1995; Stephens *et al.*, 1994) as well as MAPK/Erk mediated by Ras (Crespo *et al.*, 1994; Neer, 1995).

In addition to the chemokine receptor signaling that is dependent on G protein, some parallel G protein-independent signalings have also been identified. Activated chemokine receptors can be phosphorylated by G protein receptor kinases (GRKs) leading to the recruitment of arrestin, resulting in receptor internalization through clathrin-mediated endocytosis (Aragay *et al.*, 1998; Bohm *et al.*, 1997; Fan *et al.*, 2003; Fernandis *et al.*, 2002; Franci *et al.*, 1996; Lefkowitz & Shenoy, 2005; Mueller *et al.*, 1997). Another G protein-independent pathway triggered by chemokines is mediated by arrestin, which leads to activation of MAPK (Lefkowitz & Shenoy, 2005). As an example, ligation of CCR7 by CCL19 results in Erk activation, an effect that is inhibited by depletion of β-arrestin using small interfering RNA (Kohout *et al.*, 2004). In addition, downregulation of β-arrestin$_2$ in human embryonic kidney (HEK) 293 cells leads to an impaired activation of p38 MAP kinase and a decreased chemotactic response to CXCL12, suggesting that β-arrestin$_2$ may

mediate CXCL12 chemotactic signaling by activation of p38 (Lee *et al.*, 2002b). Moreover, activation of signal transducer and activator of transcription (STAT) by chemokines has also been shown to be G protein-independent (Mellado *et al.*, 2001a). CCL2 induces dimerisation and tyrosine phosphorylation of CCR2 and activation of JAK2/STAT3. Blocking of JAK2 kinase results in reduced Ca^{2+} mobilization and migration (Mellado *et al.*, 1998). Similarly, CXCL12 stimulation induced a transient association between CXCR4 and JAK2/JAK3, which leads to nuclear translocation of a number of STAT proteins (Vila-Coro *et al.*, 1999). Pretreatment with PTX leads to a prolonged association of JAK with CXCR4, indicating that G proteins may be involved in the receptor complex recycling (Vila-Coro *et al.*, 1999).

1.2.4 Transactivation between RTKs and GPCRs

1.2.4.1 General concept of transactivation

As discussed before, tyrosine kinase receptors (RTKs) and the G protein-coupled receptors (GPCRs) are known to initiate multiple downstream signaling cascades to regulate a wide range of cellular functions under both physiological and pathological conditions. A number of studies have shown that the signaling pathways initiated by these receptors are not activated in a linear way. They involve activation of complex interconnecting signaling networks. These observations have led to the emergence of the concept termed transactivation/crosstalk, a phenomenon that a given receptor is activated by a ligand of a heterologous receptor belonging to a different class.

There are two types of transactivation between RTKs and GPCRs. Firstly, a number of RTKs, such as the platelet-derived growth factor receptor (PDGFR) (Linseman *et al.*, 1995), the EGFR (Daub *et al.*, 1996) and the IGF-1R (Rao *et al.*, 1995). can be transactivated by GPCRs. For instance, in Rat1 and COS-7 cells, stimulation of LPA, endothelin-1 or thrombin receptors leads to tyrosine phosphorylation of EGFR and HER2, which is blocked by a dominant-inhibitory mutant of EGFR or by pharmacological inhibition of the intrinsic EGFR tyrosine kinase (Daub *et al.*, 1996; Daub *et al.*, 1997).

This type of transactivation is further demonstrated by a study showing that EGFR is phosphorylated following CCL11 (a ligand for CCR3) stimulation in bronchial epithelial cells (Adachi *et al.*, 2004). Secondly, GPCRs can be transactivated by RTKs. As an example, IGF-I stimulation results in phosphorylation of CCR5 in MCF-7 cells (Mira *et al.*, 2001). Similarly, the sphingosine 1 phosphate (S1P) receptor S1P1 has been shown to be transactivated by IGF-1R and blocking of S1P1 by a competitive antagonist leads to a significant decrease of Erk activation induced by IGF-I (El-Shewy *et al.*, 2006). Other RTKs have also been reported for their role in the transactivation of GPCRs, such as the platelet-derived growth factor receptor (PDGFR) (Hobson *et al.*, 2001; Tanimoto *et al.*, 2004) and nerve growth factor receptor (NGFR) (Toman *et al.*, 2004).

1.2.4.2 Effects of RTK-GPCR transactivation on tumourigenesis

There is an increasing body of evidence suggesting that transactivation between RTKs and GPCRs contributes to tumourigenesis through multiple signaling pathways, depending on the cellular system. In this regard, transactivation of EGFR in a series of cancer cell lines has been well-documented. LPA-induced EGFR signal transduction is associated with cell proliferation (Gschwind *et al.*, 2002; Gschwind *et al.*, 2003; Ukegawa *et al.*, 2003) and cell-cycle progression (Gschwind *et al.*, 2002; Gschwind *et al.*, 2003). Blocking the activity of EGFR by specific antibody to EGFR or by an EGFR kinase inhibitor results in decreased cell growth (Ukegawa *et al.*, 2003). EGFR transactivation has also been implicated in cancer cell migration and invasion. For instance, in bladder cancer cells, LPA receptors promote cell migration and invasion via phosphorylation of EGFR and subsequent activation of mitogen-activated protein kinase (MAPK) signaling (Schafer *et al.*, 2004). Similarly, in head and neck squamous cell carcinomas (HNSCCs) cells, gastrin-releasing peptide (GRP), through its receptor, GRPR induces rapid phosphorylation of EGFR and p42/44 MAPK activation, contributing to cell invasion (Zhang *et al.*, 2004). Moreover, gastrin-CCKB receptor stimulated EGFR phosphorylation is involved in enhanced cell migration of gastric epithelial cells (Noble *et al.*, 2003). Additionally, EGFR phosphorylation by Wnt in mammary epithelial cells is proposed to be a new mechanism for Wnt-induced oncogenesis (Civenni *et al.*, 2003).

Transactivation of GPCRs has also been studied for its roles in cancer biological processes. A study shows that insulin/IGF-I crosstalk with GPCR agonists, such as neurotensin, bradykinin and angiotension II and enhances cell growth of pancreatic cancer. This effect is inhibited by activation of AMP-activated protein kinase (AMPK), a negative regulator of mTOR using Metformin, a drug for the treatment of type 2 diabetes, suggesting that mTOR is involved in this process (Kisfalvi *et al.*, 2009). Moreover, it has been demonstrated that IGF-1R can transactivate the chemokine receptor CXCR4 in human MDA-MB-231 breast cancer cells and that this plays a key role in IGF-I-induced motility of these highly-invasive cells (Akekawatchai *et al.*, 2005).

Although most of the reports show that crosstalk between RTKs and GPCRs leads to enhanced signaling, several recent studies present the evidence supporting "inhibitory crosstalk" between the two receptors (Garcia-Sainz *et al.*, 2004; Gavi *et al.*, 2006; Gavi *et al.*, 2007; Hurley *et al.*, 2003; Rodriguez-Perez *et al.*, 2009; Strachan *et al.*, 2009; Strachan *et al.*, 2010). For example, Strachan and colleagues showed that multiple endogenous RTK receptors, including EGFR, PDGFR and ErbB4 significantly attenuate 5-HT_{2A} receptor signaling in a variety of cell types and this effect is inhibited after genetic deletion of RSK2, a downstream effector of Erk/MAPK (Strachan *et al.*, 2010). However, whether this inhibitory crosstalk contributes to tumourigenesis remains unclear.

1.2.4.3 Molecular mechanism involved in RTK-GPCR transactivation

Two distinct mechanisms by which RTKs-GPCRs transactivation is regulated have been identified so far (Delcourt *et al.*, 2007a). Firstly, signaling through one receptor leads to the synthesis and secretion of the cognate ligand of the second receptor, which in turn activates that receptor (Adachi *et al.*, 2004; El-Shewy *et al.*, 2006; Hobson *et al.*, 2001; Mira *et al.*, 2001; Ohtsu *et al.*, 2006; Toman *et al.*, 2004; Wetzker & Bohmer, 2003). A well-documented example of this ligand-dependent transactivation is EGFR, which has been shown to be activated by a variety of different GPCRs, including sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA) receptors. GPCR stimulation leads to

activation of MMPs that subsequently cause ectodomain shedding of a transmembrane RTK lignd precursor, such as proHB-EGF (Hb-EGF), which in turn activates its corresponding receptor (Asakura *et al.*, 2002; Ohtsu *et al.*, 2006; Tanimoto *et al.*, 2004; Wetzker & Bohmer, 2003; Zhao *et al.*, 2006). This ligand-dependent manner has also been implicated in GPCRs transactivation by RTKs, which involves the synthesis and secretion of a cognate ligand of the transactivated GPCRs and subsequently results in the activation of the GPCRs in an autocrine and/or paracrine manner (El-Shewy *et al.*, 2006; Hobson *et al.*, 2001; Mira *et al.*, 2001; Toman *et al.*, 2004). For instance, transactivation of CCR5 by IGF-I requires transcriptional upregulation and secretion of CCL5, a ligand of CCR5 and this transactivation is essential for IGF-I-induced chemotaxis in MCF-7 cells (Mira *et al.*, 2001). Similarly, IGF-I or IGF-II transactivates the S1P receptor via expression of S1P, to contribute the activation of Erk in HEK 293 cells (El-Shewy *et al.*, 2006).

The second mechanism by which transactivation occurs is believed to be ligand-independent. Some factors downstream of GPCRs, such as PKC (Werry *et al.*, 2005) and Src (Daub *et al.*, 1997; El Zein *et al.*, 2010; Lee & Chao, 2001; Lee *et al.*, 2002a; Luttrell *et al.*, 1997; Werry *et al.*, 2005) have been shown to be involved in ligand-independent transactivation. For example, in PC12 cells, adenosine or Pituitary adenylate cyclase-activating polypeptide (PACPA), two modulators that act through GPCRs can transactivate the Trk neurotrophin receptor in the absence of its corresponding ligand, neurotrophin. The increased activity of Trk neurotrophin receptors is inhibited by using Src family-specific inhibitor, PP1, suggesting trasactivation of RTKs by GPCRs involves a tyrosine kinase downstream of GPCRs (Lee & Chao, 2001; Lee *et al.*, 2002a). In keeping with this, Src can bind to EGFR following LPA stimulation which in turn tyrosine-phosphorylates EGFR and blocking of Src activity by dominant-negative mutant of Src inhibits GPCR-induced phosphorylation of EGFR (Lowes *et al.*, 2002). Additionally, a recent study shows that Src kinase inhibitor blocked the transactivation of EGFR by GPCRs while an inhibitor of MMP2 and MMP9 blocks the activation of both EGFR and Src, suggesting that Src exerts its effect through the signalings between ligand release and activation of the EGFR kinase (Roelle *et al.*, 2003).

Ligand-independent transactivation can also require the physical association between two distinct receptors to form functional heterodimers (Akekawatchai *et al.*, 2005; Alderton *et al.*, 2001; Delcourt *et al.*, 2007b; Waters *et al.*, 2006; Werry *et al.*, 2005; Wetzker & Bohmer, 2003). For instance, β_2-Adrenergic receptor (β_2AR) activation leads to EGFR dimerization, tyrosine autophosphorylation and internalization which require the formation of a multiple receptor complex (Maudsley *et al.*, 2000) in COS-7 cells. This direct interaction between the two receptors has also been reported in the transactivation of GPCRs induced by RTKs. Physical association between PDGFR and the S1P receptor was found in airway smooth muscle cells, which accounts for PDGFR-S1P1 transactivation (Waters *et al.*, 2006). In addition, a heterodimetic receptor complex consisting of IGF-1R and CXCR4 has been reported in highly metastatic breast cancer cell lines. This association allows IGF-I to transactivate CXCR4 inducing subsequent G-protein signaling, which partially mediates the chemotactic response of these cells to IGF-I. Interestingly, IGF-1R-CXCR4 transactivation is not observed in non-metastatic MCF-7 cells due to non-functional CXCR4 expression (Akekawatchai *et al.*, 2005) (Figure 1.8). However, a more detailed analysis of signal transduction pathways downstream of IGF-1R-CXCR4 complex following the stimulation of IGF-I is yet to be conducted.

1.3 The phosphoinositide 3- kinase signaling downstream of RTKs and GPCRs

1.3.1 Overview

Phosphoinositide 3- kinases (PI3Ks) are a family of enzymes that catalyze the phosphorylation of the third carbon of the inositol ring in phosphoinositide lipids, named phosphotidylinositol (PtdIns). The phosphorylation of PtdIns generates important second messengers, such as PtdIns-3-P (PIP), PtdIns-3, 4-P (PIP_2) and PtdIns-3, 4, 5-P (PIP_3) which are part of many signaling cascades within a cell. PI3Ks can be divided into three main groups based on structure and substrate-specificity: class I, class II and class III (Vanhaesebroeck *et al.*, 1997a). The best-characterized members of this family are the

class I PI3Ks, which are major signal transduction molecules downstream of both RTKs and GPCRs and play an important role in regulating a variety of cellular responses under both normal and pathological conditions. These class I PI3Ks are discussed in detail below.

1.3.2 Class I PI3K family structure and expression

Class I PI3Ks are divided into two sub-groups: class IA and class IB, according to the associated adaptors. Class IA PI3Ks are composed of heterodimers of a p110 catalytic subunit and p85 regulatory subunit (Cantley, 2002; Ward & Finan, 2003). There are three different isoforms of p110 catalytic subunit: p110α, p110β and p110δ, which are encoded by *PIK3CA, PIK3CB* and *PIK3CD*, respectively. The p85 regulatory subunit also has three major isoforms: p85α, p85β and p55γ that are encoded by *PIK3R1, PIK3R2* and *PIK3R3*, respectively. The *PIK3R1* codes for two shorter isoforms, p55α and p50α through alternative splicing. Class IB PI3Ks consist of either the regulatory subunit p101 (*PIK3R5*) or p84/ $p87^{PIKAP}$ (*PIK3R6*) and the catalytic subunit p110γ (*PIK3CG*) (Engelman *et al.*, 2006). The expression of class I PI3K catalytic subunits is varied. Whilst p110α and p110β are expressed ubiquitously, the expression of p110δ and p110γ is largely limited to cells of the immune system (Vanhaesebroeck *et al.*, 1997b). As is the case with the expression pattern of PI3K catalytic subunits, p85 and p55 are expressed in many cell type while p101 and p84 are mainly observed in immune cells (Suire *et al.*, 2005; Voigt *et al.*, 2006), although p84 and p110γ have been shown to be expressed in cardiac tissue (Patrucco *et al.*, 2004).

1.3.3 Class I PI3K and tumourigenesis

1.3.3.1 Mutational alterations in PI3K/Akt and cancer

PI3Ks control cell growth, proliferation, differentiation, anti-apoptosis and angiogenesis (An *et al.*, 2007; Roymans & Slegers, 2001) which constitute critical steps towards tumour formation and malignant cell dissemination. Several gene mutations related to the aberrant of this pathway have been identified and the best known genetic alterations are mutations in *PIK3CA*, *AKT1* (Berns *et al.*, 2007; Bose *et al.*, 2002; Carpten *et al.*, 2007; Dillon *et al.*, 2007; Dunlap *et al.*, 2010; Kalinsky *et al.*, 2009; Li *et al.*, 2010a; Lopez-Knowles *et al.*,

2010; Oda *et al.*, 2005; Perez-Tenorio *et al.*, 2007; Saal *et al.*, 2005; Stemke-Hale *et al.*, 2008) and the phosphatase and tension homolog deleted in chromosome ten (*PTEN*), which are discussed below.

1.3.3.1.1 Mutations in *PIK3CA*

A number of studies have provided evidence on the mutation of the *PIK3CA* gene that encodes the PI3Kα catalytic subunit and its tumourigenesis effect. While several studies show that *PIK3CA* is amplified in many tumours, such as ovarian, cervix, lung and colon cancers (Parsons *et al.*, 2005; Racz *et al.*, 1999; Shayesteh *et al.*, 1999; Zhang *et al.*, 2002), point mutations have recently been identified in a number of cancer types, including breast, brain, ovarian and lung cancers (Bader *et al.*, 2005; Samuels *et al.*, 2004). These mutations are generally located in two hotspots: the helical and the catalytic domain (Samuels *et al.*, 2004). Cells transfected with mutated p110α show constitutive activity of downstream signaling molecules, including Akt (Ikenoue *et al.*, 2005; Kang *et al.*, 2005d; Samuels *et al.*, 2005) which results in reduced apoptosis and increased proliferation (Samuels *et al.*, 2005). Moreover, these mutants can induce oncogenic transformation in fibroblasts and mammary epithelial cells (Isakoff *et al.*, 2005; Kang *et al.*, 2005d) and contribute to tumour formation in nude mice (Zhao *et al.*, 2005). More recently, a *p85α* mutation has also been found in colon cancer cells and is involved in promoting cell growth, survival and angiogenesis by abrogating the inhibitory effect of p85α on p110 (Jaiswal *et al.*, 2009; Vasudevan *et al.*, 2009).

1.3.3.1.2 Mutations of *AKT*

Functional mutation of Akt isoforms has also been reported recently. A mutation in the E17K PH domain of *AKT1* has been observed in human breast (8%), colon (6%) and ovarian cancers (2%). This mutant sufficiently transforms Rat1 cells in culture and induces leukaemia in animal models owing to its constitutive localization in the membrane (Carpten *et al.*, 2007). Gene mutations that affect Akt2 have also been identified in colon cancers (Parsons *et al.*, 2005). The detailed effects of Akt in PI3K signaling are discussed in section 1.3.4.

1.3.3.1.3 Mutations of *PTEN*

Another known genetic alteration in PI3K signaling related to tumourigenesis is in *PTEN*. Somatic mutations, gene deletion or gene inactivation in *PTEN* have been observed in a wide range of tumours, such as melanoma, prostate, breast cancer and colon cancers (Cairns *et al.*, 1997; Li *et al.*, 1997; Parsons *et al.*, 2005; Wu *et al.*, 2003). PTEN deletion and mutation lead to an increased incidence of tumours (Suzuki *et al.*, 1998) in multiple organs such as the mammary gland (Li *et al.*, 2002; Vitolo *et al.*, 2009), prostate and skin (Backman *et al.*, 2004), indicating a role for PTEN as a tumour suppressor. The detailed effects of PTEN in PI3K signaling are discussed in section 1.3.4.

1.3.3.2 PI3K isoforms in tumourigenesis

A role of PI3Ks in tumourigenesis has been reported in several studies using the well-characterized pan-PI3K inhibitors wortmannin and LY294002, which inhibit tumor activities, such as tumor growth and cell proliferation (Hu *et al.*, 2000; Itoh *et al.*, 2002; Lemke *et al.*, 1999; Schultz *et al.*, 1995). Of note, isoform-specific inhibitors for PI3K catalytic subunits have recently been described and together with various genetic approaches allow the dissection of the contribution of individual PI3K to specific processes (Camps *et al.*, 2005; Geng *et al.*, 2004; Hayakawa *et al.*, 2006; Jackson *et al.*, 2005; Knight *et al.*, 2004; Knight *et al.*, 2006; Pomel *et al.*, 2006; Sadhu *et al.*, 2003a; Sadhu *et al.*, 2003b). The effects of different isoforms on tumourigenesis are discussed below.

1.3.3.2.1 PI3Kα and PI3Kβ in tumourigenesis

Increasing data demonstrate the effect of PI3Kα in tumourigenesis. Amplification of PI3Kα has been found in a wide range of cancers, such as ovarian, lung, thyroid, cervical, gastric carcinomas (Boller *et al.*, 2008; Byun *et al.*, 2003; Fenic *et al.*, 2007; Guerreiro *et al.*, 2008; Liu *et al.*, 2008b; Ma *et al.*, 2000; Massion *et al.*, 2004; Shayesteh *et al.*, 1999; Sticht *et al.*, 2005; Zhang *et al.*, 2007). Overexpression of PI3Kα in human small lung carcinoma cells increases cell growth which involves elevated activity of Akt in response to stem cell factor (SCF) (Arcaro *et al.*, 2002). PI3Kα has also been shown to be the main provider of PI3K signaling under basal and VEGF-A-stimulated conditions and regulates

endothelial cell angiogenesis and migration *in vitro* and *in vivo* (Graupera *et al.*, 2008). Blocking of p110α by siRNA knockdown or isoform specific inhibitor induces apoptosis and decreases migratory capacity of medulloblastoma cells (Guerreiro *et al.*, 2008). However, surprisingly, PI3Kα is not required for breast cancer cell chemotaxis induced by EGF (Sawyer *et al.*, 2003).

PI3Kβ has also been implicated in multiple cancer processes. An elevated level of PI3Kβ expression has been found in various cancers (Benistant *et al.*, 2000; Carvalho *et al.*, 2010; Knobbe & Reifenberger, 2003). An expression study shows that PI3Kβ is associated with a more aggressive profile of breast cancer and highly related to HER2-overexpression and distant metastasis (Carvalho *et al.*, 2010). Transfection of p110β in chicken embryo fibroblasts can induce oncogenic transformation, which requires its lipid kinase activity (Kang *et al.*, 2006). Blocking of p110β either by siRNA-mediated p110β knockdown or anti-sense molecule inhibits invasive cell growth *in vitro* and in subcutaneous tumour model (Czauderna *et al.*, 2003). Similarly, in a murine breast cancer model, tumour formation was found to be partially blocked in p110β kinase-dead knock-in mice, which shows fewer and smaller tumours compared to the control (Ciraolo *et al.*, 2008). PI3Kβ has also been shown to be involved in cell migration in metastatic MDA-MB-231 cells (Sawyer *et al.*, 2003) and ras/TGFβ1-transfected normal mammary epithelial cells (De Laurentiis *et al.*, 2011). In contrast to PI3Kα, which is believed to regulate tumourigenesis through *PIK3CA* mutations and oncogenic RTKs/Ras, PI3Kβ appears to be the main isoform involved in PTEN-deficient tumours (Edgar *et al.*, 2010; Jia *et al.*, 2008; Torbett *et al.*, 2008; Wee *et al.*, 2008).

1.3.3.2.2 PI3Kδ in tumourigenesis

Although PI3Kδ is well-known for its role in immune system (Clayton *et al.*, 2002; Jou *et al.*, 2002; Okkenhaug *et al.*, 2002), recent studies have highlighted that PI3Kδ is also an important regulator in tumourigenesis. Overexpression of p110δ in chicken embryo fibroblasts leads to an oncogenic transformation and constitutive activation of Akt under serum-starved conditions (Kang *et al.*, 2006). One study shows that a high level of p110δ

is consistently expressed in blast cells from patients with acute myeloid leukemia (AML) compared to other isoforms and inhibition of p110δ using isoform-specific inhibitor, IC87114 can suppresses cell proliferation (Sujobert *et al.*, 2005). Reduced tumour growth has also been found after p110δ inhibition in mice with Lewis lung carcinoma or GL261 hind limb endothelial tumours under radiation treatment (Geng *et al.*, 2004). PI3Kδ is also a main isoform in regulating breast cancer cell migration in response to EGF (Sawyer *et al.*, 2003).

1.3.3.2.3 PI3Kγ in tumourigenesis

In addition to playing a similar role to p110β and p110δ to induce transformation of cultured cells (Kang *et al.*, 2006), multiple oncogenic effects of PI3Kγ have also been shown in several cancer cell types. Elevated expression and activity of p110γ are observed in chronic myeloid leukemia and expression of dominant-negative p110γ leads to decreased proliferation and increased sensitivity to cell death (Hickey & Cotter, 2006). This effect of PI3Kγ is further demonstrated in pancreatic ductal adenocarcinoma (PDAC) (Edling *et al.*, 2010). Immunohistochemical staining shows that 72% of the PDAC tissue expresses p110γ and inhibition of p110γ by selective inhibitor or siRNA knockdown inhibits cell proliferation. A study using a colitis-associated cancer model shows that PI3Kγ-deficient mice display a lower incidence of colitis-associated tumours as well as reduced tumour multiplicity and smaller tumour size than controls, suggesting that PI3Kγ controls tumour formation (Gonzalez-Garcia *et al.*, 2010). PI3Kγ also regulates colon cancer cell invasion according to a study showing that cells transfected with constitutively-active, membrane-targeted PI3Kγ display increased invasion compared to those transfected with catalytically-inactive PI3Kγ (Barbier *et al.*, 2001). In human melanoma cell lines, PI3Kγ shows an increased activity in response to autotoxin (ATX), an exo-nucleotide pyrophosphatase and phosphodiesterase, and promotes cell migration induced by ATX which can be inhibited by PI3K inhibitors and PTX (Lee *et al.*, 2002b). Similarly, melanoma cells transfected with a p110γ dominant-negative mutant results in increased adhesion induced by CXCL12 compared to control (Monterrubio *et al.*, 2009).

1.3.4 Class I PI3K signaling transduction pathways

In general, Class IA PI3Ks are activated on interaction of the regulatory subunit with phosphorylated tyrosine (Kodaki *et al.*, 1994; Rodriguez-Viciana *et al.*, 1994) (Figure 1.9). The SH2 domain of the regulatory subunit, p85 binds to phospho-tyrosine residues of the activated RTKs or adaptor proteins, such as IRSs (Songyang *et al.*, 1993) and this binding relieves the basal inhibition of p110 by p85 and recruits the p85-p110 heterodimer to its substrate, PIP_2 at the plasma membrane to generate PIP_3 (Yu *et al.*, 1998a; Yu *et al.*, 1998b). Another factor, Ras has also been demonstrated to activate PI3Kα and PI3Kδ in class IA PI3K signaling (Rodriguez-Viciana *et al.*, 2004) but not PI3Kβ. The different regulation of PI3Kβ from other class IA PI3Ks was also proposed in a study showing that PI3Kβ is activated by $G_{\beta\gamma}$ in synergy with p85-phosphotyrosine docking (Kurosu *et al.*, 1997).

PI3Kγ, the only class IB PI3K is activated and translocated to the plasma membrane after GPCR activation (Figure 1.10). Upon receptor ligation, the G_α subunit is phosphorylated thus allowing dissociation of $G_{\beta\gamma}$ subunit. Following release from G_α, the $G_{\beta\gamma}$ heterodimer directly activates PI3Kγ, resulting in PIP_3 production (Lopez-Ilasaca *et al.*, 1997; Stephens *et al.*, 1994). The p101 (Stephens *et al.*, 1997) and p84 regulatory subunits (Suire *et al.*, 2005; Voigt *et al.*, 2006) are known to be essential for PI3Kγ activation, although the precise binding site for the $G_{\beta\gamma}$ is less clear. Apart from $G_{\beta\gamma}$, GTP-Ras can also cause modest activation of PI3Kγ *in vitro* (Suire *et al.*, 2002) through binding directly to p110γ (Pacold *et al.*, 2000). The relative contribution of $G_{\beta\gamma}$ and GTP-Ras to the regulation of PI3Kγ has been further assessed by using $p101^{-/-}$ and knock-in mutation in p110γ mice and the results shows that both $G_{\beta\gamma}$ and GTP-Ras are involved in PI3Kγ activity in response to fLMP and C5a in neutrophils (Suire *et al.*, 2006).

Activated Class I PI3Ks generate PIP_3 as an important second messenger to regulate the activity of a subset of proteins by binding to their pleckstrin-homology (PH) domains. Among a number of PH-domain-containing targets, Akt has been shown to be one of the

main regulators of cellular functions downstream of PI3Ks (Figure 1.11). Akt, also known as protein kinase B is recruited to the cellular membrane, whereby it is phosphorylated by phosphoinostide-dependent kinase 1 (PDK1) at Thr 308 (Engelman *et al.*, 2006; Sarbassov *et al.*, 2005). The full activation of Akt is achieved after phosphorylation of Akt in the hydrophobic C-terminal domain (Ser 473) by PDK2 (Blume-Jensen & Hunter, 2001; Hennessy *et al.*, 2005). Activated Akt subsequently initiates the activation of multiple downstream effectors to regulate cellular responses. For instance, Akt can enhance cell survival through the inhibition of pro-apoptotic proteins such as Forkhead Homeobox type O (FOXO) and Bcl2-antaginist of cell death (BAD) (Song *et al.*, 2005) and the induction of survival proteins such as Bcl2, IkappaB kinase (IKK) and human double minute 2 (HDM2) (Condliffe *et al.*, 2005; Engelman *et al.*, 2006) as well as degradation of the tumour suppressor protein p53 (Hirsch *et al.*, 2008). Akt can also increase glycogen synthesis and cell metabolism through the inactivation of FOXO family of transcription factors and glycogen synthase kinase 3 (GSK3) (Christian *et al.*, 2002; Cong *et al.*, 1997; Summers *et al.*, 1999). Akt is also involved in regulating cell-cycle progression through blocking FOXO-mediated transcription of cell-cycle inhibitors, such as p27Kip1 or directly inactivate p27Kip1 by phosphorylation to promote the G_1-S phase transition (Burgering & Medema, 2003). Akt induces cell proliferation by blocking the GTPase-activating proteins (GAPs) activity of the tuberous sclerosis complex 1 (TSC1) and TSC2 which results in the activation of the mammalian target of rapamycin (mTOR)-raptor kinase complex and subsequent activation of p70S6 Kinase (S6K) and the phosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), targeting eukaryotic elongation factor 2 (eEF2) and eukaryotic initiation factor 4E (eIF4E), respectively, to regulate protein synthesis (Engelman *et al.*, 2006; Martelli *et al.*, 2009; White-Gilbertson *et al.*, 2009). Of note, the absence of TSC1/2 leads to reduced activity of Akt which can be subsequently restored by depletion of S6K (Radimerski *et al.*, 2002). Subsequent studies show that S6K can phosphorylate and inhibit IRS-1 and therefore block the PI3K/Akt signal (Chalhoub & Baker, 2009; Chitnis *et al.*, 2008; Loi *et al.*, 2009). These studies suggest that a negative feedback mechanism is involved in the regulation of this complex signaling network. Moreover, Akt has been implicated in cancer cell migration and invasion whereby Akt1 and Akt2 play opposing roles with Akt1 acting as an

inhibitor while Akt2 exerts promigratory effects (Arboleda *et al.*, 2003; Dillon *et al.*, 2009; Hutchinson *et al.*, 2004; Iliopoulos *et al.*, 2009; Irie *et al.*, 2005; Ju *et al.*, 2007; Maroulakou *et al.*, 2007; Yoeli-Lerner *et al.*, 2005). The precise mechanism by which Akt exerts distinct biological roles in breast cancer migration have yet to be elucidated, although several studies have proposed hypotheses, such as differences in activation levels (Brognard *et al.*, 2007), interacting partners (Brognard *et al.*, 2007; Figueroa *et al.*, 2003; Lyons *et al.*, 2007; Pekarsky *et al.*, 2000), downstream substrates (Gonzalez & McGraw, 2009; Jiang *et al.*, 2008; Li *et al.*, 2005; Roberts *et al.*, 2004; Zhou *et al.*, 2006) or subcellular localization (Gonzalez & McGraw, 2009; Zhou *et al.*, 2006).

In addition to Akt, other PH-domain-containing targets such as Tec family tyrosine kinases, guanine nucleotide exchange factors (GEF) for Rac, adenosine diphophae (ADP)-ribosylating factor 6 (ARF6) and GAPs (Engelman *et al.*, 2006; Hennessy *et al.*, 2005; Ward & Finan, 2003) and novel binding partners for PIP_3 induced by PI3Ks have been identified including FYVE, Phox (PX), C1 and C2 domains (Itoh & Takenawa, 2002) have also been implicated downstream of PI3Ks, adding more complexity to PI3K signaling.

The activation of the PI3K signal is negatively regulated by two phosphoinositide phosphatases, PTEN and the SH2 domain-containing inositol phosphatase (SHIP). PTEN converts PIP_3 to PIP_2 (Kalesnikoff *et al.*, 2003; Kisseleva *et al.*, 2000; Rohrschneider *et al.*, 2000) (Stambolic *et al.*, 1998). PTEN is also known as a tumour suppressor and often mutated, deleted or down-regulated in various types of tumours, which results in a constitutive activation of the PI3K pathway (Cully *et al.*, 2006; Vivanco & Sawyers, 2002; Wymann & Marone, 2005). It has been shown that the progressive reduction of PTEN results in increasingly aggressive mouse prostate cancer (Trotman *et al.*, 2003). Reconstitution of PTEN inhibits prostate cancer cell proliferation *in vitro* and also inhibits tumour growth and angiogenesis *in vivo*. This effect is abrogated by overexpression of PTEN mutant lacking phosphatase activity (Fang *et al.*, 2007). Similar to PTEN,

dyregulation of SHIP has also been found to be associated with different tumours, such as breast cancer (Prasad *et al.*, 2008) and leukaemia (Luo *et al.*, 2003; Luo *et al.*, 2004).

1.4 The research project

1.4.1 Significance and rational of the research

IGF-1R and CXCR4 have been shown to play an important role in metastasis of breast cancer through initiating multiple signaling pathways upon the binding of their cognate ligands. Recent data suggest that transactivation between the two distinct receptors contributes to breast cancer cell migration and adds complexity to the signaling network. However, the underlying molecular mechanisms by which IGF-1R-CXCR4 transactivation regulate breast cancer cell migration remain unclear. Therefore, the aim of this study was to investigate the downstream signaling of IGF-1R-CXCR4 transactivation, focusing on PI3K pathway, which has been shown to be an important regulator of cell migration and is known to be involved in signaling through both RTKs and GPCRs. The understanding of the molecular mechanisms underlying IGF-1R-CXCR4 transactivation including PI3K pathway in the progression of breast cancer metastasis and invasion may lead to development of more effective diagnostic and therapeutic strategies.

1.4.2 The central hypothesis to be tested

PI3Kγ and its specific effectors downstream of IGF-1R/CXCR4 transactivation play a key role in breast cancer cell migration.

To address the hypothesis the following aims were investigated:

Aim 1: To investigate the expression of class I PI3K isoforms in breast cancer cells.

Aim 2: To investigate PI3K utilization by IGF-1R in breast cancer cells in response to IGF-I.

Aim 3: To identify PI3Kγ specific effectors downstream of IGF-1R-CXCR4 transactivation.

Table 1.1: Functional classification of the chemokine system

Chemokines	Chemokine receptors
Inflammatory	
CCL1/I-309	CCR8
CCL2/MCP-1	CCR2
CCL3/MIP-1α	CCR1,CCR5
CCL4/MIP-1β	CCR5
CCL5/RANTES	CCR2,CCR3,CCR5
CCL7/MP-3	CCR1,CCR2
CCL8/MCP-2	CCR3,CCR3,CCR5
CCL11/eotaxin-1	CCR1, CCR2,CCR3
CCL13/MCP-4	CCR2
CCL17/TARC	CCR4
CCL20/MIP-3α	CCR6
CCL22/MDC	CCR4
CCL24/ eotaxin-2	CCR3
CCL26/ eotaxin-3	CCR3
CCL27/CTACK	CCR10
CXCL1/GROα	CXCR2
CXCL2/GROβ	CXCR2
CXCL3/GROγ	CXCR2
CXCL5/ENA-78	CXCR2
CXCL6/GCP-2	CXCR1

Table 1.2: Functional classification of the chemokine system (continued)

Chemokines	Chemokine receptors
Infammatory	
CXCL7/NAP-2	CXCR1
CXCL8/IL-8	CXCR1
CXCL9/MIg	CXCR3
CXCL10/IP-10	CXCR3
CXCL11/I-TAC	CXCR3
CXCL16	CXCR6
XCL1/Lymphotactin	XCR1
CX_3CL1/fractalkin	CX_3CR1
Homeostatic	
CCL18/DC-CK1	Unknown
CCL19/ELC	CCR7
CCL21/SLC	CCR7
CCL25/TECK	CCR11
CCL28/MEC	CCR10
CCL20/MIP-3α	CCR6
CXCL12/SDF-1	CXCR4
CXCL13/BLC	CXCR5

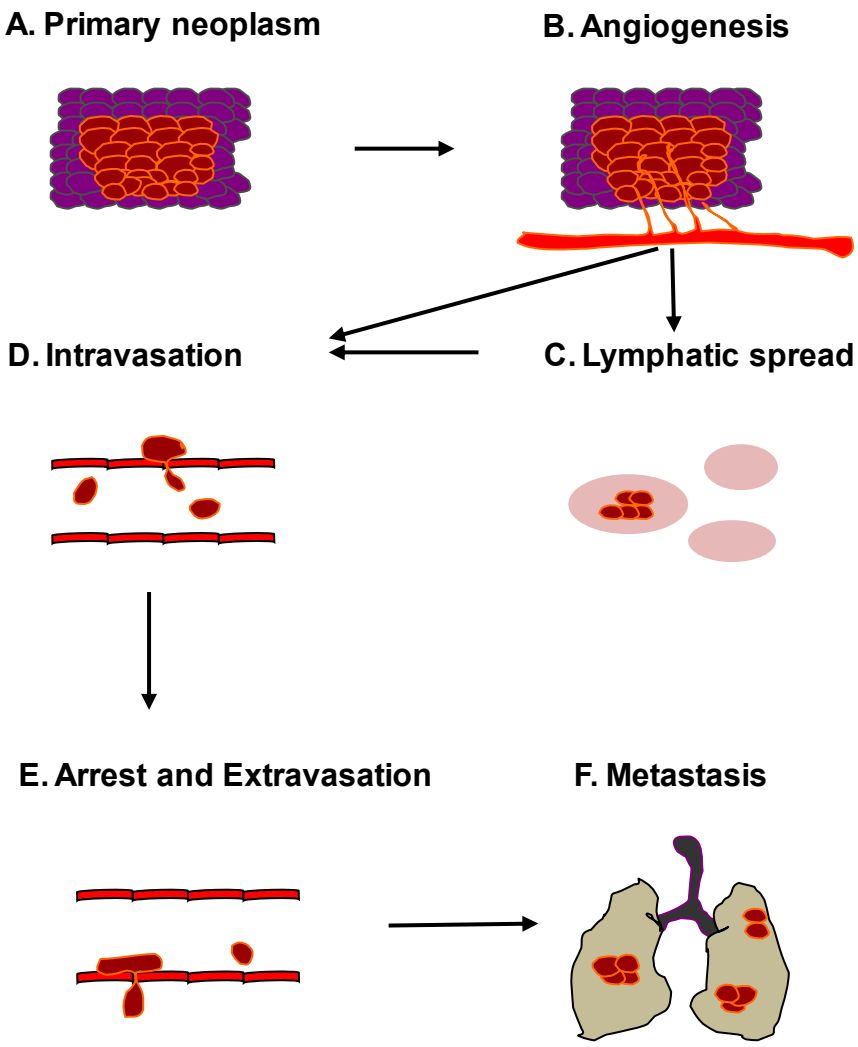

Figure 1.1: The multi-step process of tumour metastasis

(A) Cellular transformation and proliferation occur at the primary sites. (B) Tumour cells acquire the ability to invade through the basement membrane and ECM and form new blood vessels to facilitate metastatic dissemination. (C) and (D) Tumour cells entre via lymphatic or directly entre the circulation. (E) Tumour cells arrest selectively in the blood vessels of target organs and adhere to vascular endothelial cells and migrate to the surrounding tissues. (F) Tumour cells interact with the microenviroment in the target organs to establish metastasis. (Adapted from Mundy, 2002 and Steeg, 2002)

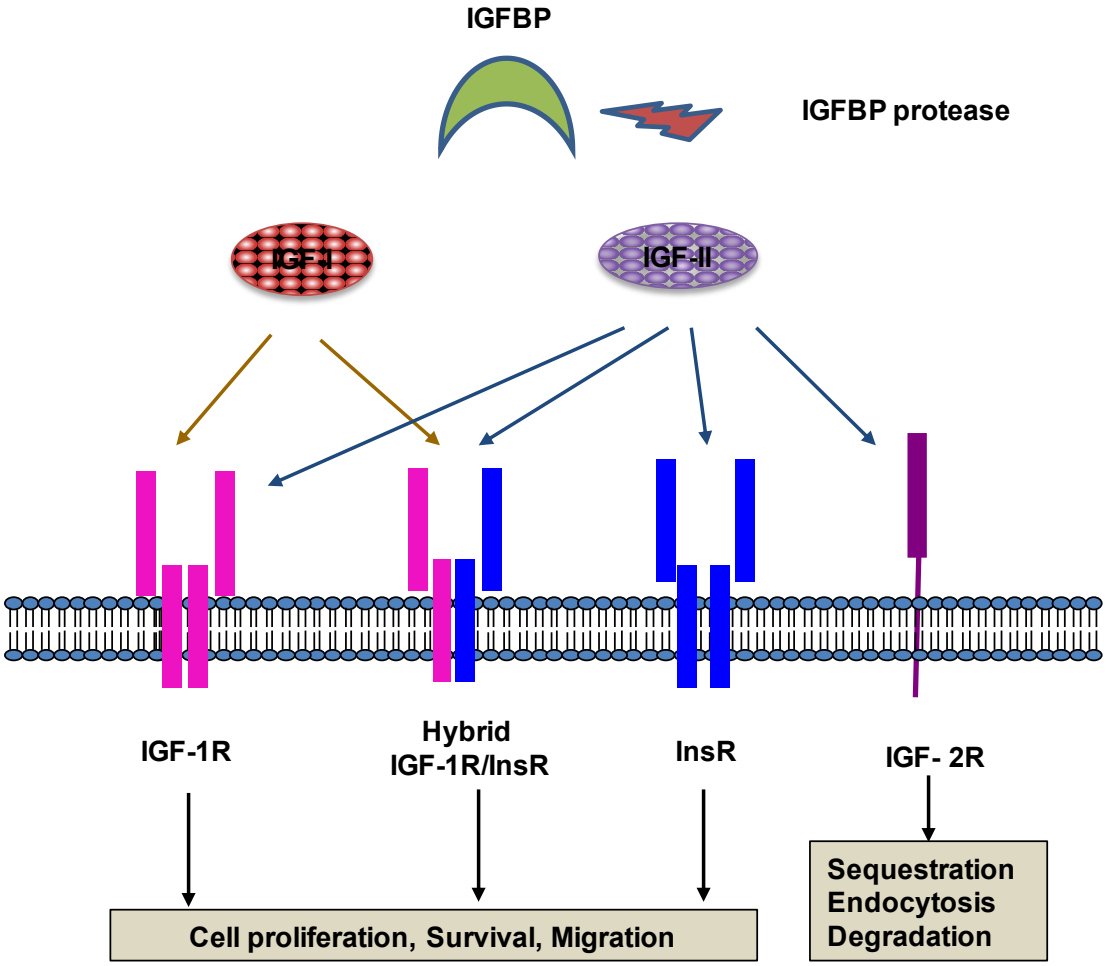

Figure 1.2: Schematic representation of the IGF system

The IGF system is composed of three peptide hormones: IGF-I, IGF-II and insulin, four cell surface receptors: IGF receptor type 1 (IGF-1R), IGF receptor type II (IGF-2R)/mannose 6-phosphate receptor (M6P-R), insulin receptor (IR) and hybrid receptor of IGF/insulin, six high-affinity binding proteins (IGFBPs 1-6) and IGFBP protease. The IGF system regulates various cellular responses including cell proliferation, survival, and migration through interaction with these components (adapted from Adams, 2000; Sachdev, 2001).

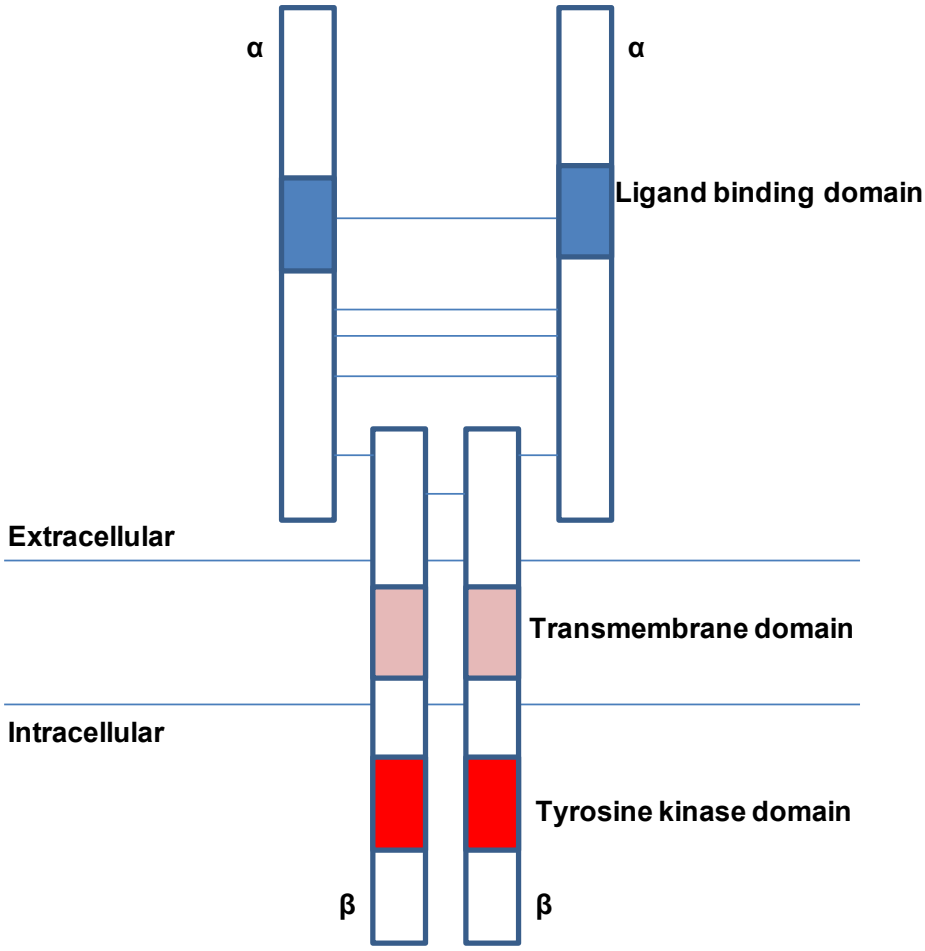

Figure 1.3: Schematic representation of the structure of IGF-1R

IGF-1R is a heterotetramer tyrosine kinase receptor. The mature cell membrane-bound IGF-1R consists of two 130- to 135-kDa α–chains and two 90- to 95-kDa β–chains, with several α-α and α-β disulfide bridges. The α subunits, which are entirely extracellular, form the ligand-binding domain that binds one ligand molecule whereas β subunits containing a single transmembrane domain and an intracellular segment, with tyrosine kinase activity, form multiple binding sites for signaling substrates (Adapted from Adams, 2000).

Figure 1.4: Simplified schematic diagram of the IGF-1R signaling.

The binding of IGF-I to the IGF-1R generally leads to receptor homodimerisation and autophosphorylation, followed by recruitment of scaffold proteins, such as IRS and Shc to activate two key signaling pathways components, PI3K/Akt and Ras-Raf-Erk/MAPK, thereby regulating various cellular responses. The scheme presented is a summary of the major pathways rather than a complete depiction of all the signaling. (Adapted from Tao, 2007) Abbreviations: Janus kinase (JAK); insulin receptor substrate (IRS); src-homology 2/2 α-collagen-related (Shc); growth factor receptor-bound protein 2 (Grb2); son of sevenless (Sos); Ras activated factor (Raf), mitogen-activated protein kinases (MAPK), Extracellular Signal-Regulated Kinase (Erk); phosphatidylinositol 3-kinase (PI3K); 3-phosphoinositide-dependent protein kinase (PDK1); protein kinase B (PKB/Akt).

C	NH_2……C…C…..COOH
CC	NH_2….CC…C…C…..COOH
CXC	NH_2…CXC….C…..COOH
CX_3C	NH_2..CXXXC..C...C….COOH

Figure 1.5: Schematic representation of the structural classification of chemokines.

Four major subfamilies of chemokines are classified based on the number and arrangement of the conserved cysteines, indicated as C. X represents an amino acid other that cysteine. Amino and carboxy tails are marked as NH_2 and COOH respectively.

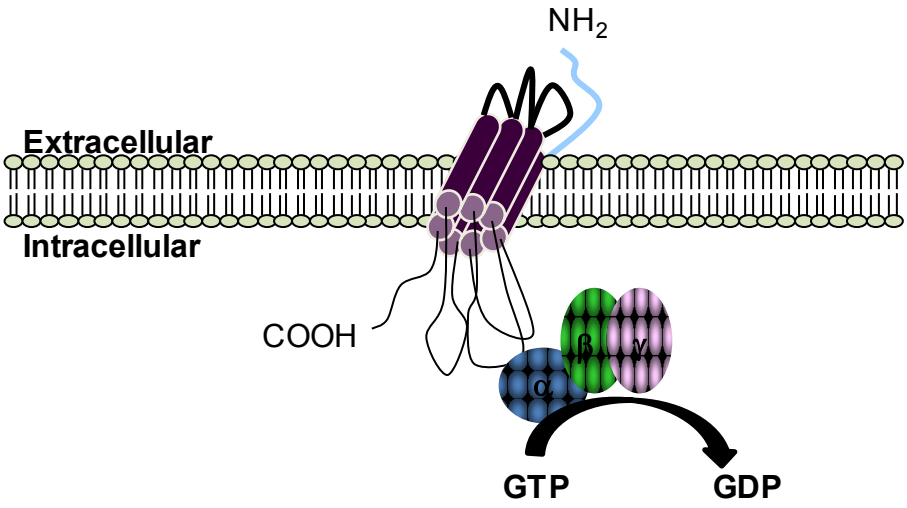

Figure 1.6: Schematic representation of the structure of typical chemokine receptors.

Chemokine receptors belong to the seven transmembrane G protein-coupled receptor family. Most of the chemokine receptors contain approximately 350 amino acids and have a molecular weight of around 40-50. These receptors comprise two main parts functionally. Three extracellular loops with NH_2-terminus act as a binding site for chemokine ligands whereas three intracellular loops with COOH-terminus are associated with intracellular signalling transduction. A conserved 10-amino acid sequence in the second intracellular loop in most of the receptors is critical for heterotrimeric G-protein coupling and a cysteine residue in each of the extrcellular loops help to maintain their three-dimensional structure by forming disulfide bridges.

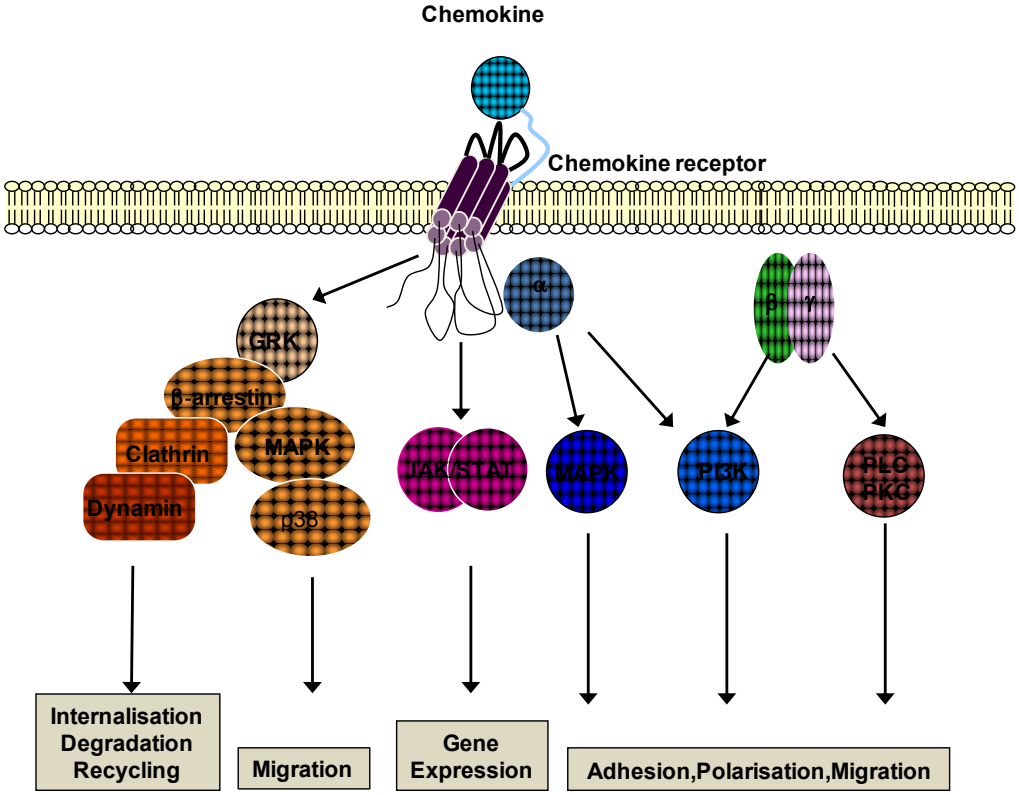

Figure 1.7: Simplified schematic diagram of the chemokine receptor signalings.

Upon chemokine binding, a conformational change of the receptor leads to the activation of the G protein by facilitating the exchange of bound GDP with GTP, as shown in Figure 1.6. The activated G protein subsequently dissociates from the receptor as active G_{α} and $G_{\beta\gamma}$, binding to GTP to trigger downstream signaling cascades. Through these G-protein-dependent signalings as well as G-protein-independent signaling, chemokines regulate a variety of cellular processes, such as chemotaxis, cell survival and intracellular calcium. The scheme presented is a summary of the major pathways rather than a complete decipition of all the signaling. (Adapted from Mellado, 2001) Abbreviations: G protein-coupled receptor kinase (GRK); mitogen-activated protein kinases (MAPK); Janus kinase/signal transducer and activator of transcription (JAK/STAT); phosphatidylinositol 3-kinase (PI3K); phospholipase C/ protein kinase C (PLC /PKC).

Figure 1.8: IGF-1R-CXCR4 transactivation in highly metastatic MDA-MB-231 but not in non-metastatic MCF-7 cells.

(A) In MDA-MB-231 cells, IGF-1R can transactivate the CXCR4 signal transduction pathway that works coordinately with tyrosine kinase-dependent pathways of IGF-1R to induce chemotaxis. (B) In MCF-7 cells, no transactivation occurs by IGF-I due to non-functional CXCR4 expression. Chemotaxis of these cells is independent of CXCR4 signaling (Adapted from Akekawatchai, 2005).

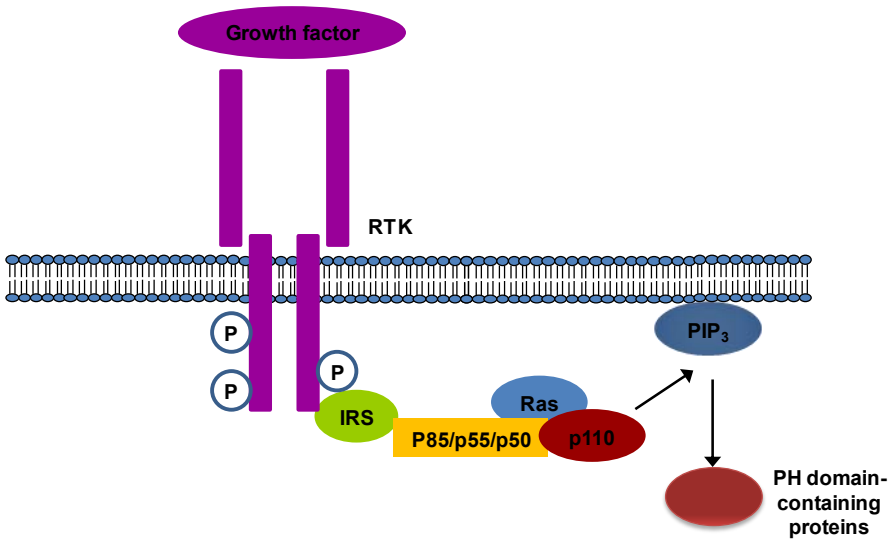

Figure 1.9: Schematic diagram of the class IA PI3K signaling.

The binding of IGF-I to the IGF-1R leads to receptor homodimerisation and autophosphorylation. The SH2 domain of the regulatory subunit of PI3Ks, p50, p55 or p85 binds to phospho-tyrosine residues of the activated RTKs or adaptor proteins, such as IRSs and this binding relieves the basal inhibition of p110 by p85 and recruits the PI3K heterodimer to its substrate, PIP_2 at the plasma membrane to generate PIP_3, which acts as an important second messenger to activate a subset of proteins by binding to their pleckstrin-homology (PH) domains. Abbreviations: insulin receptor substrate (IRS); phospha- tidylinositol 3-phosphate (PIP_3).

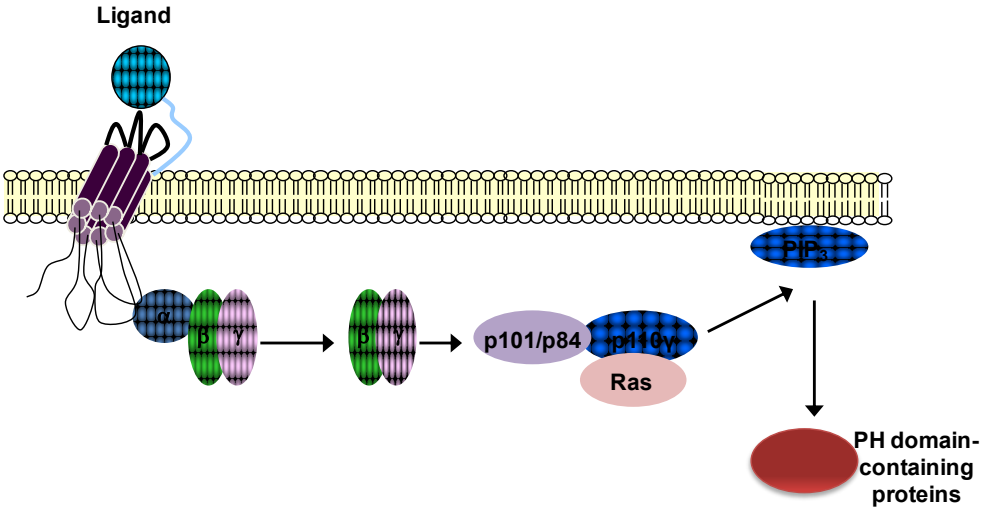

Figure 1.10: Schematic diagram of the class IB PI3K signaling.

PI3Kγ, the only class IB PI3K is activated and translocated to the plasma membrane by βγ subunit of trimeric G proteins after GPCR activation. GTP-Ras can also binds directly to p110γ (Pacold *et al.*, 2000) and cause modest activation of its catalytic activity. Activated p110γ generate PIP_3, which acts as an important second messenger to activate a subset of proteins by binding to their pleckstrin-homology (PH) domains. Abbreviation: phosphatidylinositol 3-phosphate (PIP_3).

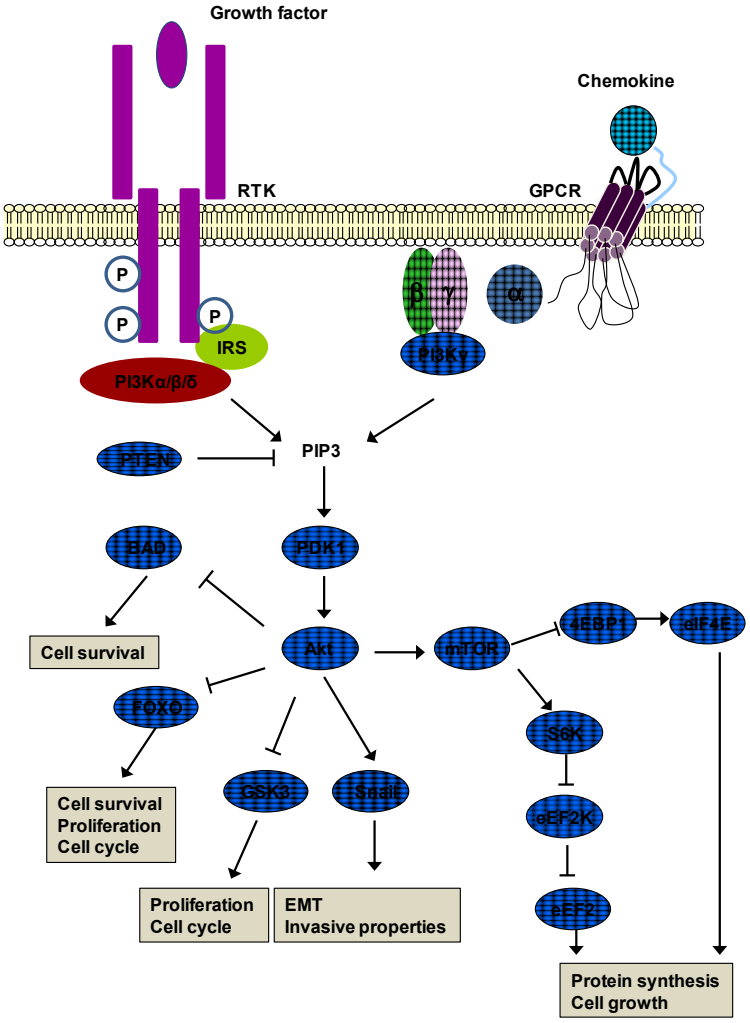

Figure 1.11: Combined schematic diagram of the PI3K/Akt signaling downstream of RTKs and GPCRs showing potential interaction at the level of PIP_3 generation.

Ligation of RTKs and GPCRs by their corresponding ligands leads to activation of class IA or class IB PI3K, respectively. Activity of PI3K is negatively-regulated by PTEN, which converts PIP_3 to PIP_2. Akt activation results in phosphorylation of a number of key substrates to regulate cell survival, cell cycle, cell growth and metabolism. (Adapted from Tao, 2007 and Castaneda,2010) Abbreviations: tyrosine kinase receptor (RTK); G-protein coupled receptor (GPCR); insulin receptor substrate (IRS); phosphatidylinositol 3-kinase (PI3K); phospha- tidylinositol 3-phosphate (PIP_3); phosphatase and tension homolog deleted in chromosome ten (PTEN); 3-phosphoinositide-dependent protein kinase (PDK1); protein kinase B (PKB/Akt); bcl-associated death promoter (BAD); Forkhead Homeobox type O (FOXO); glycogen synthase kinase-3 (GSK3); mammalian target of rapamycin (mTOR); p70S6 Kinase (S6K); eukaryotic elongation factor 2 kinase (eEF2K); eukaryotic elongation factor 2 (eEF2); eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1); eukaryotic initiation factor 4E (eIF4E).

CHAPTER 2

Materials and Methods

Chapter 2: Materials and methods

2.1 General materials

2.1.1 General chemicals, solutions, buffers and antibodies

General chemicals and reagents provided from various main suppliers or manufactures are listed in Table 2.1. Table 2.2 shows preparation of general solutions and buffers used throughout this study. Lists of antibodies, suppliers and applications in immunoprecipitation and Western blot are shown in Table 2.3 and Table 2.4.

2.1.2 Synthetic peptide and inhibitors

IGF-I was obtained from GroPrep Pty Ltd, Adelaide, Australia. Wortmannin was purchased from Sigma-Aldrich (St Louis, USA). AS605240 was supplied by Echelon Biosciences Inc. (Utah, USA). IC87114 was from Australia Centre for Blood Diseases, Monash University, Australia. Detailed information for these inhibitors is listed in Table 2.5.

2.2 Cell culture

2.2.1 Cell lines

Human breast cancer cell lines, the non-metastatic MCF-7 and highly metastatic MDA-MB-231 cells, were obtained from American Type Culture Collection (ATCC; Manassa, VA, USA). B300-19/huCXCR4 cells, murine pre-B cells over-expressing human CXCR4, were kindly provided by Professor Ian Clark-Lewis, Biomedical Research Centre, University of British Columbia, Vancouver, Canada. P6 cells, BALB/c3T3 derivative overexpressing human IGF-1R were kindly provided by Dr. Broiny Fobes, University of Adelaide, South Australia, Australia. MDA-MB-231 cells with CXCR4 knockdown were provided by Dr. Marina Kochetkova, University of Adelaide, South Australia, Australia.

2.2.2 Cell culture maintenance

All basic solutions for cell culture were obtained from suppliers or manufacturers listed in Table 2.6. All cell lines used throughout this study were treated according to standard tissue culture procedures, including thawing, sub-culturing and freezing. Cells in vials stored in liquid nitrogen were thawed rapidly at 37ºC in a water bath followed by dilution in 10 ml of culture medium, centrifugation (300×g for 4 minutes), resuspension in related growth medium shown in Table 2.6 and culture at 37ºC in 5% (v/v) CO_2 atmosphere. To subculture adherent cells, confluent monolayer cells were washed twice with PBS. A sufficient volumn of 1% trypsin was added to cover the cell monolayer for 3-5 minutes at room temperature or 37ºC in the incubator if necessary. Detached cells were resuspended in medium containing 10% fetal calf serum (FCS). Cell suspensions containing appropriate numbers of cells were transferred to fresh 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 a splitting ratio of 1:10, subculture was performed once a week for MCF-7 cells and twice a week for MDA-MB-231 cells. For long term storage, cells in exponential growth phase were washed, harvested and resuspended in freezing medium (Table 2.7) before being transferred to cryogenic vials. The vials were placed in a cryogenic container containing isopropanol at -80ºC overnight, and subsequently transferred to a liquid nitrogen tank. Viable cell counts were 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 subjected to extraction of total RNA. The pellet was mixed with Trizol (Life Technologies, Gilbertsville, PA, USA) (1 ml per 5-10×10^6 cells) and left at room temperature for 5 minutes before addition of chloroform (200 µl per 1 ml of the mixture). The mixture was shakened vigorously by hand for 15 seconds and incubated for 2-3 minutes at room temperature prior to centrifugation at 12,000×g for 15 minutes at 4ºC. The upper aqueous phase containing RNA was

transferred to a fresh microcentrifuge tube. The RNA was then precipitated by addition of 500 µl of isopropanol followed by incubation at room temperature for 10 minutes. The precipitate was then spun down at 12,000×g for 10 minutes at 4ºC. The supernatant was then discarded, leaving a gel-like pellet containing the RNA. The RNA pellet was washed by adding 1 ml of 75% ethanol and spinning down at 7,500×g for 5 minutes at 4ºC. The supernatant was subsequently drained and the pellet was air-dried for 5-10 minutes. The RNA precipitate was then dissolved in 20µl of DEPC-treated water and incubated at 55-60ºC 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 = A_{260}/A_{280}]. A recommended purity is over 1.5. The concentration of RNA was calculated using the following formula. [RNA concentration (µg/µl) = A_{260} × dilution factor × 0.04]

2.3.2 Synthesis of cDNA by reverse-trascriptase enzyme

Prior to synthesis of cDNA from isolated RNA, the RNA was treated by DNase I to remove contaminating chromosomal DNA following the instruction provided by Promega, Madison, WI, USA. Briefly, 5 µg of RNA was diluted to a final volume of 17 µl in DEPC-treated water to which 2 µl of 10× reaction buffer and 1 µl of DNase were added. The reaction was performed at 37ºC for 1 hour and terminated by addition of 2 µl of 10× stop buffer and heat-inactivation at 65ºC for 20 minutes. Generation of first strand cDNA from RNA was conducted using Superscript II reverse-transcriptase and associated buffer as provided (Life technologies, Gilbertsville, PA, USA). 2.5 µg of RNA in 11 µl was combined with 1 µl of oligo (dT) or random primer (500 µg/ml) and heated to 70ºC for 5 minutes to denature the template-primer mixture. After immediate cooling the mixture down to 4ºC, the following reagents were added: 4 µl of first strand buffer (5×), 2 µl of DTT (DL-Dithiothreitol) (0.1 M) and 1 µl of deoxynucleoside triphosphate (dNTP) mixture (10mM each dATP, dTTP, dCTP and dGTP diluted in DEPC-treated water (Amersham Pharmacia Biotech)), and incubated at 42ºC for 2 minutes. Finally, 1 µl of Superscript II (200 units/µl) was added and reverse transcription was allowed to proceed at 42ºC for 50 minutes before final inactivation at 70ºC for 15 minutes. The cDNA products were stored at -20ºC for 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). Sequences for PI3K isoforms, including regulatory subunits, p101, p85α and p85β as well as catalytic subunits, p110α, p110β, p110δ and p110γ were amplified using specific primers (Table 2.8). In general, each PCR was set up in a 25 µl reaction mix containing 5 µl of forward primer and reverse primer respectively, 1 µl of 50 mM $MgCL_2$(2 mM), 1 µl of 10 mM dNTP mixture, 2.5 µl of 10×Mg^{2+} free NH_4 buffer, 0.25 µl of DyNAzyme (Finnzymes) and 1.25 µl of template cDNA. The reactions were cycled in a hot-bonnet thermal cycler. The PCR condition was set up as follows: 95ºC for 12 minutes, 35 cycles of 95ºC for 30 seconds, 54ºC for 30 seconds, 72ºC for 30 seconds, and a final 5 minutes extension at 72ºC. All reactions were held at 4ºC until analyzed.

2.3.4 Agarose gel electrophoresis

Agarose gels (2%) were prepared by dissolving in 1× TAE buffer. After heating, the gels were allowed to settle in a horizontal gel apparatus. The gels were submerged in 1× TAE buffer in an electrophoresis tank. DNA samples and markers were mixed with 6× DNA loading buffer to a final concentration of 1× and loaded onto the gels. The marker was 100 bp DNA markers provided by Invitrogen, Life Technologys, USA. The gels were electrophoresed in 1× TAE buffer at 80 V. Following electrophoresis, gels were stained with 5 µg/ml ethidium bromide in TAE for 5-10 minutes, visualized and analyzed 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 4ºC for 15 minutes in a 200-1000 µl of NP40 lysis buffer (Table 2.2) supplemented with inhibitors (2 mM Na_3VO_4, 5 mM NaF, 1 mM phenylmethylsufonyl fluoride (PMSF) and 1:100 protease inhibitor cocktail (Sigma-Aldrich)). Cell lysates were then spun at 14,000 rpm at 4ºC for 10 minutes to remove insoluble material and the supernatants collected. The total protein concentration in cell

lysates were determined using a colorimetric method, utilizing 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 sample, and the plate was incubated at 37ºC for 30 minutes. Absorbance was measured at 560 nm using a microplate reader (Amersham Biotrack reader II) and analyzed for protein concentration (µg/ml). For protein analysis in Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), the whole cell lysates were prepared by boiling at 95ºC for 5 minutes in 2× SDS loading buffer (Table 2.2). Generally, the lysates were subjected to analysis in SDS-PAGE at 50 µg per well.

2.3.6 Co-immunoprecipitation

Cell lysates (1 mg of total protein) were incubated with antibody for co-immunoprecipitation (see Table 2.3 for details) overnight at 4ºC with constant agitation (spin-dragon) after being pre-cleared with 20 µl of protein A/G (slurry 1:1 in lysis buffer) for one hour at 4ºC (spin dragon). The immunocomplex was then precipitated with 50 µl of Protein A/G (GE Healthcare) for one hour at 4ºC (spin dragon) followed by three washes with 1ml of lysis buffer freshly supplemented with inhibitors (2 mM Na_3VO_4, 5 mM NaF, 1 mM PMSF and 1:100 protease inhibitor cocktail (Sigma-Aldrich)). The immunoprecipitate was then resuspended with 50 µl of 2× loading buffer (Table 2.2) and heated to 95ºC for 5 minutes for SDS-PAGE and Western blot analysis.

2.3.7 Extraction of membrane fraction

Approximately 4×10^6 cells were lysed for 10 minutes at 4ºC in 3 ml of hypotonic buffer A (Table 2.2) supplemented with inhibitors (2 mM Na_3VO_4, 5 mM NaF, 1 mM PMSF and 1:100 protease inhibitor cocktail (Sigma-Aldrich)). Cell lysates were then homogenized with a glass denounce homogenizer and spun at 500×g for 5 minutes at 4ºC to remove the nuclear fraction. The post-nuclear supernatant was subsequently transferred to a Beckman ultracentrifuge tube (Beckman Instruments, Inc, USA) to be further centrifuged at 100,000×g (49,000 rpm) for one hour at 4ºC. The pellet containing the membrane fraction

was then resuspended in 30µl of 2× loading buffer (Table 2.2) and heated to 95ºC for 5 minutes for SDS-PAGE analysis.

2.3.8 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted according to the procedure provided by the manufacturer (Bio-rad, CA, USA). Polyacrylamide gels consisting of 4% stacking gel and 8% resolving gel compartments (Table 2.2) were prepared using a gel pouring apparatus. Protein samples and protein markers were loaded on the polyacylamide gel in 1× electrode buffer (Table 2.2) and electrophoresis was performed at 120 V in the stacking gel and at 150 V in the running gel until the icon front reached the bottom of the gel. 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 PVDF membrane (Hybond™ P, Amersham Pharmacia Biotech) by wet transfer system (Bio-rad, CA, USA) in 1× working Western transfer buffer (Table 2.2) and analyzed by Western blotting. Briefly, membrane was blocked in 5% skim milk powder in TBS-T for 1 hour at room temperature. After 10 minutes wash in TBS-T for 3 times, the membrane was then incubated overnight with primary antibody at the appropriate concentration (Table 2.3) at 4ºC. The membrane was then washed for 3 times in TBS-T followed by incubation with horseradish peroxidise (HRP)-conjugated secondary IgG (Table 2.4) in 1% skim milk powder in TBS-T for 1 hour at room temperature. Another series of washes in TBS-T were performed and proteins on the membrane were detected with an ECL detection solution kit (Sigma, USA) and visualized by exposure to X-ray film, developed using an Ilfospeed 2240 X-Ray processor (Ilford, Switzerland). Gel bands were quantified using Imagequant software (GE Healthcare, USA). All values were normalized to the appropriate loading control (β-actin) and then expressed as a value relative to the 10 minute control-treated values essentially as previously described (Saavedra *et al.*, 2010).

2.3.9 Retroviral-mediated siRNA knockdown

siRNA construct in pLKO.1 was purchased from Open Biosystems Inc (Huntsville, AL, USA). The target sequence for p110γ is as follows:

CCGGGCAGAGCTTCTTCACCAAGATCTCGAGATCTTGGTGAAGAAGCTCTGCT
TTTTG

To produce retroviral supernatants, HEK293T packaging cells were transfected with 2 μg of specific or control expression vectors, 1.33 μg each of psPAX2, pREV and pMD2-G packaging vectors and 16.67 μl of Lipofectamine 2000 reagent (Invitrogen, USA) in 1 ml Opti-MEM medium (Gibco) in 60-mm tissue culture dishes without fetal calf serum and without antibiotics. The medium was replaced 6 hours later with 10% FCS-supplemented Opti-MEM and then replaced with complete RPMI medium 16 hours later. Virus-containing supernatants were harvested at 48 hours post-transfection. Supernatants were filtered through a 0.45 μm Minisart syringe filter (Sartorius AG, Germany) and polybrene (Sigma) was added to a final concentration of 8 μg/ml.

For transduction, MDA-MB-231 cells were plated in 6-well trays at $1.5x10^5$cells/well. The culture medium was removed 24 hours later and replaced with neat viral supernatant. The supernatant was replaced by cell growth medium after 6 hours of infection. After 16 hours, the medium was changed once more and cells were incubated at 37ºC, 5% CO_2 for a further 48 hours before adding puromycin (5 ng/ml) to the medium. Transduced cells were selected for one week with puromycin. Cells transduced with retroviral vectors expressing scrambled siRNA were used as controls.

2.4 Assays for assessment of receptor function

2.4.1 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). Briefly, approximately 2.5×10^5 cells/well were cultured overnight in 24-well flat-bottom culture plates. The culture medium was replaced by serum-free medium (RPMI-1640, GibcoBRL, Grand Island, NY, USA) with 0.5% BSA for 4 hours before being incubated with various concentrations of IGF-I. After 10 minutes stimulation, cell lysates were prepared by addition of Triton-X100 lysis buffer pH 7.5 (20 mM HEPES, 150 mM NaCL, 1.5 mM $MgCL_2$, 1 mM 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 pre-coated with anti-IGF-1R Abs (mAb 24-31) diluted in 50 mM $NaHCO_3/Na_2CO_3$, pH 9.6 (0.25 μg/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-labeled anti-phosphotyrosine PY20 (PerkinElmer, Turku, Finland) diluted in ligand binding buffer (Table 2.2) (7.6 ng/well) for 2 hours at room temperature. After washing the plate 5 times with distilled water, 100 μl 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 BMGlab Technologies Polarstar Fluorometer. The level of phosphorylated receptor complex formed in IGF-I-stimulated cells was expressed as fold-increase compared with unstimulated cells.

2.4.2 Chemotaxis Assay

Chemotaxis was measured in a modified Boyden Chamber using polycarbonate filters (8 μM for MDA-MB-231 cells, Neuroprobe, Gaithersburg, MD, USA) coated with 25 μg/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) were preincubated with calcein-AM (1 μg/ml of final concentration, Molecular Probes, Eugene, OR, USA) for 30 minutes before being loaded in the upper chamber (5×10^4 viable cells /well), whereas the lower chamber contained various concentration of stimulant (IGF-I), diluted in serum-free medium. After the chamber was incubated in a tissue culture incubator at 37ºC for 2 hours, the membrane was 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 Image® Fx (BioRad Laboratories, USA), and expressed as a migration index, representing the fluorescent signal of stimulated cells compared with that of non-stimulated cells.

2.5 Differential 2D fluorescence gel electrophoresis (2D-DIGE)

2.5.1 Sample preparation

Approximately 5×10^6 cells of control and p110γ knockdown MDA-MB-231 cells either unstimulated or stimulated with IGF-I for 5 minutes were lysed in 500 µl of hypotonic buffer B (Table 2.2) for 10 minutes at 4ºC. The cell lysates were then homogenized with a glass dounce homogenizer and centrifuged at 800×g for 10 minutes at 4ºC. The pellet was washed with the hypotonic buffer and the supernatants were combined to generate the cytosolic fraction. These samples were then precipitated with a Clean-up kit (BioRad) according to the manufacture's protocol and resuspended in labeling buffer (7 M Urea, 2 M Thiourea, 4% (w/v) CHAPS, 30 mM Tris, pH 8.5). Protein concentration was estimated by an EZQ protein quantitation assay (Invitrogen/Molecular Probes) against an ovalbumin standard curve, performed according to the manufacturer's instruction. Each of the tested conditions (non-IGF-I-stimulation and IGF-I-stimulation) were repeated in triplicate.

2.5.2 Protein labeling

Fifty micrograms of proteins, from each sample were labeled with 200 pmol of CyDye DIGE fluor minimal dyes, Cy3 or Cy5 (GE Healthcare) and a reverse-labeling approach was used to avoid dye-labeling bias. The gel-to-gel variation was controlled using an internal standard sample (IPS), obtained by mixing equal amounts of proteins from the tested condition. As common practice, the Cy2 minimal dye (GE Healthcare) was used to label the IPS (Table 2.9). The labeling reaction was carried out incubating samples for 30 minutes on ice in dark, and then stopped by adding 1 µl of 10 mM lysine per 200 pmol of CyDye and incubated for further 10 minutes on ice in dark. After labeling, 2% DTT (0.167 g/100µl H_2O) and 2% Pharmalyte 3-10 (GE Healthcare) were added to the samples and incubated for 60 minutes on ice in dark.

2.5.3 2D gel electrophoresis

Six individual samples from the control and p110γ knockdown cells under each of the tested conditions (non-IGF-I-stimulation and IGF-I-stimulation) were co-resolved in 3 different 2D-DIGE gels. Isoelectric focusing (IEF) was performed on an immobilized non-

linear pH 3-11 gradient of 24 cm length (GE Healthcare), using an Ettan IPGphor II system (GE Healthcare) with the current limited to 50 μA per strip. The proteins were focused for 27,000 Volt-hours at 8.000 V (Table 2.10).

Following IEF, the strips were equilibrated in equilibration buffer (Gel Company) with added 6 M urea containing 10 mg/ml of DTT for 15 minutes followed by the exchange of solution for equilibration buffer containing 25 mg/ml iodoacetamide (IAA) in place of DTT. Afterwards, SDS-PAGE in the second dimension was carried out using 12.5% 2D gel DALT NF precast polyacrylamide gels (Gel Company). Electrophoretic separation was performed using an Ettan Dalt 12 Seperation Unit (GE Healthcare) in the electrohporesis buffer provided with the pre-cast gels at 25ºC using the following conditions: 50 V, 5 mA/gel, 0.5 W/gel, for 1 hour, 110 V, 10 mA/gel, 0.5 W/gel, for 1 hour, 250 V, 30 mA/gel, 2.5 W/gel until the dye-front emerged from the bottom of the gel.

2.5.4 2D-DIGE image acquisition and analysis

Fluorescence signals were imaged using the Typhoon Trio (GE Healthcare) at 100 μm resolution with exposures according to Table 2.11. The resultant images were cropped to show only the relevant regions of the gel.

Image analysis was undertaken using DeCyder 2D software (version 7, GE Healthcare) to compare the control and p110γ knockdown cells to generate lists of differentially-expressed proteins. Each gel image was processed separately in the differential In-gel Analysis (DIA) module of DeCyder prior to export to the Biological Variation Analysis (BVA) module. In the DIA module, spot detection was performed based on an estimated 5,000 spots. Exclusion filters were set to reject spots with a slope of > 1.1, an area of < 600, a volume of < 30,000 and a peak height of < 80 and > 65,000. The resulting spot maps were inspected manually and regions that showed poorly resolved spot patters were excluded from further processing. The three DIA workspaces were then imported into BVA for spot matching and comparative analysis. To aid in the spot matching process, a

selected number of spots from different areas across the gels were matched manually to provide "landmarks". The automatic matching function was then applied and the results were evaluated. Any errors in the automatically assigned spot matches were re-matched manually.

The control and test samples were compared using a two-tailed Students t-test to detect spots that are differentially expressed, correction for false discovery rate (FDR) was turned on. Those spots that returned a p-value of < 0.05 were accepted and were subjected to manually inspection to remove what deemed unsuitable.

2.6 Liquid chromatography-electrospray ionisation ion-trap mass spectrometry (LC-eSI-I MS/MS)

2.6.1 Sample preparation

Selected spots of differentially-expressed proteins were excised from the gel by Ettan Spot Picker (GE Healthcare), washed in 500 µl of 50 mM ammonium bicarbonate (NH_4HCO_3) and processed as follows: first, they were digested with 100 ng of sequencing grade modified trypsin (Promega) in 5 mM ammonium bicarbonate with 10% acetonitrile (ACN), then extracted with 1% formic acid (FA) in water, 1% FA in 50% ACN and 100% ACN. The volumes of the resulting peptide extracts were reduced by vacuum centrifugation to approximately 1 µl.

2.6.2 Data acquisition

Vacuum concentrated samples were resuspended with 0.1% FA in 2% ACN to a total volume of 8 µl. LC-eSI-IT MS/MS was performed using an online 1100 series HPLC system (Agilent Technologies) and HCT Ultra 3D-ion-Trap mass spectrometer (Bruker Daltonics). The LC system was interfaced to the MS using an Agilent Technologies Chip Cube operating with a ProtID-Chip-150 (II), which intergrates the enrichment column (Zorbax300SB-C18, 4 mm, 40 nl), analytical column (Zorbax300 SB-C18,150 mm × 75 µm), and nanospray emitter. Five microlitres of samples were loaded on the enrichment

column at a flow rate of 4 µl /min in Mobile Phase A (0.1% FA in 2% (v/v) ACN) and resolved with 1-30% gradient of Mobile Phase B (0.1% FA in 98% (w/v) ACN) over 32 minutes at 300 nl/min. Ionizable species (300 < m/z < 1,200) were trapped and the two most intense ions eluting at the time were fragmented by collision-induced dissociation. Active exclusion was used to exclude a precursor ion for 30 seconds following the acquisition of the two spectra.

2.6.3 Protein identification

MS and MS/MS spectra were subjected to peak detection and de-convolution using DataAnalysis (Version 3.4, Bruker Daltonics, Billerica, MA, USA). Compound lists were exported into BioTools (Version3.1, Bruker Daltonics) then submitted to the in-house MASCOT database-searching engine (Version 2.2, Matrix Science, Boston, MA, USA) using the following specifications: Taxonomy: Mammalia, Database: SwissProt 57.7, Enzymes: Trypsin, Fixed modifications: Carbamidomethyl (C), Variable modifications: Oxidation (M), Gln- > pyro-Glu (N-term Q), Glu- > pyro-Glu (N-term E), Mass tol MS: 0.3 Da, MS/MS tol: 0.4 Da, Peptide charge: 1+, 2+ and 3+, Missed cleavages: 2.

Protein identifications were made on the basis of having at least two matching unique peptides with individual ion scores above the specified threshold. These unique peptides were required to have different sequences or different variations of the same sequence, for example, containing a modified residue or missed cleavage site. Multiple charge states were not considered as unique. Identities assigned based on only a single peptide hit should be considered as tentative and further experimental evidence is required to confirmed when redundancy in the protein identity was observed, for example, when different isoforms of a protein were matched to identical mass, only the most suitable database entry corresponded to the full length sequence and/or the entry to which additional masses were matched to isoform-specific regions of the protein, were reported.

2.7 Statistics

Statistical analyses were conducted by unpaired Student's t-test or 2-way ANOVA with Bonferroni post-tests, as stated in the figure legends, using Graphpad Prism Software (GraphPad software, Inc, 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	Heideburg, Germany
Ammonium persulfate (APS)	Sigma, St. Louis, MO, USA
Bovine serum albumin (BSA)	Sigma, St. Louis, MO, USA
Chloroform	BDH Chemicals, Klisyth, Vic, Australia
Diethylpyrocarbonate (DEPC)	Sigma, St. Louis, MO, USA
DL-Dithiothreitol (DTT)	Sigma, St. Louis, MO, USA
Ethylendiaminotetra-acid (EDTA)	BDH Chemicals, Klisyth, Vic, Australia
Ethylene glycol-bis (β-aminoethyl ether) N, N, N'-tetraacetic acid ($C_{14}H_{24}N_2O_{10}$) (EGTA)	Sigma, St. Louis, MO, USA
Ethanol	BDH Chemicals, Klisyth, Vic, Australia
Ethidium bromide	Molecular Probes, Oregon, USA
Glacial acetic acid	BDH Chemicals, Klisyth, Vic, Australia
Glycerol	Ajax Chemicals, Auburn, NSW, Australia
Glycine	Sigma, St. Louis, MO, USA
Hydrochloric acid (HCL)	BDH Chemicals, Klisyth, Vic, Australia
HEPES ($C_8H_{18}N_2O_4S$)	BDH Chemicals, Klisyth, Vic, Australia
Igepal CA 630 (NP40)	Fluka Chemie GmbH, Switzerland
Isopropanol	BDH Chemicals, Klisyth, Vic, Australia
Magnesium Chloride ($MgCL_2$)	Ajax Chemicals, Auburn, NSW, Australia
N, N, N′, N′-tetramethyl ethylenediamine (TEMED)	Sigma, St. Louis, MO, USA
Polyoxyethylene sorbitan monolaurate (Tween-20)	Sigma, St. Louis, MO, USA
Potassium Chloride (KCL)	Ajax Chemicals, Auburn, NSW, Australia
Sodium Chloride (NaCl)	BDH Chemicals, Klisyth, Vic, Australia

Table 2.1: General chemicals and reagents (continued)

Name	Main Suppliers or Manufacturers
Sodium dodecyl sulphate (SDS)	Sigma, St. Louis, MO, USA
Tris amiomethane	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) APS (freshly prepared)
DEPC-treated water	0.1% (v/v) DEPC in MilliQ water
DNA loading buffer	30% (w/v) sucrose, 0.35% (w/v) Orange G
Electrode (Running) buffer	30.3 g/l Tris base, 144.0 g/l glycine and 10.0 g/l SDS
Ligand binding buffer	100 mM HEPES, 100 mM NaCL, 0.05% Tween20 and 2 µM DTPA, pH 8.0
Hypotonic buffer A	10 mM Tris-Hcl (pH 8.0), 1.5 mM $Mgcl_2$, 10 mM KCL, 0.5 mM Dithiothreitol, 0.1% Nonidet P-40
Hypotonic buffer B	10 mM Hepes, pH 7.9, 133 mM sorbitol
NP-40 lysis buffer	50 mM Tris pH 7.5, 200 mM NaCL, 1% Igepal CA-630, 2.5 µl/ml EDTA, 1.5 µl/ml $MgCL_2$
Phosphate buffer saline (PBS)	0.137 M NaCL, 2.7 mM KCL, 1.46 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4
PBS-T	PBS containing 0.05% (v/v) Tween20
Resolving gel (8%)	27% (v/v) of 30% Acrylamide/Bis, 25% (v/v) resolving gel buffer, 1% (v/v) of 10% SDS, 0.07%(v/v) TEMED and 0.7% of 10% APS in distilled water
Resolving gel buffer	1.5 M Tris-HCL, pH 8.8
SDS loading buffer (2×)	100 mM Tris-HCL (pH 6.8), 200 mM DTT, 2% SDS, 0.01% bromphenol blue, 20% glycerol

Table 2.2: General solutions and buffers (continued)

Name	Content
Stacking gel (4%)	6% of 30% Acrylamide/Bis, 25% stacking gel buffer, 0.02% (V/V) 10% SDS, 0.14% TEMED and 0.7% of 10% APS in distilled water
Stacking gel buffer	0.5 M Tris HCL, pH 6.8
TBS	25mM Tris pH 7.4, 137mM NaCL, 2.7 mM KCL
TBS-T	TBS containing 0.1% (v/v) Tween-20
Tris/acetic acid/EDTA (TAE)	40mM Tris, 40mM Glacial acetic acid, 1mM EDTA (pH 8.0)
Western transfer buffer (10× stock)	30.3 g/l Tris base, 144.0 g/l Glycine
Western transfer buffer (1× working buffer)	1× Western transfer buffer with 20% ethanol

Table 2.3: Primary antibodies used in immunoprecipitation, Western blot and KIRA

Name	Conc/dilution	Application	Sources
Anti-hu-p101,	19 μg/reaction	IP	Home-made
Anti-hu-p101	1:500	WB	Home-made
Anti-hu-p84,	1:500	WB	Home-made
PI3-Kinase p110δ,	1:500	WB	Home-made
PI3-Kinase p110γ	1:500	WB	Cell Signaling
Anti-Phospho-Akt (Ser 473)	1:1000	WB	Cell Signaling
Anti-Phospho-Akt (Ser 473)	1:100	IP	Cell Signaling
Anti-β-actin	1:5000	WB	Sigma
Anti-Akt1	1:1000	WB	Cell Signaling
Anti-Akt2	1:1000	WB	Cell Signaling
Anti-Akt	1:1000	WB	Cell Signaling
Anti-eEF2	1:1000	WB	Cell Signaling
Anti-Phospho-eEF2(Thr56)	1:1000	WB	Cell Signaling
Anti-cadherin	1:500	WB	Sigma
Anti-phosphotyrosine py20	76 ng/ml	KIRA	PerkinElmer Life Sciences
Anti-IGF-1R (24-31)	2.5 μg/ml	KIRA	N/A

Table 2.4: Secondary antibodies used in Western Blot analysis

Name	Conc/dilution	Application	Sources
HRP-conjugated goat anti-mouse IgG (2 mg/ml)	1: 10,000	WB	Rockland
HRP-conjungated goat anti-rabbit IgG (1 mg/ml)	1: 25,000	WB	Thermo Fisher Scientific

Table 2.5: Inhibitors

Inhibitor	Source	Catalogue number	Concentration	Diluent
Wortmannin	Sigma-Aldrich	W-1628	0.1 μM	DMSO
AS605240	Echelon Biosciences	B-0301	2 μM , 10 μM	DMSO
IC87114	Monash University	N/A	10 μM	DMSO

Table 2.6: 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	GibcoBRL, Grand Island, NY, USA
Fetal calf serum (FCS) (heated inactivated at 56ºC for 1 hour)	JPH Bioscience Ltd, Hampshire, UK
G418, 100 mg/ml	Life Technologies, Gilbertsville, PA, USA
HEPES ($C_8H_{18}N_2O_4S$),1 M	Infectious Disease Laboratories Media Production Unit (IMVS), SA, Australia
β-mercaptoethanol, 27 mM	Sigma-Aldrich, St Louis, MO, USA
Penicillini/Gentamycin	Infectious Disease Laboratories Media Production Unit (IMVS), SA, Australia
RPMI (Roswell Park Memorial Institute) medium 1640	GibcoBRL, Grand Island, NY, USA
Sodium pyruvate, 100 mM	Sigma-Aldrich, St Louis, MO, USA
Trypan blue	Sigma-Aldrich, St Louis, MO, USA
Trypsin, 1 M	Infectious Disease Laboratories Media Production Unit (IMVS), SA, Australia

Table 2.7: 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% penicillini/gentamycin and 10% FCS
MDA-MB-231	RPMI 1640 with 20 mM HEPES, 1% penicillini/gentamycin and 10% FCS and 1 mM sodium pyruvate
B300-19/huCXCR4	RPMI 1640 with 20 mM HEPES, 1% penicillini/gentamycin, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM non-essential amino acid, 0.054 mM β-mercaptoethanol and 10% FCS
p6	DMEM with 20 mM HEPES, 1% penicillini/gentamycin, 250 μg/ml G418 and 10% FCS
Freezing medium	Serum-free medium with 20% DMSO an 30% FCS

Table 2.8: Primers used in this study

Name	Sequence (5'-3')	µl/reaction	Final conc
p85α-F	TCC GTG GAC TTG GAA ATG AT	5	1 pmol/µl
p85α-R	TCT CCC CAG TAC CAT TCA GC	5	1 pmol/µl
p85β-F	CTG CAG GAT GCT GAG TGG TA	5	1 pmol/µl
p85β-R	CAG CAG GAT CCT TTG CAT CT	5	1 pmol/µl
p110α-F	GAC TTA TTG AGG TGG TG	5	1 pmol/µl
p110α-R	GGC ATG CTG TCG AAT AG	5	1 pmol/µl
p110β-F	GCT AAT GTG TCA AGT CG	5	1 pmol/µl
p110β-R	CCG ATT ACC AAG TGC TC	5	1 pmol/µl
p110δ-F	GTG TCC CAT TCA TCC TCA CC	5	1 pmol/µl
p110δ-R	TCA GTG CCT CCT CCT CTG TT	5	1 pmol/µl
p110γ-F	CCT GCA GAA TTC TCA AC	5	1 pmol/µl
p110γ-R	CAC AAT CTC GAT CAT TC	5	1 pmol/µl
p101-F	GTC TCA GGC CTC TCT GAT GG	5	1 pmol/µl
p101-R	TGG GAT CCT CAT CTC CAC TC	5	1 pmol/µl

Table 2.9: Summary of sample loading strategy for 2D-DIGE

Replicate	CyDye		
	Cy3	**Cy5**	**Cy2**
Gel 1	Control	p110γ KD	IPS
Gel 2	p110γ KD	Control	IPS
Gel 3	Control	p110γ KD	IPS

Table 2.10: Summary of isoelectric focusing program

Step	Voltage (V)	Type	Duration (hours)
1	150	Step-and-hold	1
2	300	Step-and-hold	1
3	600	Step-and-hold	2
4	1000-8000	Gradient	1
5	8000	Step-and-hold	27,000 Vhrs
6	300	Step-and-hold	Until end

Table 2.11: Summary for the settings for Typhoon scanner

Emission Filter and Bandpass (BP)	PMT-Value (V)	Laser (nm)
520 nm Bandpass 40 nm	550	Blue (488)
580 nm Bandpass 30 nm	550	Green (532)
670 nm Bandpass 30 nm	550	Red (633)

CHAPTER 3

Regulation of Cell Migration by PI3Kγ Downstream of IGF-1R-CXCR4 Transactivation in Human Metastatic MDA-MB-231 Breast Cancer Cells

Chapter 3: Regulation of cell migration by PI3Kγ downstream of IGF-1R-CXCR4 transactivation in human metastatic MDA-MB-231 breast cancer cells

3.1 Introduction

Breast cancer metastasis is a multi-step process regulated by a number of homeostatic factors. Various RTKs and GPCRs have been shown to play an important role in this process through activation of their cognate receptors to trigger multiple downstream signaling cascades (Dong *et al.*, 2007; Dunn *et al.*, 1998; Liang *et al.*, 2005; Muller *et al.*, 2001; Pennisi *et al.*, 2002; Qian *et al.*, 2007). Moreover, accumulating evidence suggest that RTKs and GPCRs may also regulate cell migration through transactivation between these two distinct families of receptors. For example, transactivation of EGFR by GPCRs has been shown to promote cell migration in a wide range of cancer cell lines (Gschwind *et al.*, 2002; Gschwind *et al.*, 2003; Hart *et al.*, 2005; Schafer *et al.*, 2004). Additionally, recent data from our laboratory have demonstrated that IGF-1R can transactivate CXCR4 in MDA-MB-231 cells and this plays a key role in IGF-I-induced motility of these highly-invasive cells (Akekawatchai *et al.*, 2005).

Despite these previous studies, the precise mechanisms by which IGF-1R-CXCR4 transactivation regulates breast cancer cell migration still remains unknown. However, as PI3Kδ and PI3Kγ are known effectors for cell migration following activation of CXCR4 (Saudemont *et al.*, 2009), the effect of these isoforms on the cell migration upon IGF-1R-CXCR4 transactivation was assessed by using highly selective inhibitors for p110δ (IC87114) and p110γ (AS605240) as well as gene-silencing technique. The main aim of these experiments was to provide proof for an essential role of PI3K and to identify the PI3K isoforms, which regulates migration of MDA-MB-231 cell in response to IGF-I.

3.2 Results

3.2.1 Expression of PI3K isoforms in MDA-MB-231cells

The expression of PI3Kδ and PI3Kγ in MDA-MB-231 cells was investigated by Western blot analysis. Because the results of our previous studies also showed that IGF-I-induced migration of non-metastatic MCF-7 cells does not require transactivation of CXCR4 by IGF-I (Akekawatchai *et al.*, 2005), those cells were included in the analysis for comparison. With the exception of p110δ and p110γ in MDA-MB-231 cells, the level of the proteins was too low to be detected by Western blot using any of the currently available antibodies. Therefore, MDA-MB-231 cell lysates were subjected to immunprecipitation prior to Western blot analysis to enhance detection. Under these conditions, all three PI3Kγ subunits (p101, p84 and p110γ,) were detected in MDA-MB-231 cells (Figure 3.1). Interestingly, while the two PI3Kγ regulatory subunits, p101 and p84 were expressed at the similar level in both MBA-MB-231 cells and MCF-7 cells, a high level of p110γ expression was only observed consistently in MDA-MB-231 cells. This was confirmed at the level of gene expression by RT-PCR (Figure 3.2). The PI3Kδ catalytic subunit p110δ was detected at the protein level in both MDA-MB-231 and MCF-7 cells (Figure 3.1).

3.2.2 Effect of PI3Kδ on IGF-I-induced chemotaxis

To determine whether PI3Kδ plays a role in IGF-I-induced migration of MDA-MB-231 cells, cells were tested for their chemotatic response to various concentration of IGF-I using a modified Boyden Chamber assay after being pretreated with IC87114. IC87114 is one of the isoform-specific inhibitors for p110δ which has no known off-target effects on other protein kinases such as Akt1, PKCα, PKCβII, p38MAPK and significantly inhibits p110δ catalytic function and the generation of PIP_3 (Sadhu *et al.*, 2003a). The doses of inhibitor used in this study (10 μM) were chosen from the results of previous studies (Sadhu *et al.*, 2003a). As previously observed (Akekawatchai *et al.*, 2005), IGF-I dose-dependently induced migration of MDA-MB-231 cells, IC87114 treatment had no statistically significant effect on IGF-I-induced cell migration (Figure 3.3), indicating that PI3Kδ is not involved in IGF-I-induced cell migration in MDA-MB-231 cells.

3.2.3 Activation of PI3Kγ by IGF-I in MDA-MB-231 cells

PI3Kγ is known to be predominant PI3K responsible for cell migration after activation of GPCRs, including CXCR4 (Adams *et al.*, 2000; Barbier *et al.*, 2001; Brock *et al.*, 2003; Del Prete *et al.*, 2004; Heit *et al.*, 2008; Hirsch *et al.*, 2000; Lee *et al.*, 2002b; Liu *et al.*, 2007; Monterrubio *et al.*, 2009; Naccache *et al.*, 2000; Oak *et al.*, 2007; Procko & McColl, 2005; Puri *et al.*, 2004; Sasaki *et al.*, 2000b; Saudemont *et al.*, 2009; Ward & Marelli-Berg, 2009). To determine whether PI3Kγ could be activated by IGF-I in MDA-MB-231 cells, the translocation of the catalytic subunit p110γ to the cell membrane was investigated (Lopez-Ilasaca *et al.*, 1997; Stephens *et al.*, 1994). The cells were incubated with IGF-I for 5 minutes and membrane fractions were compared for the presence of p110γ by Western blot analysis. Under resting conditions, p110γ was not observed in the membrane fraction. In contrast, p110γ was detected in the membrane fraction after IGF-I stimulation (Figure 3.4). Western blotting for pan-cadherin expression showed equal protein loading. The results of these experiments clearly indicate that IGF-I induces translocation of p110γ to the cell membrane.

3.2.4 Effect of AS605240 on IGF-I-induced chemotaxis and IGF-1R activation

To determine whether PI3Kγ is essential for IGF-I-induced migration of MDA-MB-231 cells, cells were tested for their chemotactic response to various concentrations of IGF-I using a modified Boyden Chamber assay after being treated with AS605240. AS605240 is highly specific for the class IB p110γ catalytic isoform (Camps *et al.*, 2005). Published studies using AS605240 have shown that this compound is capable of blocking neutrophil chemotaxis *in vitro* and *in vivo*, minimizing progression of joint destruction due to the selective inhibition effect on PI3Kγ (Camps *et al.*, 2005). The doses of inhibitor used in this study (2 μM, 10 μM) were chosen from the results of previous studies (Camps *et al.*, 2005). As previously observed (Akekawatchai *et al.*, 2005), IGF-1 dose-dependently induced migration of MDA-MB-231 cells, AS605240 inhibited the response of the cells to IGF-I. Inhibition was observed at all IGF-I doses when the cells were treated with 10 μM AS605240, and at the highest dose of IGF-I when treated with 2 μM AS605240 (Figure 3.5). Because the results of previous studies indicate that the selectivity of AS605240 for p110γ is greater at 2 μM than at 10 μM, all subsequent experiments were conducted using

2 μM. Overall, these data indicate that PI3Kγ is required for cell migration downstream of IGF-1R-CXCR4 transactivation in MDA-MB-231 cells.

To rule out the possibility that blocking PI3Kγ with AS605240 directly inhibits the activation of IGF-1R by IGF-I, the lysates of cells either untreated or treated with 2 μM AS605240 were assayed for the level of tyrosine-phosphorylated IGF-1R complex formed in response to various concentrations of IGF-I using the KIRA assay (Chen *et al.*, 2003; Denley *et al.*, 2004; Sadick *et al.*, 1999). The result indicates that activity of IGF-1R was not significantly altered after AS605240 treatment (Figure 3.6) indicating that PI3Kγ is not involved in IGF-I-induced formation of the activated IGF-1R complex in MDA-MB-231 cells.

3.2.5 Effect of p110γ knockdown on IGF-I-induced chemotaxis and IGF-1R activation

To confirmed the results obtained using the p110γ-specific inhibitor, p110γ knockdown cells were used. To generate siRNA-mediated p110γ knockdown cells, MDA-MB-231 cells were transduced with lentiviral vectors expressing either p110γ-siRNA or scrambled siRNA as a control. Knockdown of protein expression was confirmed by immunopreciptation and Western blot analysis (Brazzatti, 2011). Afterwards, these cells were tested for their chemotactic response to IGF-I using the modified Boyden Chamber assay as described above. The chemotactic response of siRNA-mediated p110γ knockdown cells to IGF-I was significantly decreased compared to that of control cells (Figure 3.7), confirming that PI3Kγ is involved in the cell migration in response to IGF-I.

The effect of siRNA-mediated p110γ knockdown on IGF-1R activation in MDA-MB-231 cells was also determined. Using the KIRA assay, the lysates from p110γ knockdown cells and control cells were tested for the levels of tyrosine-phosphorylated IGF-1R complex formed in response to various concentrations of IGF-I. As observed with the p110γ-specific inhibitor, there was no significant difference in the levels of activated IGF-1R between p110γ knockdown cells and control cells (Figure 3.8), indicating that p110γ

knockdown does not affect the IGF-I mediated formation of activated IGF-1R complex in MDA-MB-231 cells.

3.2.6 Effect of PI3Kγ on Akt activation in response to IGF-I

One of the earliest detectable events occuring downstream of PI3K activation is phosphorylation of Akt. In fact, Akt phosphorylation on S473 is often used as a surrogate readout of PI3K activation. Therefore, Akt phosphorylation upon IGF-1R-CXCR4 transactivation in response to IGF-I was investigated by Western blot analysis using phospho-Akt antibody. Cell lysates from MDA-MB-231 cells pretreated with AS605240 and stimulated with IGF-I for an increasing period of time were subjected to Western blot analysis. The level of inducible phospho-Akt in response to IGF-I was significantly decreased after AS605240 treatment (Figure 3.9).

The effect of PI3Kγ in Akt phosphorylation was further investigated using p110γ knockdown cells. The p110γ knockdown cells and control cells were stimulated with IGF-I for an increasing period of time and then tested for the level of phosphorylated Akt using Western blot analysis. As observed with experiments using AS605240, loss of p110γ significantly reduced Akt phosphorylation in response to IGF-I (Figure 3.10).

3.2.7 Effect of CXCR4 on PI3Kγ activation in response to IGF-I

As previously reported CXCR4 activation is required in IGF-I-induced migration of MDA-MB-231 cells (Akekawatchai *et al.*, 2005). To determine whether CXCR4 is essential for activation of PI3Kγ by IGF-I, the effect of AS605240 on chemotaxis of MDA-MB-231 cells in response to IGF-I was assessed in cells in which CXCR4 had been knocked down by siRNA. CXCR4 knockdown cells were produced using a retrovirus expressing either RNAi specific to CXCR4 or target sequences from Renilla luciferase as negative control. The target site for CXCR4 was 21 nucleotides at the position of 470 to 490 of human CXCR4 cDNA sequence (5 GGT GGT CTA TGT TGG CGT CTG 3). The oligonucletides containing specific sequences for *Renilla* luciferase were used to produce an expression vector for the negative control (5 AAA CAU GCA GAA AAU GCU G 3) (Elbashir *et al.* 2001).

Individual clones were characterized for CXCR4 surface expression by flow cytometry analysis (C. Akekawatchai, unpublished data). These cells were pretreated with AS605240 and then tested for their ability to migrate towards various concentration of IGF-I. AS605240 failed to alter the chemotactic response of the cells to IGF-I in CXCR4 deficient cells (Figure 3.11), indicating that CXCR4 is required for PI3Kγ activation in response to IGF-I.

The essential role of CXCR4 in the activation of PI3Kγ/Akt in response to IGF-I was further confirmed by Western blot analysis. The level of phosphorylated Akt induced by IGF-I was decreased in CXCR4 knockdown cells compared to the control at all time points tested (Figure 3.12). To further confirm the role for CXCR4 in PI3Kγ/Akt signaling downstream of IGF-I, the effect of AS605240 on the level of phospho-Akt in MCF-7 cells, which has been reported not express functional CXCR4 (Akekawatchai *et al.*, 2005) was examined. As shown in Figure 3.13, AS605240 failed to alter the level of phosphorylated Akt after IGF-I stimulation in these cells.

3.2.8 MAPK/Erk signaling in response to IGF-I

Apart from PI3K, MAPK/Erk has been previously implicated in signaling transduction involving growth factor and chemokine receptors to regulate cell migration (Du *et al.*, 2010; Li *et al.*, 2010b; Taylor *et al.*, 2010). To determine whether it plays a role in IGF-I-induced migration of MDA-MB-231 cells, Erk1/2 phosphorylation upon IGF-1R-CXCR4 transactivation in response to IGF-I was investigated by Western blot analysis using phospho-Erk1/2 antibodies. Cell lysate from MDA-MB-231 cells stimulated with IGF-I for an increasing period of time were analyzed in Western blot to detect the level of phosphorylated Erk. As shown in Figure 3.14, constitutive activation of Erk was observed in these cells and this constitutive activation could not be elevated further by stimulation with IGF-I. Western blotting for β-actin in the lysates demonstrated equal protein loading.

3.3 Summary

In the experiments described in this chapter, analysis of expression and function of PI3Kγ in breast cancer cell migration was addressed in order to investigate downstream signaling following IGF-1R-CXCR4 transactivation. The data show that highly-metastatic MDA-

MB-231 cells expressed both PI3Kδ and PI3Kγ. However, blocking of PI3Kδ activity with the isoform-specific inhibitor, IC87114 failed to affect the chemotactic ability of these cells, suggesting PI3Kδ is not involved in IGF-I-induced migration of MDA-MB-231 cells. In contrast, PI3Kγ is activated by IGF-I as the catalytic subunit p110γ was translocated to cell membrane in response to IGF-I stimulation. Moreover, inhibition of PI3Kγ using either isoform-specific inhibitor, AS605240 or siRNA-mediated knockdown of p110γ led to a significant decrease in the chemotactic response to IGF-I and the decreased level of phosphorylated-Akt, without affecting IGF-I-induced IGF-1R activation. Furthermore, the inhibition effect of AS605240 on chemotactic response to IGF-I was not observed in RNAi-mediated CXCR4 knockdown cells, indicating that IGF-1R-CXCR4 transactivation is required in PI3Kγ activation. Compared to PI3Kγ, Erk1/2 was constitutively activated in both control and p110γ knockdown cells and this constitutive activation could not be elevated further by stimulation with IGF-I.

In conclusion, the data presented in this chapter indicate that PI3Kγ regulates cell migration in response to IGF-I downstream of IGF-1R-CXCR4 transactivation in MDA-MB-231 cells.

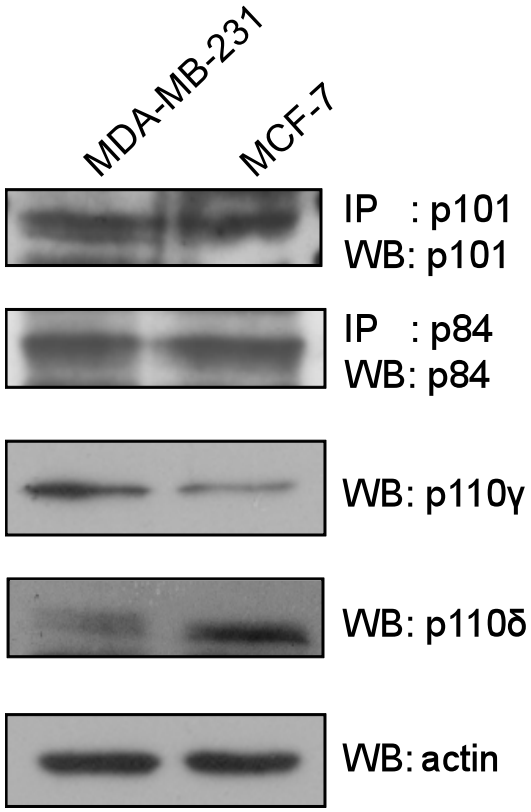

Figure 3.1: Western blot analysis of expression of PI3K isoforms in MDA-MB-231 and MCF-7 cells.

Lysates from MDA-MB-231 and MCF-7 cells were subjected to immunoprecipitation using anti-p101 or anti-p84 followed by SDS-PAGE and Western blot to detect p101 and p84, respectively. Whole lysates from the breast cancer cells were used for Western blot analysis for p110γ and p110δ detection. These data are representative of at least 3 independent experiments performed with the similar results.

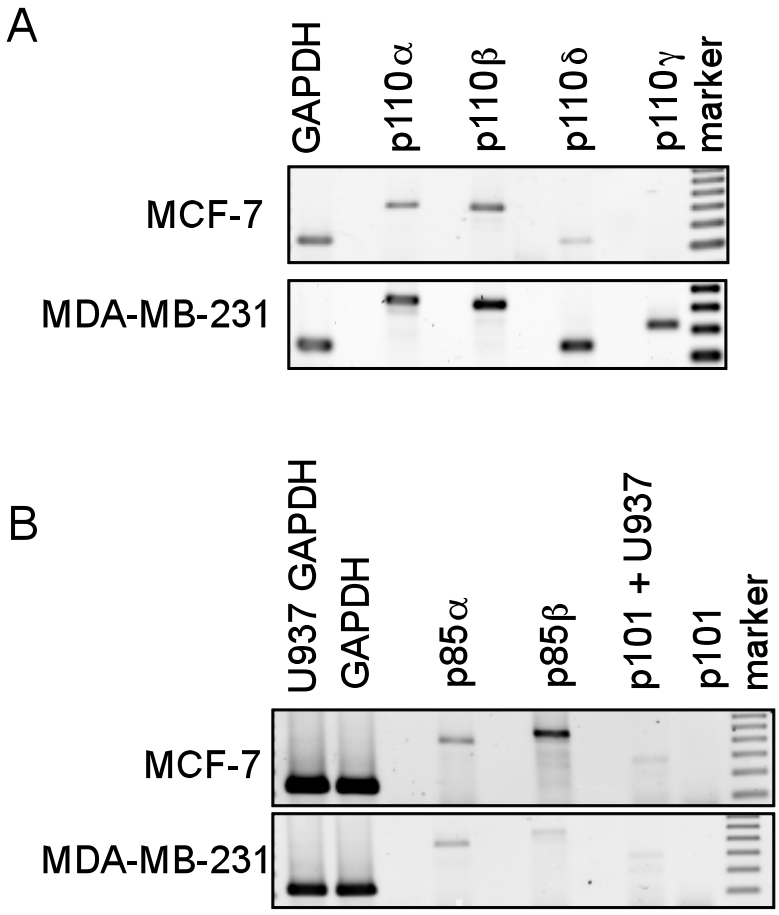

Figure 3.2: PCR analysis of class I PI3K isoform expression in MDA-MB-231 and MCF-7 cells.

RNA was extracted from breast cancer cell lines, DNase-treated and reverse-transcribed. Full-length oligonucleotide primers were used to amplify the complete gene products of the catalytic or regulatory subunits of PI3Ks, the GAPDH housekeeping gene control was included as a loading control. The PCR-amplified products were resolved on a 2.0% agarose gel and detected by ethidium bromide staining. The data shown are representative of 2 independent experiments performed with similar results (C. Akekawatchai, unpublished data).

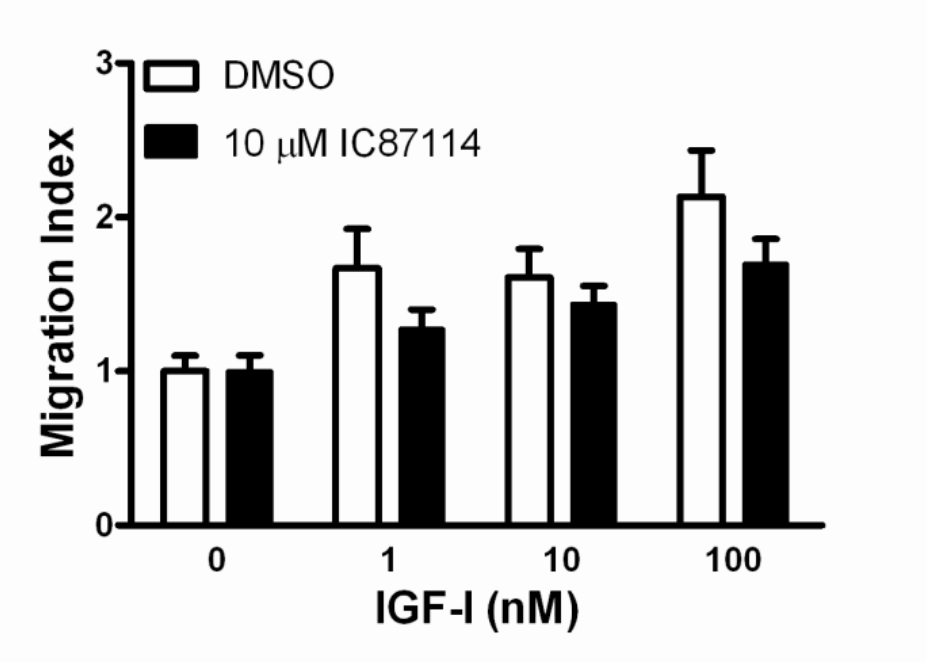

Figure 3.3: Effect of the p110δ inhibitor, IC87114, on IGF-I-induced chemotaxis in MDA-MB-231 cells.

MDA-MB-231 cells were treated with DMSO or 10 μM IC87114 for 1 hour prior to testing their chemotactic response to various concentrations of IGF-I using a 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 the mean ± S.E.M. of the migration index from at least three separate experiments, each performed in triplicate.

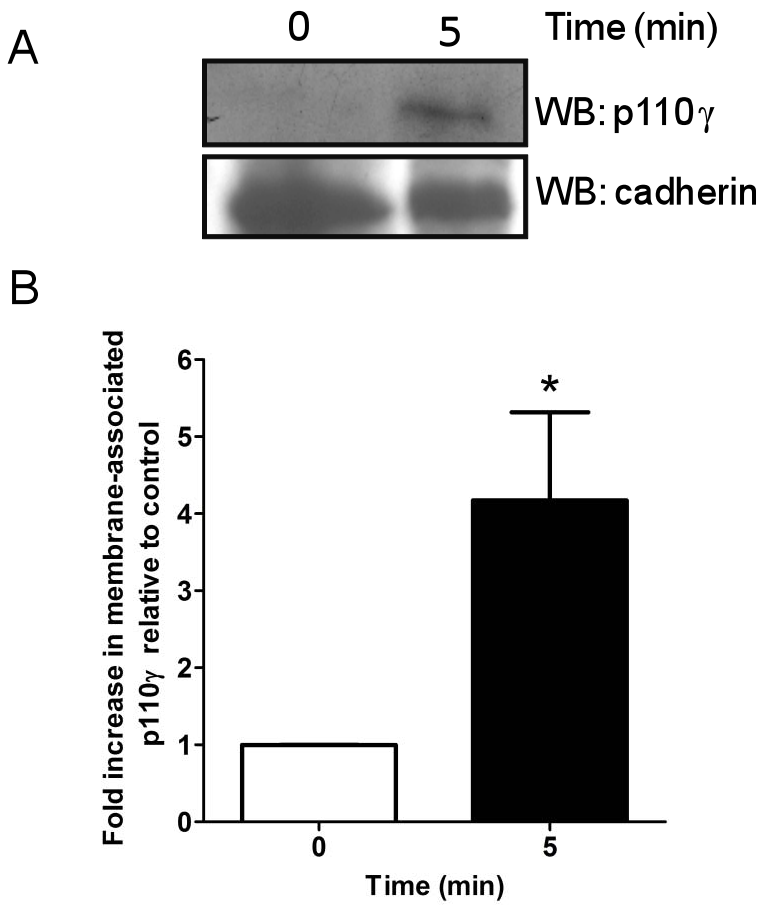

Figure 3.4: Translocation of p110γ to the cell membrane after IGF-I stimulation in MDA-MB-231 cells.

(A) MDA-MB-231 cells were incubated in serum-free medium for 1 hour before being stimulated with 0.1 nM IGF-I for 5 minutes. Cell membrane fractions were obtained as described in Materials and Methods, followed by SDS-PAGE and Western blot analysis, pan-cadherin was included as the loading control. These data are representative of 3 independent experiments performed with similar results. (B) Quantitation of Western blot densitometry from panel A. The level of p110γ was quantified by densitometry, normalized according to the level of pan-cadherin, and the level present in the membrane fraction following stimulation of the cells with IGF-I was expressed as fold-increase relative to the unstimulated level (mean ± S.E.M. of three independent experiments). Asterisks indicate significantly different from the control values (Student's unpaired t test) at *, $p < 0.05$.

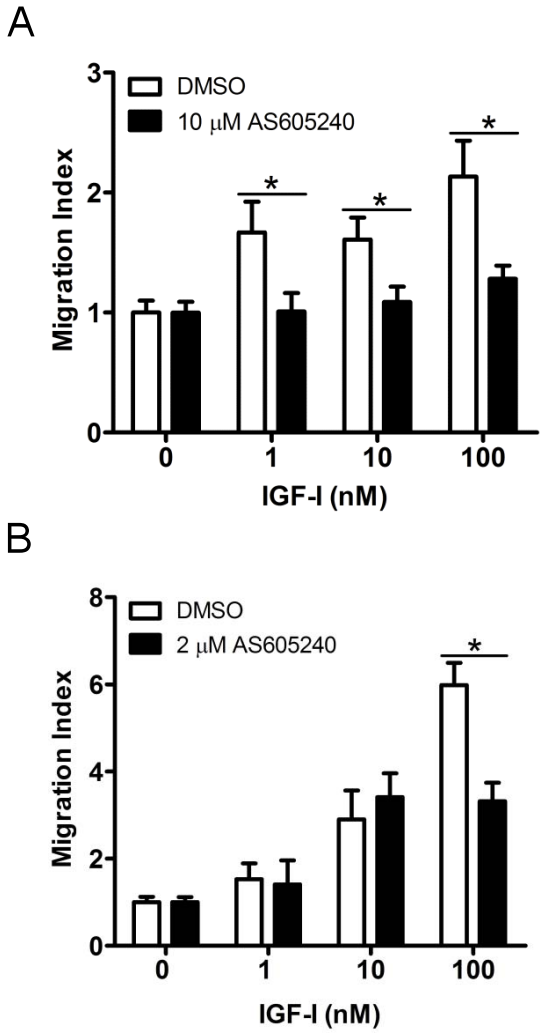

Figure 3.5: Effect of the p110γ inhibitor, AS605240, on the chemotactic response of MDA-MB-231 cells.

MDA-MB-231 cells were treated with DMSO or (A) 10 μM (B) 2 μM AS605240 for 1 hour prior to testing their chemotactic response to various concentrations of IGF-I using a 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 the mean ± S.E.M. of the migration index from at least three separate experiments, each performed in triplicate. Asterisks indicate significantly different from the control values (Student's unpaired t test) at *, $p < 0.05$.

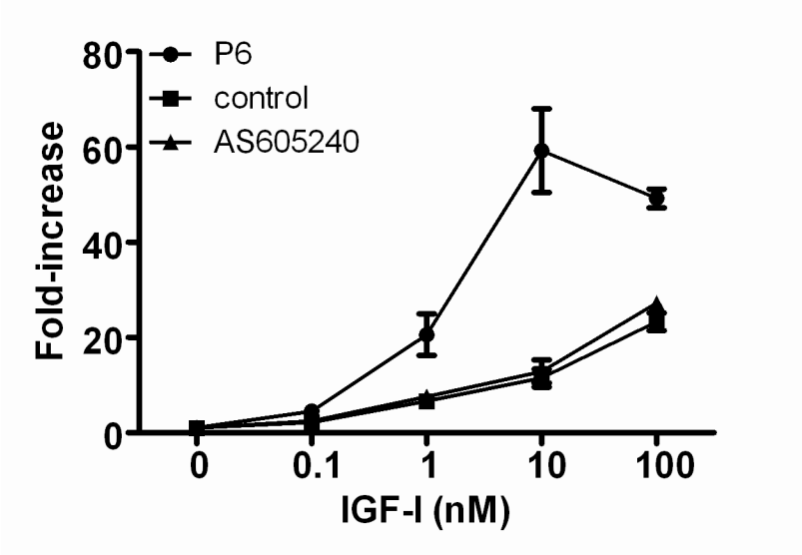

Figure 3.6: Lack of effect of the p110γ inhibitor, AS605240, on IGF-1R phosphorylation induced by IGF-I.

Cells were either untreated or treated with 2 μM AS605240 for 1 hour and the level of tyrosine-phosphorylated IGF-1R complex formed after the stimulation with different doses of IGF-I was quantified using the KIRA assay. Fold-increase represents the level of activated IGF-1R complex in the stimulated cells relative to that observed in the unstimulated cells. Data are presented as the mean ± S.E.M. from at least three independent experiments, each performed in triplicate.

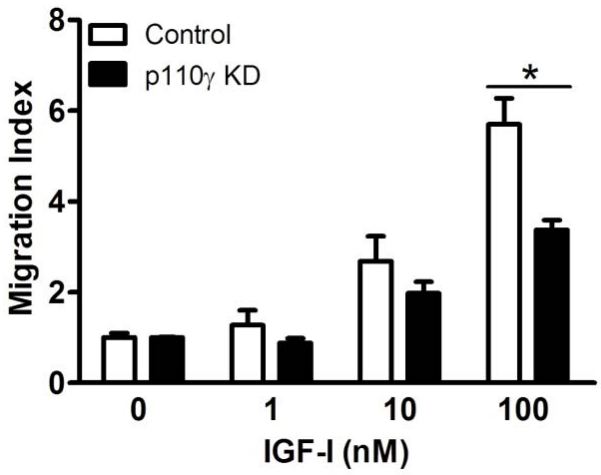

Figure 3.7: Effect of p110γ knockdown on the chemotactic response of MDA-MB-231 cells to IGF-I.

Cells were transfected with the lentivirus producing either siRNA to knock down p110γ or scrambled control siRNA, and knockdown of protein expression was confirmed by immunopreciptation and Western blot analysis (Brazzatti et al, 2011). These cells were evaluated for their chemotactic response to various concentrations of IGF-I using a 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 the mean ± S.E.M. of the migration index from at least three separate experiments, each performed in triplicate. Asterisks indicate significantly different from the control values (Student's unpaired t test) at *, $p < 0.05$.

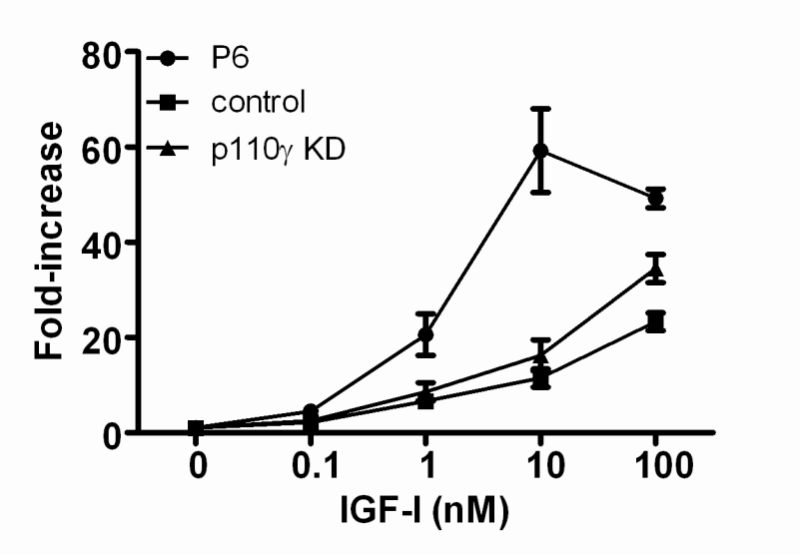

Figure 3.8: Lack of effect of p110γ knockdown on IGF-1R phosphorylation induced by IGF-I.

Cells were serum-starved for 4 hours before the level of tyrosine-phosphorylated IGF-1R complex formed after the stimulation with different doses of IGF-I was qauntified using the KIRA assay. Fold-increase represents the level of activated IGF-1R complex in the stimulated cells relative to that observed in the unstimulated cells. Data are presented as the mean± S.E.M. from at least three independent experiments each performed in triplicate.

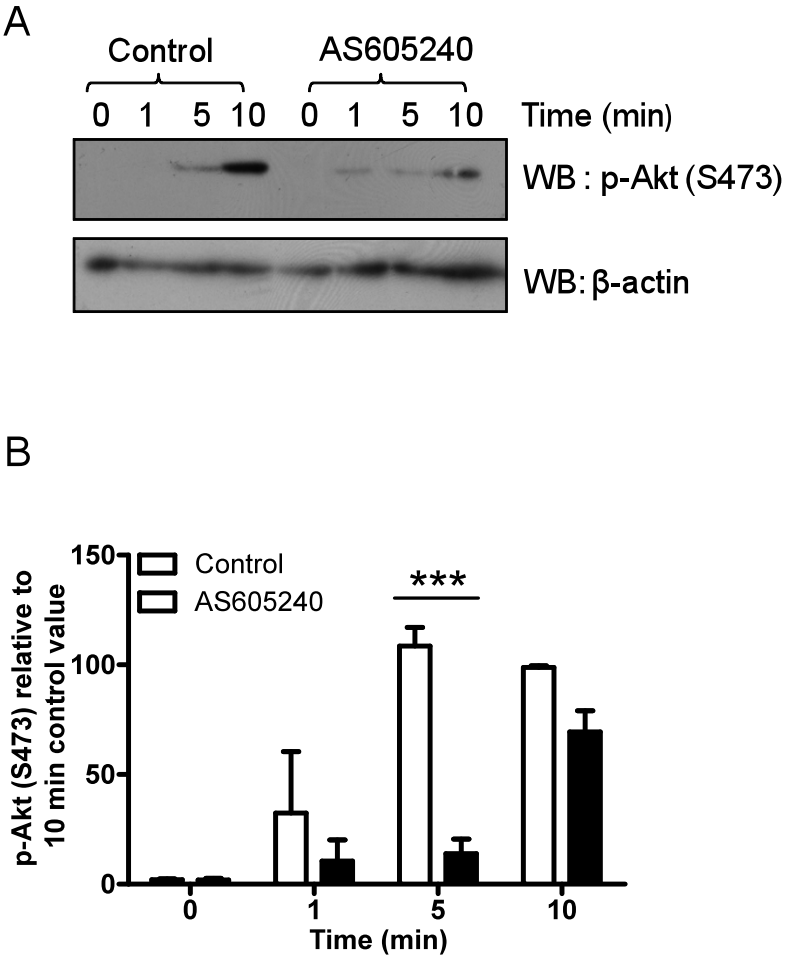

Figure 3.9: Effect of the p110γ inhibitor, AS605240, on Akt activation induced by IGF-I in MDA-MB-231 cells.

(A) MDA-MB-231 cells were either untreated or treated with 2 μM AS605240 for 1 hour before being stimulated with 0.1 nM IGF-I for increasing periods of time 0, 1, 5, and 10 minutes. Cell lysates were prepared and subjected to SDS-PAGE and Western blot to detect phosphorylated Akt. β-actin was included as the loading control. (B) Quantitation of Western blot densitometry from panel A. Akt phosphorylation was quantified by densitometry, normalized to the level of β-actin and expressed as a value relative to the 10 minute control-treated values (mean ± S.E.M. of three independent experiments) as described in Materials and Methods. Asterisks indicate significantly different from the control values (2-way ANOVA with Bonferroni post-test) at ***, $p < 0.001$.

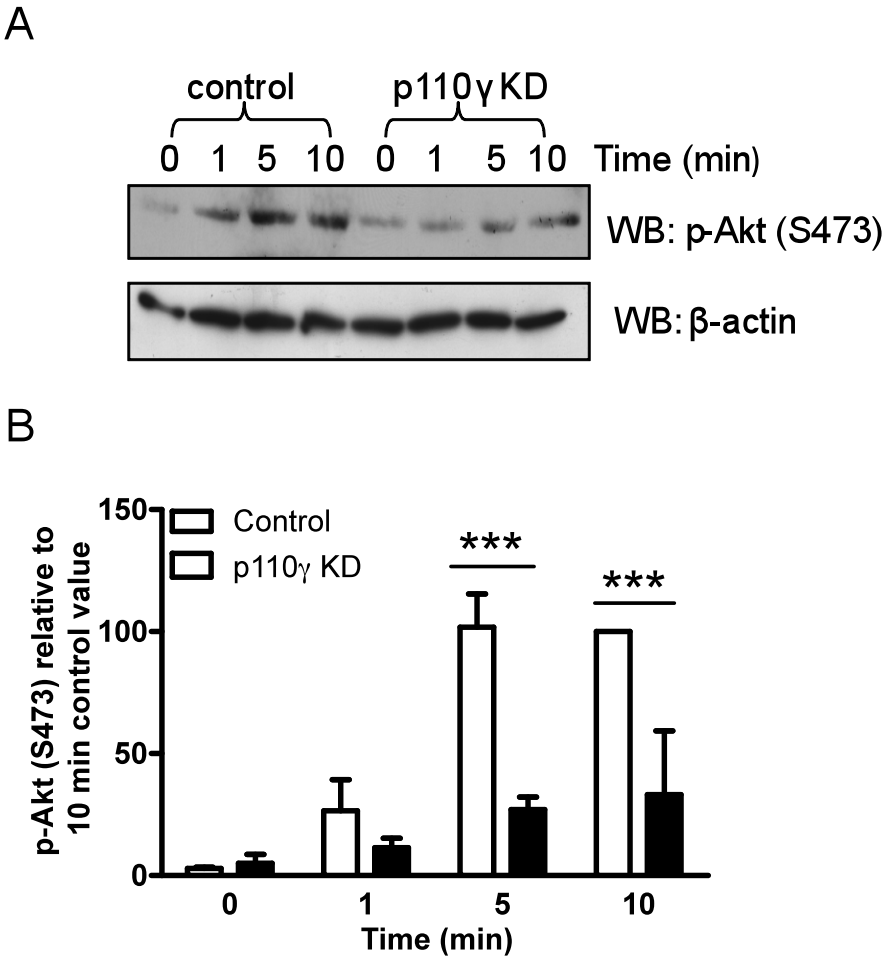

Figure 3.10: Effect of p110γ knockdown on Akt activation induced by IGF-I in MDA-MB-231 cells.

(A) Cells were incubated in serum-free media for 1 hour before being stimulated with 0.1 nM IGF-I for increasing periods of time 0, 1, 5, and 10 minutes. Cell lysates were prepared and subjected to SDS-PAGE and Western blot to detect phosphorylated Akt and β-actin was included as the loading control. (B) Quantitation of Western blot densitometry from panel A. Akt phosphorylation was quantified by densitometry, normalized to the level of β-actin and expressed as a value relative to the 10 minute control-treated values (mean ± S.E.M. of three independent experiments) as described in Materials and Methods. Asterisks indicate significantly different from the control values (2-way ANOVA with Bonferroni post-test) at ***, $p < 0.001$.

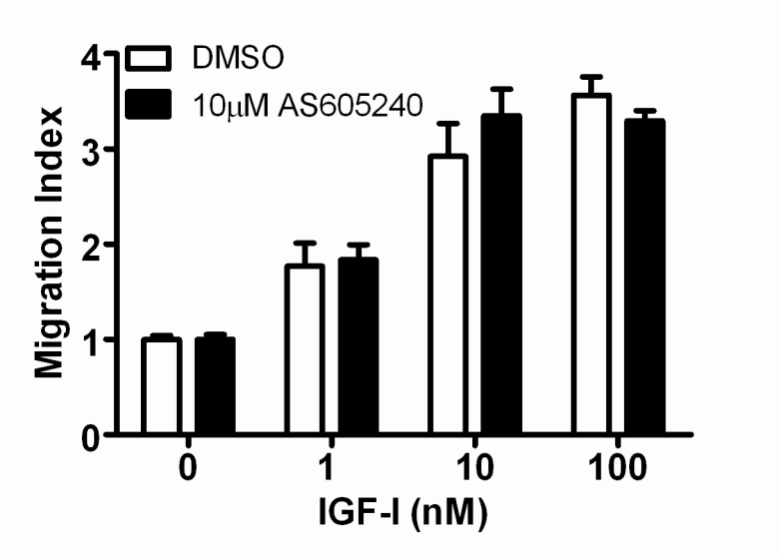

Figure 3.11: Effect of the p110γ inhibitor, AS605240, on IGF-I-induced chemotaxis in CXCR4 knockdown cells

CXCR4 knockdown cells were produced by a retrovirus expressing either RNAi specific to CXCR4 or target sequences from Renilla luciferase as a negative control. Individual clones were characterised for CXCR4 surface expression by flow cytometry (C. Akekawatchai). These cells were treated with DMSO or 2 μM AS605240 for 1 hour prior to testing their chemotactic response to various concentrations of IGF-I using a 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 the mean ± S.E.M. of the migration index from at least three separate experiments, each performed in triplicate.

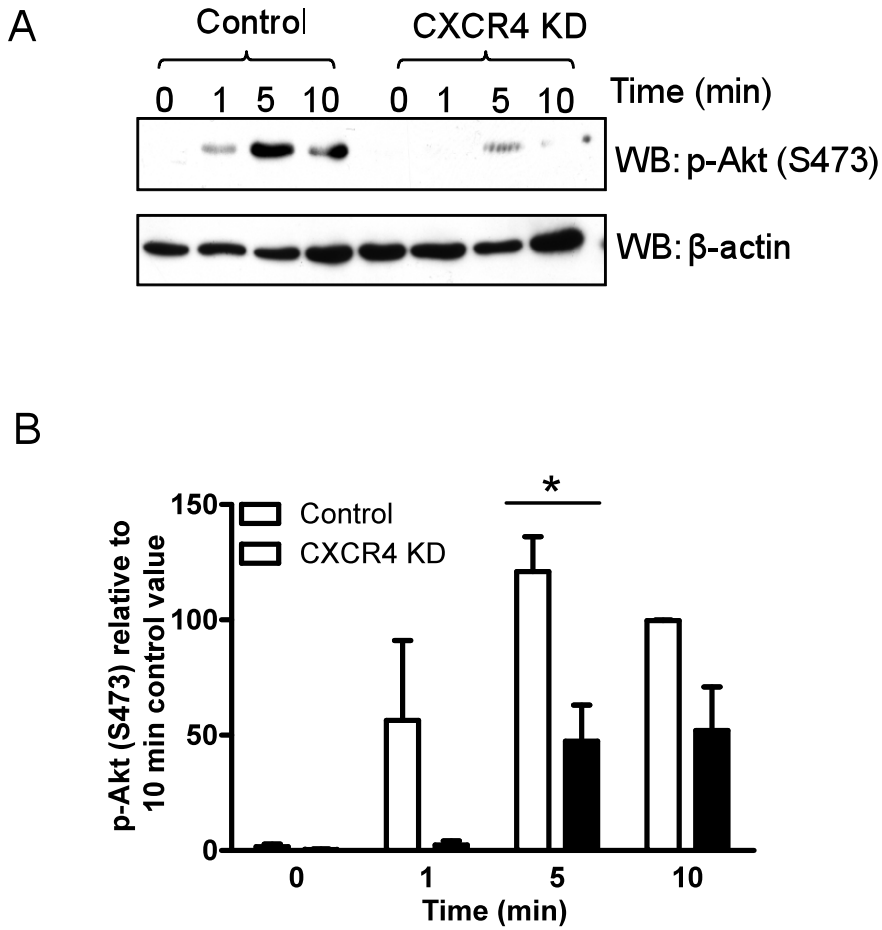

Figure 3.12: Effect of CXCR4 knockdown on Akt activation induced by IGF-I.

(A) Cells were infected with a retrovirus producing either siRNA to knockdown CXCR4 or specific sequences for Renilla Luciferase as a negative control. Cells were incubated in serum-free media for 1 hour before being stimulated with 0.1 nM IGF-I for increasing periods of time 0, 1, 5 and 10 minutes. Cell lysates were prepared and subjected to SDS-PAGE and Western blot to detect phosphorylated Akt. β-actin was included as the loading control. (B) Quantitation of Western blot densitometry from panel A. Akt phosphorylation was quantified by densitometry, normalized to the level of β-actin and expressed as a value relative to the 10 minute control-treated values (mean ± S.E.M. of three independent experiments) as described in Materials and Methods. Asterisks indicate significantly different from the control values (2-way ANOVA with Bonferroni post-test) at *, $p < 0.05$.

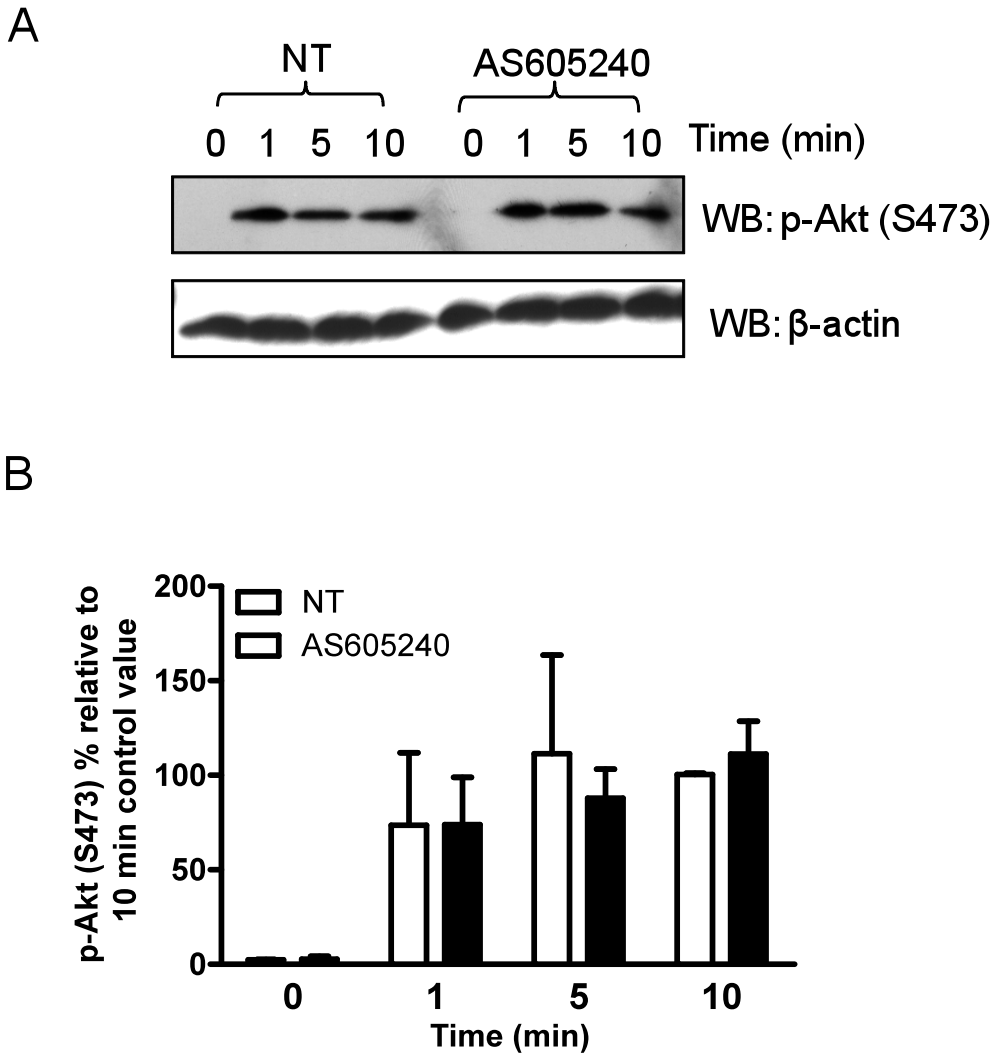

Figure 3.13: Effect of the p110γ inhibitor, AS605240, on Akt activation induced by IGF-I in MCF-7 cells.

(A) MCF-7 cells were either untreated or treated with 2 μM AS605240 for 1 hour before being stimulated with 0.1 nM IGF-I for increasing periods of time 0, 1, 5, and 10 minutes. Cell lysates were prepared and subjected to SDS-PAGE and Western blot to detect phosphorylated Akt, β-actin was included as the loading control. (B) Quantitation of Western blot densitometry from panel A. Akt phosphorylation was quantified by densitometry, normalized to the level of β-actin and expressed as a value relative to the 10 minute control-treated values (mean ± S.E.M. of three independent experiments) as described in Materials and Methods.

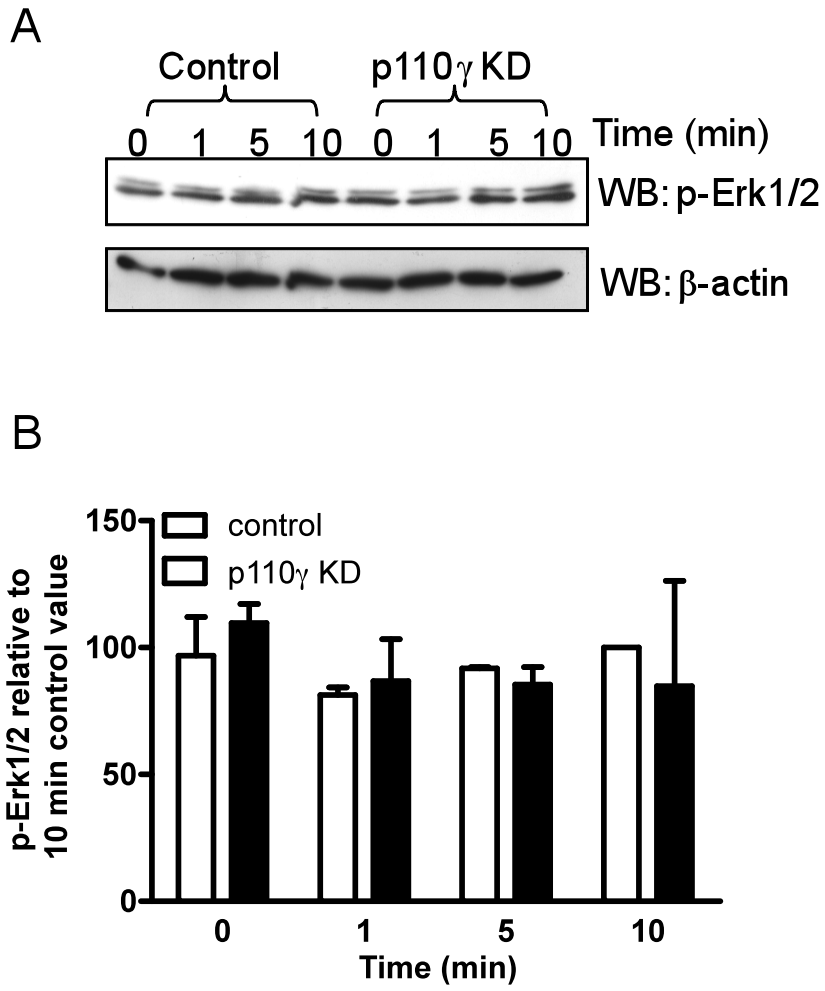

Figure 3.14: Constitutive activation of Erk in MDA-MB-231 cells.

(A) Cells were incubated in serum-free media for 1 hour before being stimulated with 0.1nM IGF-I for increasing periods of time 0, 1, 5, and 10 minutes. Cell lysates were prepared and subjected to SDS-PAGE and Western blot to detect phosphorylated Erk. β-actin was included as the loading control. (B) Quantitation of Western blot densitometry from panel A. Erk1/2 phosphorylation was quantified by densitometry, normalized to the level of β-actin and expressed as a value relative to the 10 minute control-treated values (mean ± S.E.M. of three independent experiments) as described in Materials and Methods.

CHAPTER 4

Identification of PI3Kγ-specific Targets Downstream of IGF-1R-CXCR4 Transactivation in MDA-MB-231 cells Using 2D Fluorescence Differential Gel Electrophoresis (DIGE) and Mass Spectrometry (MS)

Chapter 4: Identification of PI3Kγ-specific targets downstream of IGF-1R-CXCR4 transactivation in MDA-MB-231 cells using 2D Fluorescence Difference Gel Electrophoresis (DIGE) and mass spectrometry (MS)

4.1 Introduction

PI3Ks exert their effects through downstream molecules which trigger a series of signaling cascades to regulate cell survival, growth and migration (Arboleda *et al.*, 2003; Barber & Welch, 2006; Bastian *et al.*, 2006; Cantley, 2002; Engelman *et al.*, 2006; Engelman, 2009; Shukla *et al.*, 2007; Stephens *et al.*, 2005; Wong *et al.*, 2010). A number of molecules involved in PI3K signaling have been shown to be frequently altered in cancers, including Akt/PKB, small GTPases of the Rho and PTEN (Yuan & Cantley, 2008). However, signaling events following PI3K activation are complex and are yet to be fully elucidated. In the previous chapter clear evidence for the expression and functional involvement of PI3Kγ in breast cancer cell migration downstream of IGF-1R-CXCR4 transactivation was established. However, downstream effectors of PI3Kγ remain unknown. As a result, the aim of this chapter was to identify specific targets of PI3Kγ following IGF-1R-CXCR4 transactivation by comparing the differentially-expressed proteins between the control and p110γ knockdown cells and investigate their potential roles in breast cancer cell migration.

Proteomics studies offer the potential to identify proteins, which is essential to the full understanding of molecular events occurring in cancer cells. Rapid and high throughput proteomic technologies are currently being developed and applied to breast cancer research (Wulfkuhle *et al.*, 2001). Among these techniques, 2D Fluorescence Difference Gel Electrophoresis (DIGE) has become a powerful technique to detect and quantify the proteome of paired samples (Unlu *et al.*, 1997). The basis of the technique is to use the distinct excitation and emission spectra of the fluorescent cyanine dyes Cy3, Cy5, and Cy2, which are mass- and charge-matched N-hydroxy succinimidyl ester derivatives. Samples labeled with these Cydyes are mixed on the same gel and proteins are separated according

to the charge in the first dimension by isoelectric focusing (IEF) and size in the second dimension by SDS-PAGE. The samples are then analyzed using fluorescence imaging to detect the difference between the experimental pairs. Published studies using this technique have successfully examined the protein profiles in various tissues and cell lines including cancer cells (Cristea *et al.*, 2004; Friedman *et al.*, 2004; Gharbi *et al.*, 2002; Lee *et al.*, 2003; Seike *et al.*, 2003; Sekhar *et al.*, 2003; Somiari *et al.*, 2003; Von Eggeling *et al.*, 2001; Zhou *et al.*, 2002a). With regards to breast cancer cells, multiple proteins possibly involved in cell transformation mediated by ErbB-2 have been identified using this approach (Gharbi *et al.*, 2002).

Based on the current literature, 2D DIGE is a suitable approach for protein identification. Therefore, it was used to identify specific targets of PI3Kγ following IGF-1R-CXCR4 transactivation. The proteome of control and p110γ-knockdown MDA-MB-231 cells with and without IGF-I stimulation were compared in 2D DIGE and proteins with different expression levels were identified using mass spectrometry analysis.

4.2 Results

4.2.1 Identification of PI3Kγ targets independent of IGF-I stimulation

4.2.1.1 Differential protein abundance in control and p110γ knockdown cells

In order to identify the downstream molecules which are dependent on PI3Kγ expression in MDA-MB-231 cells, the proteomics of control and p110γ knockdown MDA-MB-231 cells were compared in 2D DIGE under resting conditions. Cytosolic fractions from triplicate samples were labelled with Cy3 or Cy5. The pool of these 6 samples was labelled with Cy2, which serves as an internal standard control for normalization and quantitation of the Cy3- and Cy5-labelled samples. These labelled samples were then combined and resolved on 2D electrophoresis and 2D DIGE images were obtained. Gel images of Cy2, Cy3, and Cy5 were scanned using Typhoon Trio at 100 μm resolution (Figure 4.1). Image analysis was undertaken using DeCyder 2D software. Control and p110γ knockdown MDA-MB-231 cells were compared using a two-tailed Student's t-test to detect spots that were differentially expressed. Those spots that returned a p value of <

0.05 were accepted. Up to 427 protein spots were visualized and 10 of them exhibited significant differences in protein abundance (Figure 4.2).

4.2.1.2 Identification of proteins with differential expression in control and p110γ knockdown cells

Protein spots that exhibited significant differences in protein abundance in the p110γ knockdown MDA-MB-231 cells compared to the control cells were selected for automated spot picking and identified by MS (Table 4.1). These spots represented 4 distinct proteins. Spot 122 was identified as pyruvate kinase isozymes M1/M2 (KPYM), an isoenzyme of the glycolytic enzyme pyruvate kinase, expressed in different tissues and all cells, especially tumour cells (Brinck *et al.*, 1994; Corcoran *et al.*, 1976; MacDonald & Chang, 1985; Reinacher & Eigenbrodt, 1981; Schering *et al.*, 1982; Steinberg *et al.*, 1999; Tolle *et al.*, 1976) that catalyzes the last step in glycosis (Vaupel *et al.*, 2004). Spot 222 was identified as phosphoglycerate kinase 1 (PGK1), which is an ATP-generating enzyme in the glycolytic pathway and affects DNA replication and repair (Gavi *et al.*, 2007). Serum albumin protein was present in multiple spots. Keratin, type II cytoskeletal 1 (K2C1) was also identified. Enlarged regions of images and three-dimensional fluorescence intensity profiles of the individual spots of these identified proteins were also shown with the Decyder-matched spots of interest highlighted in Figure 4.3-4.6. Since these identified proteins have not been implicated in PI3K signaling, the focus of the study shifted to identify the differentially-expressed proteins in p110γ knockdown cells in response to IGF-I.

4.2.2 Identification of PI3Kγ targets mediated by IGF-I

4.2.2.1 Differential protein abundance in control and p110γ knockdown cells

To identify downstream events that are dependent on PI3Kγ activation in response to IGF-I, the control and p110γ knockdown cells were stimulated with IGF-I for 5 minutes, conditions that consistently lead to the maximum involvement of PI3Kγ in Akt phosphorylation. In these experiments, the initial focus was on the phosphoproteome. The cytosolic fractions from triplicate samples were labelled with Cy3 or Cy5 and the internal standard control from the pool of these 6 samples was labelled with Cy2. These samples

were combined and resolved as described in the previous section (Figure 4.7) and proteins were analysed using DeCyder 2D software. Results from the 2-D DIGE analysis demonstrated that there were up to 1207 protein spots observed in the gel and 38 of them exhibited significant alteration in protein abundance (Figure 4.8),. Of these 38, 28 spots were upregulated while 10 spots were downregulated in p110γ knockdown MDA-MB-231 cells.

4.2.2.2 Identification of proteins with differential expression in control and p110γ knockdown cells

Thirty-eight of protein spots identified as being either upregulated or downregulated in the p110γ knockdown MDA-MB-231 compared to the control cells were selected for automated spot picking and identified by MS. These spots represented 7 distinct proteins, as some of proteins were present in multiple spots. As shown in Table 4.2, spot 100, 101 and 106 were identified as eukaryotic elongation factor 2 (eEF2), an important translation factor governing protein synthesis. Spot 449 was identified as alpha-enolase (ENOA), a glycolytic enzyme known as an autoantigen associating with several diseases, such as asthma (Nahm *et al.*, 2006) and Hashimoto's encephalopathy (Yoneda *et al.*, 2007). Spot 734 and 746 were identified as L-lactate dehydrogenase A chain (LDHA), an enzyme regulating anaerobic glycolysis. Spot 837 was identified as purine nucleoside phosphorylase (PNPH), an enzyme involved in purine metabolism. KPYM was identified in spot 265, 267, 275 and PGK1 was identified in spot 541, expression of which has also been shown to be altered in p110γ knockdown MDA-MB-231 cells without IGF-I stimulation. Different types of Keratin were also identified in multiple protein spots. Enlarged regions of images and three-dimensional fluorescence intensity profiles of the individual spots of several identified, differentially-expressed proteins were also shown with the Decyder-matched spots of interest highlighted in Figure 4.9-4.14. Among these identified proteins, eEF2 became the focus of further study because of its prior implication in PI3K signaling (Woo & Kim, 2006). The characteristics of the expression pattern of this protein as well as the short stimulation time used in the experiment indicated that the difference observed may be due to alteration of a posttranslational modification. Based on the shift from acidic to basic site of the gel, it is speculated that the protein is less

phosphorylated in the p110γ knockdown cells when compared to the control cells, and the subsequent focus of experimentation was on this aspect.

4.2.3 Regulation of phosphorylation of eEF2 by PI3Kγ after IGF-1R-CXCR4 transactivation

4.2.3.1 Effect of p110γ knockdown on phosphorylation of eEF2 in response to IGF-I

To confirm the involvement of eEF2 in PI3Kγ signaling in MDA-MB-231 cells, cell lysates from the control and p110γ knockdown cells stimulated for increasing periods of time with IGF-I were immunoblotted with anti-phospho-eEF2, followed by stripping and reprobing with total eEF2 antibody. As shown in figure 4.15, phosphorylation of eEF2 induced by IGF-I was attenuated in p110γ knockdown cells compared to that in the control cells, whereas the total eEF2 protein was not affected.

4.2.3.2 Effect of AS605240 on phosphorylation of eEF2 in response to IGF-I

The effect of AS605240 on eEF2 phosphorylation induced by IGF-I in MDA-MB-231cells was also determined. The lysates from parental MDA-MB-231 cells pretreated with the p110γ isoform-specific inhibitor, AS605240 followed by stimulation with IGF-I were immunoblotted with phopho-eEF2 and total eEF2 antibodies. The results demonstrated that the level of phosphorylated eEF2 in response to IGF-I was significantly decreased after AS605240 treatment compared to the control cells (Figure 4.16). Taken together, these data indicate that eEF2 is phosphorylated downstream of activation of the IGF-1R-CXCR4 heterodimer in response to IGF-I and that this is dependent on PI3Kγ.

4.3 Summary

As discussed in the last chapter, PI3Kγ plays a key role in MDA-MB-231 breast cancer cell migration in response to IGF-I upon IGF-1R-CXCR4 transactivation. However, the downstream effectors dependent on PI3Kγ during this process have yet to be determined. To this end, 2D DIGE and MS analysis were performed to identify molecules that are regulated by PI3Kγ. The data indicated that 10 distinctive protein spots showed consistent differences in expression levels in control and p110γ knockdown cells under resting

conditions. Subsequent MS analysis demonstrated that most of the spots were identified as serum albumin while others were pyruvate kinase isozymes M1/M2 (KPYM), phosphoglycerate kinase 1(PGK1) and keratin, type II cytoskeletal 1(K2C1). Since these identified proteins have not been implicated in PI3K signaling, the focus of the study shifted to identify differentially-expressed proteins in p110γ knockdown cells induced by IGF-I. The data indicated that 38 protein spots exhibited significant differences in protein abundance in control and p110γ knockdown cells upon stimulation by IGF-I. These proteins were identified as eukaryotic elongation factor 2 (eEF2), alpha-enolase(ENOA), L-lactate dehydrogenase A chain (LDHA), purine nucleoside phosphorylase (PNPH), keratins, KPYM and PGK1. In view of the previous studies suggesting that eEF2 may be an important molecule downstream of PI3K signaling, eEF2 was further investigated for its involvement in PI3Kγ signaling and breast cancer cell migration. The data of these investigations showed that the level of phosphorylated eEF2 induced by IGF-I was decreased after the blockade of PI3Kγ by either p110γ knockdown or isoform selective inhibitor, AS605240, indicating that PI3Kγ regulates phosphorylation of eEF2 in response to IGF-I in MDA-MB-231 cells.

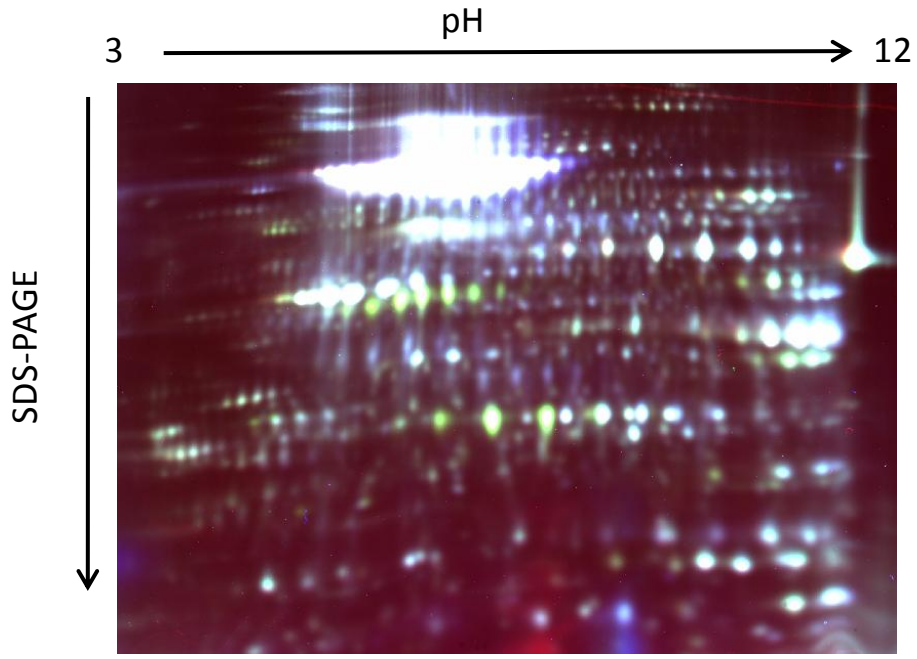

Figure 4.1: Overlay map of DIGE comparing protein abundance in control and p110γ knockdown cells without IGF-I stimulation

Control and p110γ knockdown cells were incubated in serum-free media for 1 hour. Cytosolic fractions were obtained and subjected to 2D clean-up and protein concentration estimation. 50 µg of the total protein from the samples were labelled with Cy3 or Cy5 while the pool of the samples as the internal pooled standard was labelled with Cy2. These samples were then combined and subjected to 2D gel electrophoresis, followed by scanning at 100 µm resolution using the Typhoon Trio. These data are representative of 3 gels run with similar results.

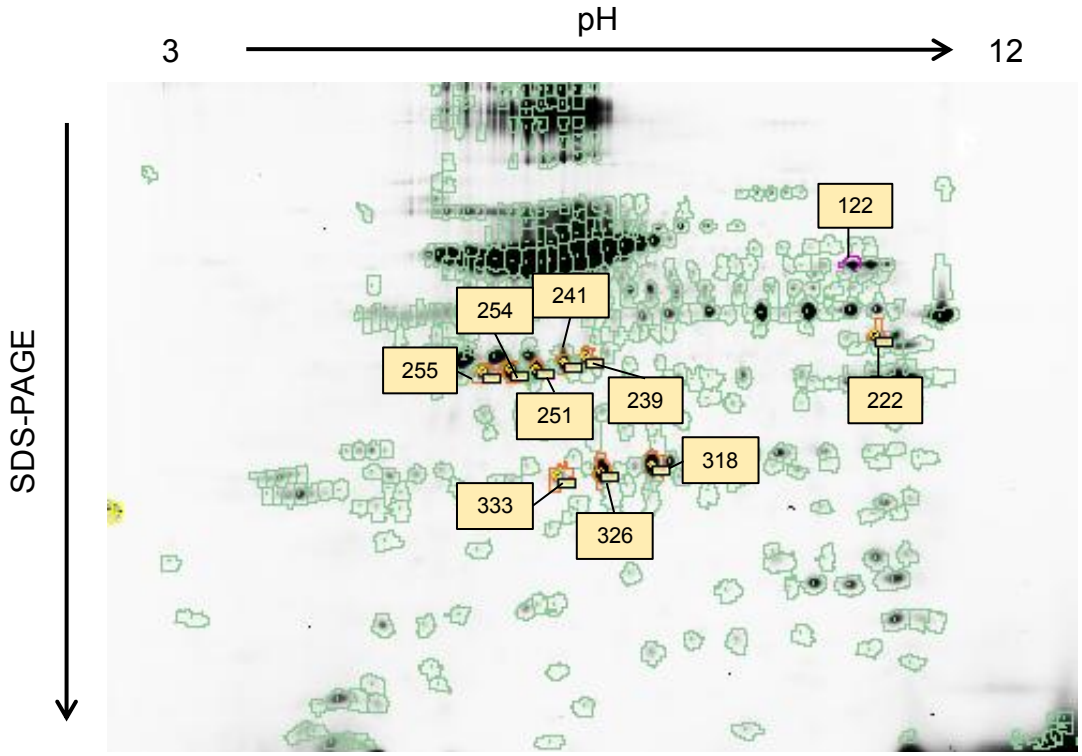

Figure 4.2: Differentially-expressed protein spots identified by 2D-DIGE.
Shown is the Cy5-labeled master gel of the protein fractions from p110γ knockdown MDA-MB-231 cells. Number-labeled rectangles indicate the protein spots with altered abundance. The pH values of the first dimension gel system are indicated on the top.

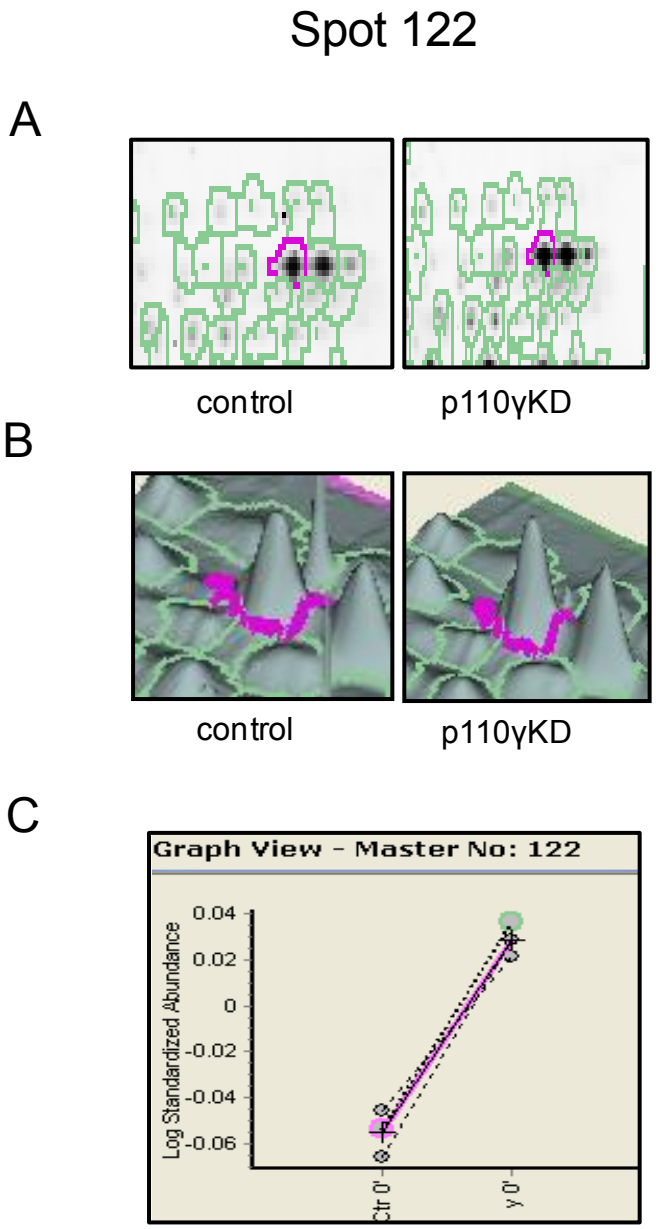

Figure 4.3: Enlarged regions of images and three-dimensional fluorescence intensity profiles of the individual spots of several identified, differentially-expressed proteins.

Image analysis was undertaken using DeCyder 2D software. (A) Spot map of differentially-expressed proteins, the spot circled with pink line represents spot 122. (B) Spot 122 shown in three-dimensional view. (C) Graph view of spot 122. These data are representative of 3 gels run with similar results.

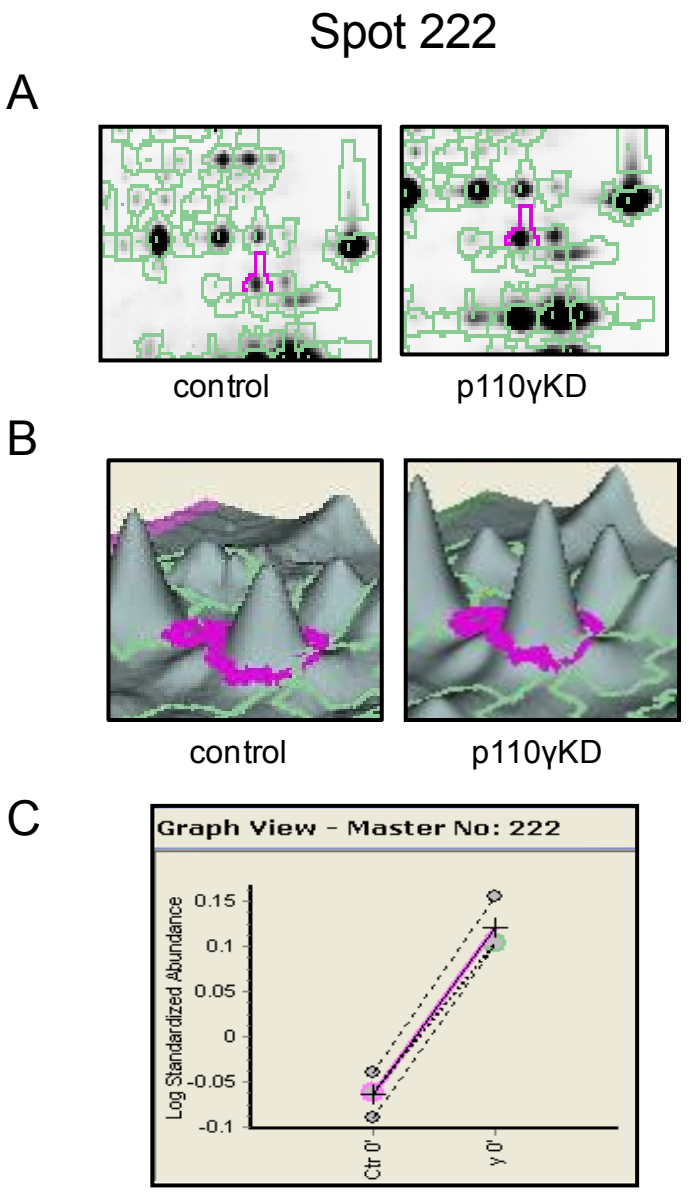

Figure 4.4: Enlarged regions of images and three-dimensional fluorescence intensity profiles of the individual spots of several identified, differentially-expressed proteins.

Image analysis was undertaken using DeCyder 2D software. (A) Spot map of differentially-expressed proteins, the spot circled with pink line represents spot 222. (B) Spot 222 shown in three-dimensional view. (C) Graph view of spot 222. These data are representative of 3 gels run with similar results.

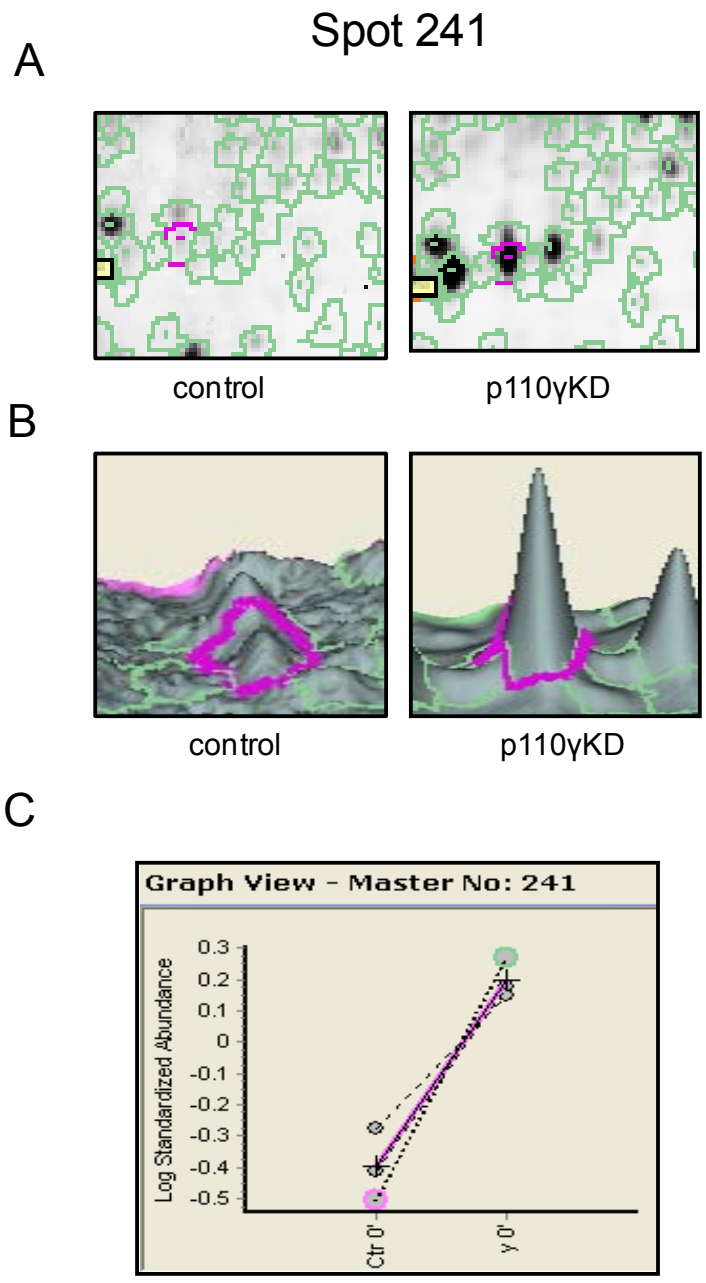

Figure 4.5: Enlarged regions of images and three-dimensional fluorescence intensity profiles of the individual spots of several identified, differentially-expressed proteins.

Image analysis was undertaken using DeCyder 2D software. (A) Spot map of differentially-expressed proteins, the spot circled with pink line represents spot 241. (B) Spot 241 shown in three-dimensional view. (C) Graph view of spot 241. These data are representative of 3 gels run with similar results.

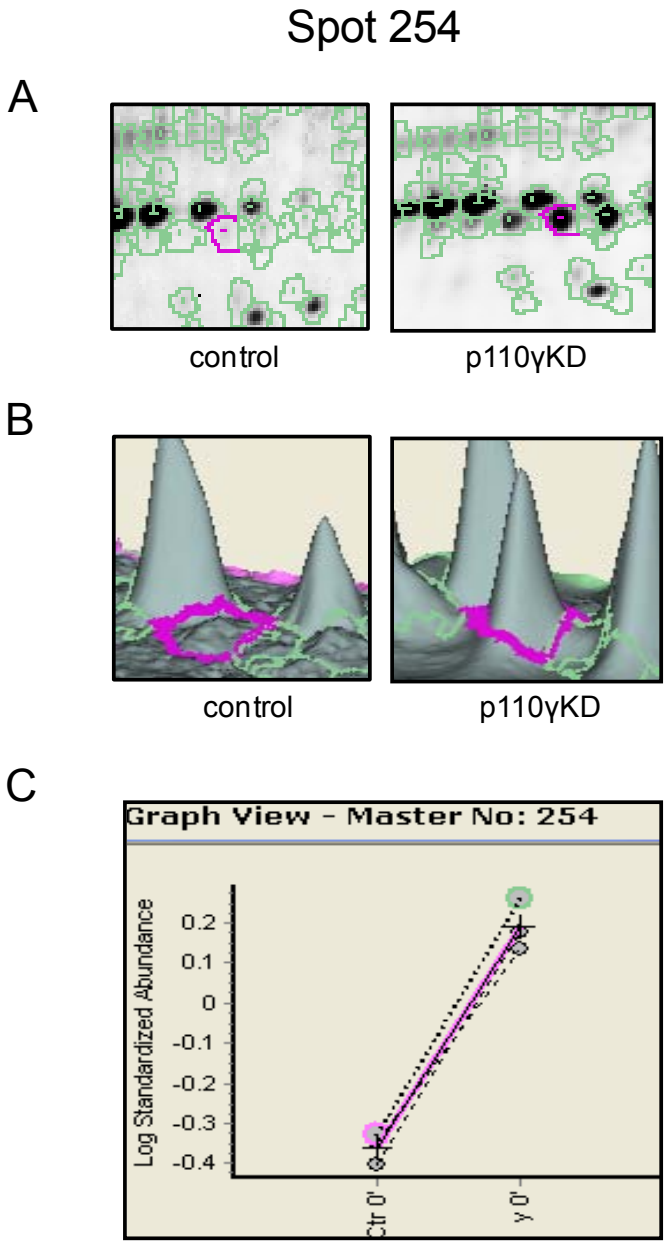

Figure 4.6: Enlarged regions of images and three-dimensional fluorescence intensity profiles of the individual spots of several identified, differentially-expressed proteins.

Image analysis was undertaken using DeCyder 2D software. (A) Spot map of differentially-expressed proteins, the spot circled with pink line represents spot 254. (B) Spot 254 shown in three-dimensional view. (C) Graph view of spot 254. These data are representative of 3 gels run with similar results.

Table 4.1: List of differentially-expressed proteins in control and p110γ knockdown cells without IGF-I stimulation determined by DIGE and MS

Name	Accession	MW (kDA)/pI	Spot No.	Fold change	Mascot search results		
					ID/Total queries	Sequence coverage (%)	Combined IonScore
Pyruvate kinase isozyme M1/M2	KPYM_HUMAN	58.5/7.96	122	1.2	62/566	49	1121
Phosphoglycerate kinase 1	PGK1_HUMAN	45.0/8.30	222	1.5	39/540	44	607

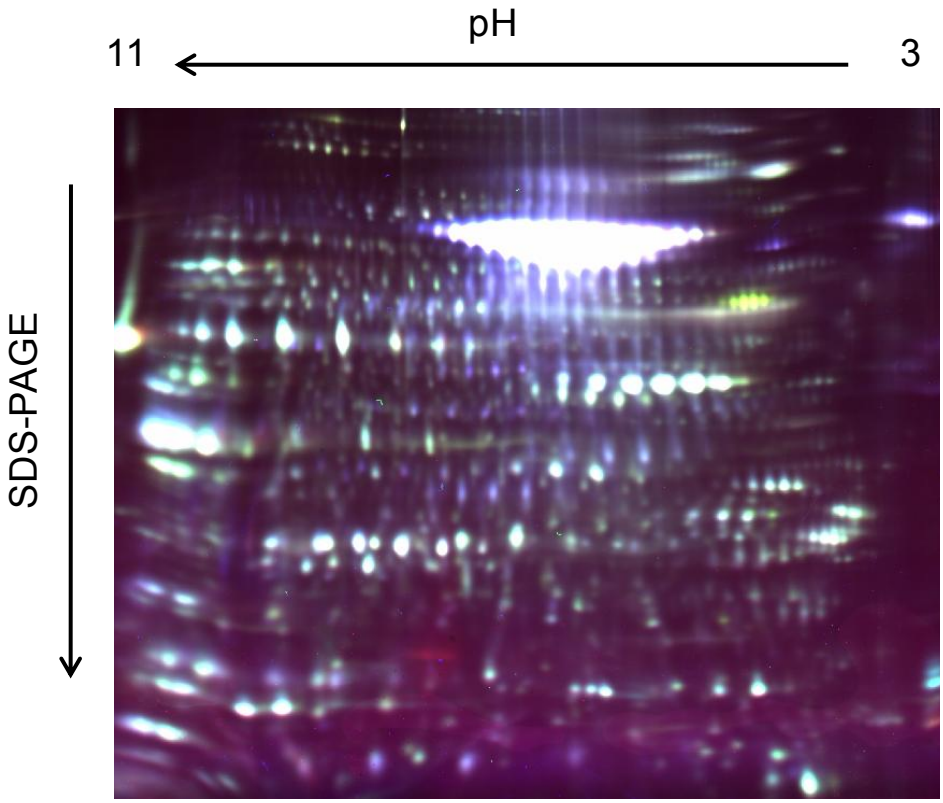

Figure 4.7: Overlay map of DIGE comparing protein abundance in control and p110γ knockdown cells after 5 min IGF-I stimulation

Control and p110γ knockdown cells were incubated in serum-free media for 1 hour. Cytosolic fractions were obtained and subjected to 2D clean-up and protein concentration estimation. 50 µg of the total protein from the samples were labelled with Cy3 or Cy5 while the pool of the samples as the internal pooled standard was labelled with Cy2. These samples were then combined and subjected to 2D gel electrophoresis, followed by scanning at 100 µm resolution using the Typhoon Trio. These data are representative of 3 gels run with similar results.

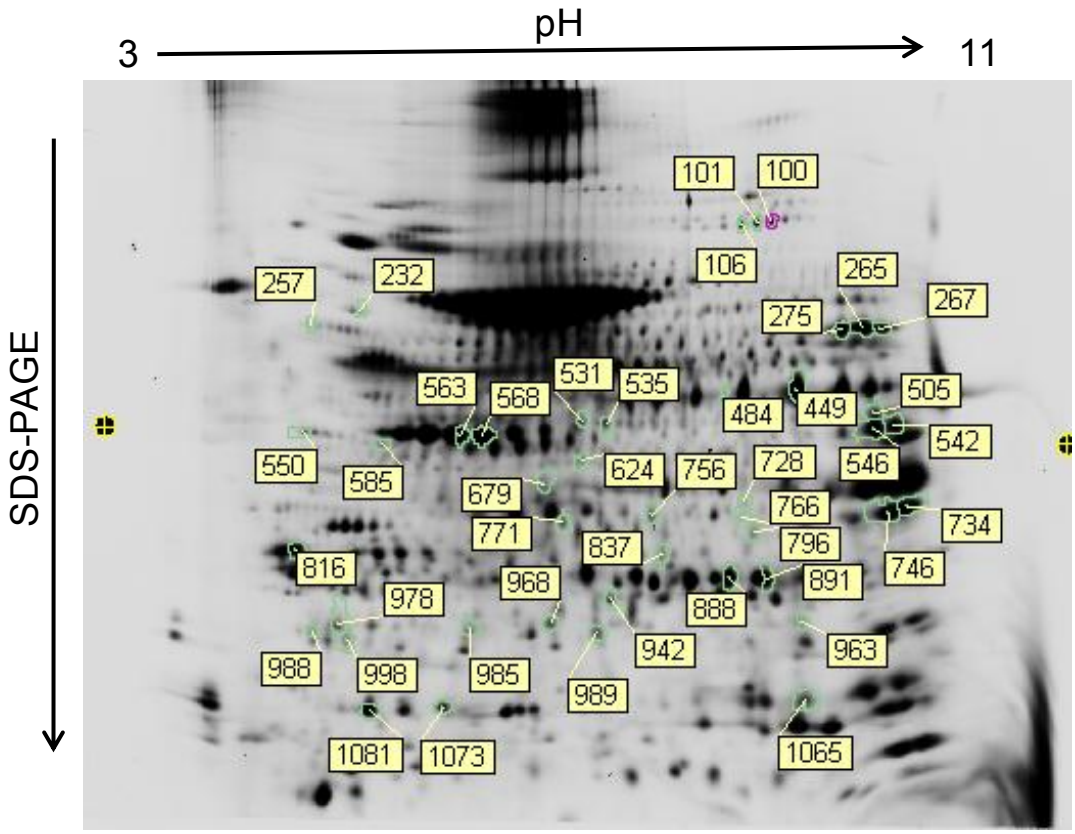

Figure 4.8: Differentially-expressed protein spots identified by 2D-DIGE.

Shown is the Cy5-labeled master gel of the protein fractions from p110γ knockdown MDA-MB-231 cells. Number-labeled rectangles indicate the protein spots with altered abundance. The pH values of the first dimension gel system are indicated on the top.

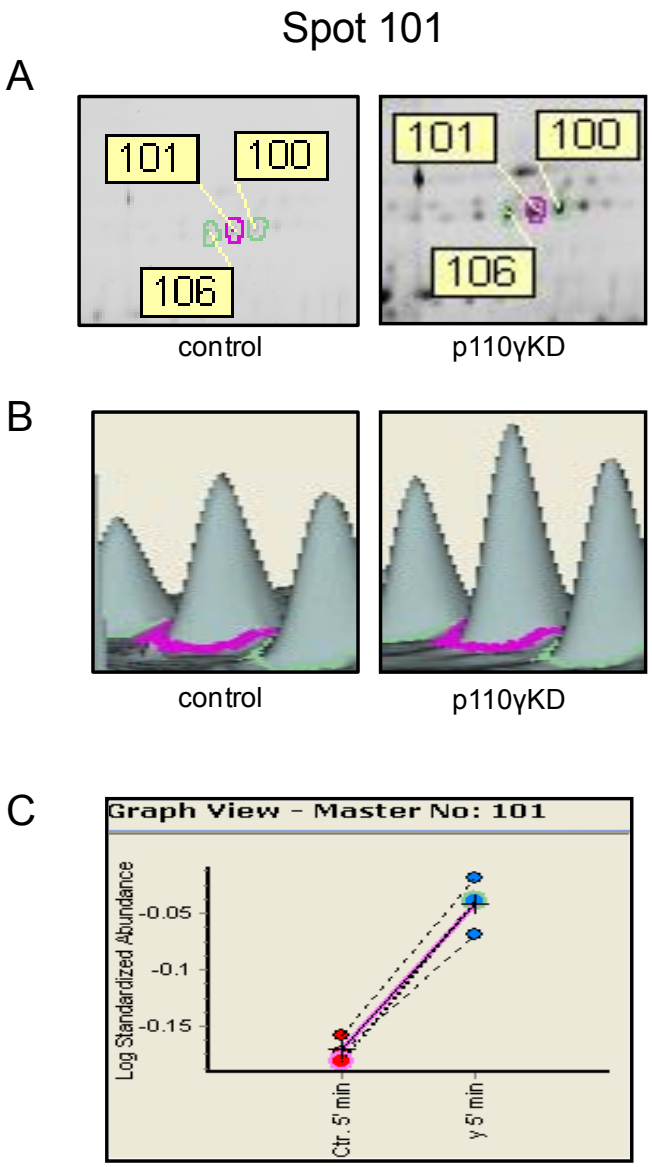

Figure 4.9: Enlarged regions of images and three-dimensional fluorescence intensity profiles of the individual spots of several identified, differentially-expressed proteins.

Image analysis was undertaken using DeCyder 2D software. (A) Spot map of differentially-expressed proteins, the spot circled with pink line represents spot 101. (B) Spot 101 shown in three-dimensional view. (C) Graph view of spot 101. These data are representative of 3 gels run with similar results.

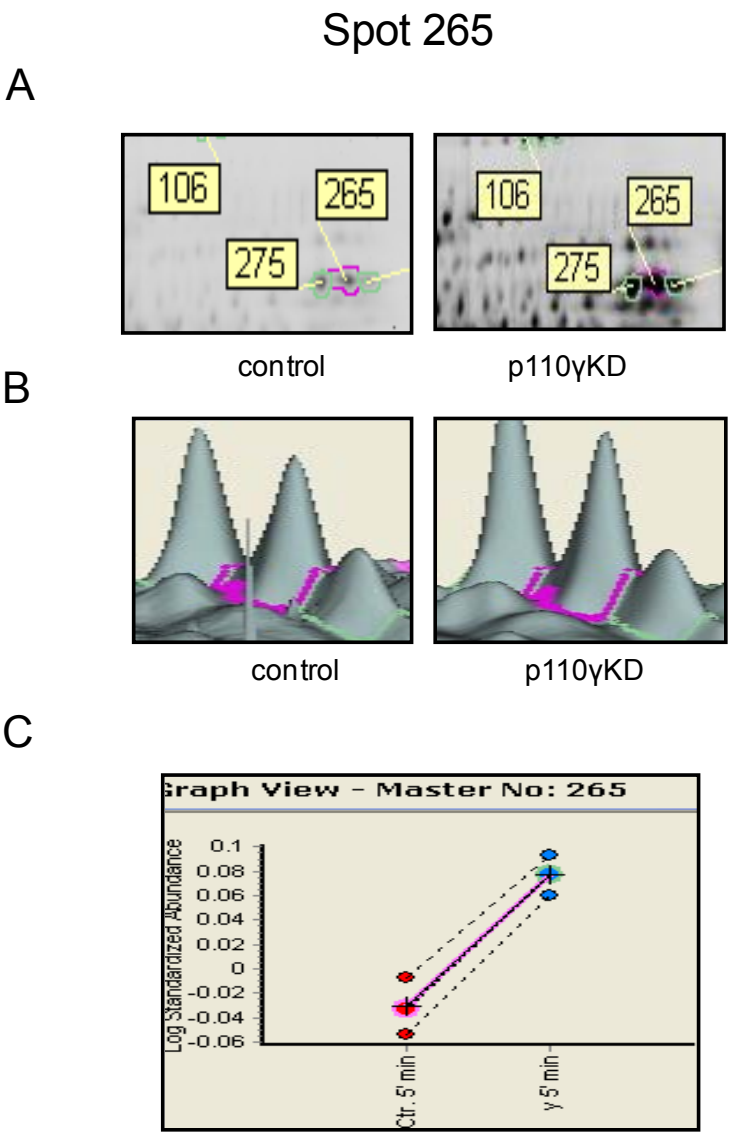

Figure 4.10: Enlarged regions of images and three-dimensional fluorescence intensity profiles of the individual spots of several identified, differentially-expressed proteins.

Image analysis was undertaken using DeCyder 2D software. (A) Spot map of differentially-expressed proteins, the spot circled with pink line represents spot 265. (B) Spot 265 shown in three-dimensional view. (C) Graph view of spot 265. These data are representative of 3 gels run with similar results.

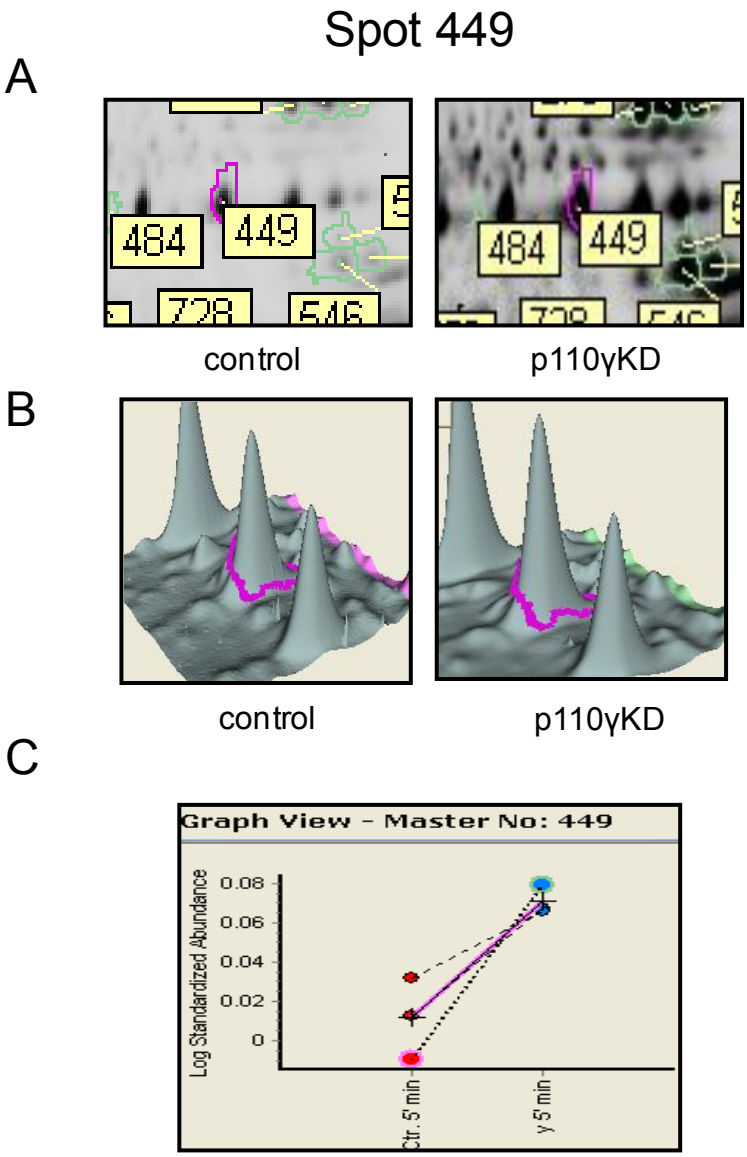

Figure 4.11: Enlarged regions of images and three-dimensional fluorescence intensity profiles of the individual spots of several identified, differentially-expressed proteins.

Image analysis was undertaken using DeCyder 2D software. (A) Spot map of differentially-expressed proteins, the spot circled with pink line represents spot 449. (B) Spot 449 shown in three-dimensional view. (C) Graph view of spot 449. These data are representative of 3 gels run with similar results.

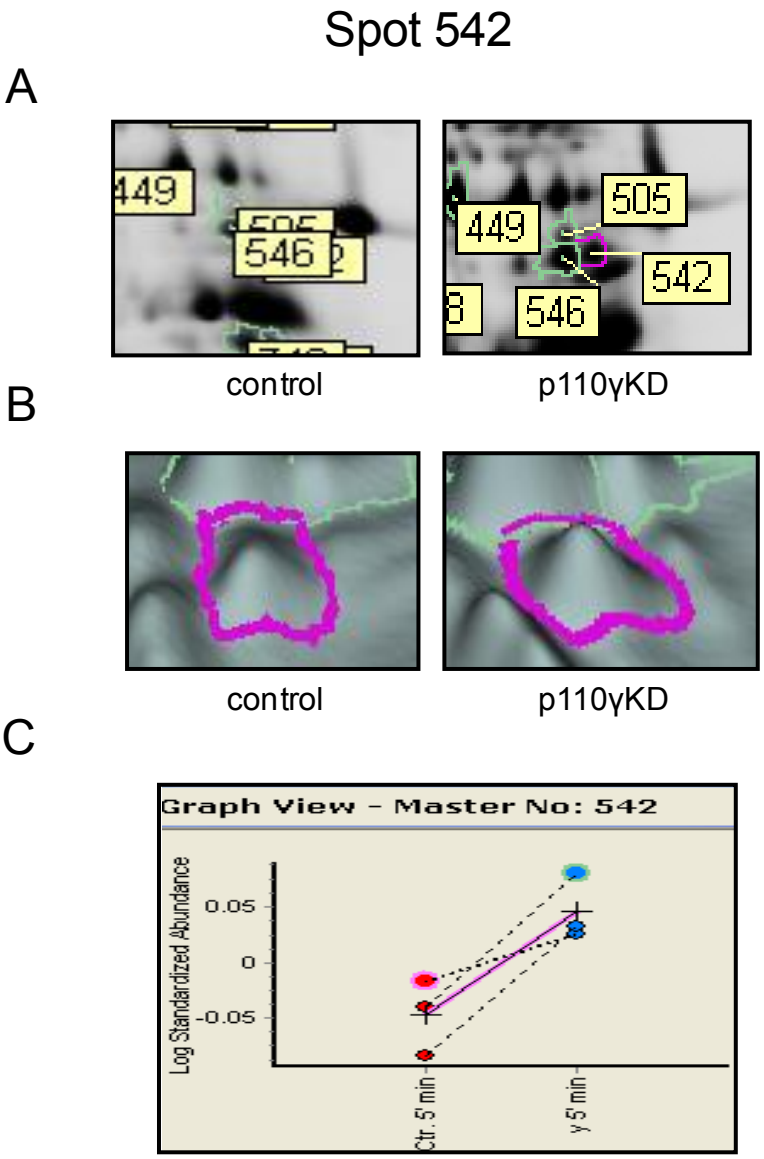

Figure 4.12: Enlarged regions of images and three-dimensional fluorescence intensity profiles of the individual spots of several identified, differentially-expressed proteins.

Image analysis was undertaken using DeCyder 2D software. (A) Spot map of differentially-expressed proteins, the spot circled with pink line represented spot 542. (B) Spot 542 shown in three-dimensional view. (C) Graph view of spot 542. These data are representative of 3 gels run with similar results.

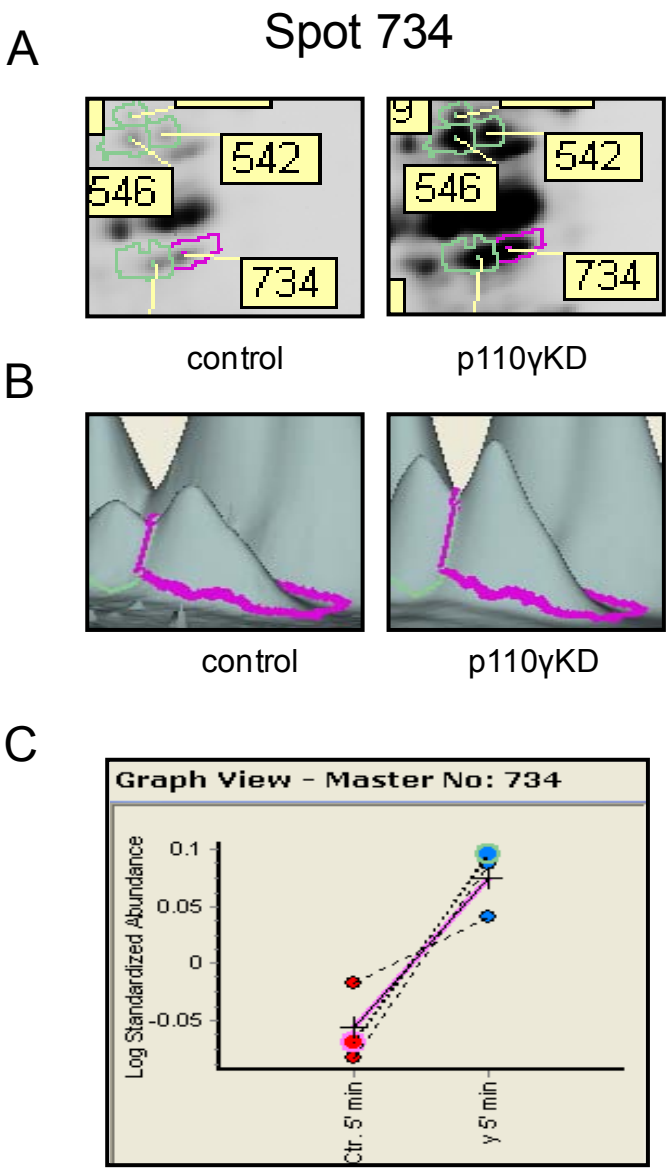

Figure 4.13: Enlarged regions of images and three-dimensional fluorescence intensity profiles of the individual spots of several identified, differentially-expressed proteins.

Image analysis was undertaken using DeCyder 2D software. (A) Spot map of differentially-expressed proteins, the spot circled with pink line represents spot 734. (B) Spot 734 shown in three-dimensional view. (C) Graph view of spot 734. These data are representative of 3 gels run with similar results.

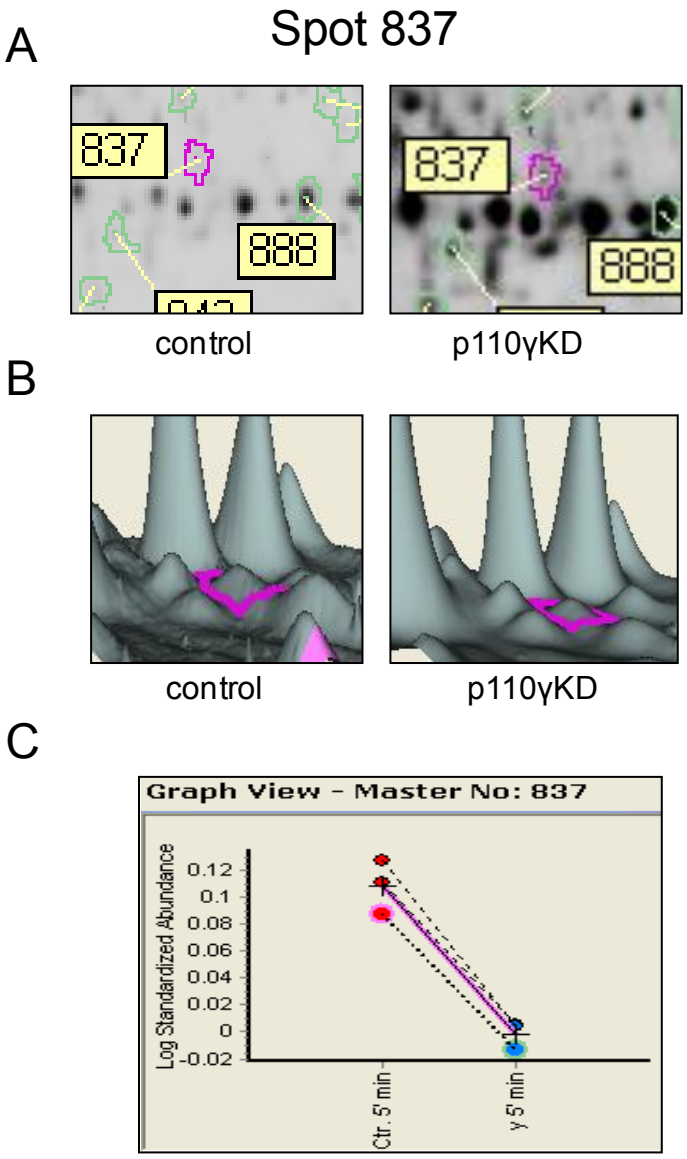

Figure 4.14: Enlarged regions of images and three-dimensional fluorescence intensity profiles of the individual spots of several identified, differentially-expressed proteins.

Image analysis was undertaken using DeCyder 2D software. (A) Spot map of differentially-expressed proteins, the spot circled with pink line represents spot 837. (B) Spot 837 shown in three-dimensional view. (C) Graph view of spot 837. These data are representative of 3 gels run with similar results.

Table 4.2: List of differentially-expressed proteins between control and p110γ knockdown cells after 5 min IGF-I stimulation determined by DIGE and MS

Name	Accession	MW (kDA)/pI	Spot No.	Fold change	Mascot search results		
					ID/Total queries	Sequence coverage (%)	Combined IonScore
Eukaryotic elongation factor 2	eEF2_HUMAN	96.2/6.4	100	1.4	22/545	18	395
			101	1.3	14/547	16	215
			106	1.3	10/534	9	137
Pyruvate kinase isozyme M1/M2	KPYM_HUMAN	58.5/8.0	265	1.3	45/462	41	882
			267	1.3	52/560	47	1112
			275	1.3	78/572	69	1471
Alpha-enolase	ENOA_HUMAN	47.5/7.0	449	1.2	27/481	45	556
Phosphoglycerate kinase 1	PGK1_HUMAN	45.0/8.3	542	1.2	17/519	34	387
L-lactate dehydrogenase A chain	LDHA_HUMAN	37.0/8.4	734	1.4	11/603	23	185
			746	1.4	8/507	15	112
Purine nucleoside phosphorylase	PNPH_HUMAN	32.3/6.4	837	-1.3	3/585	9	63

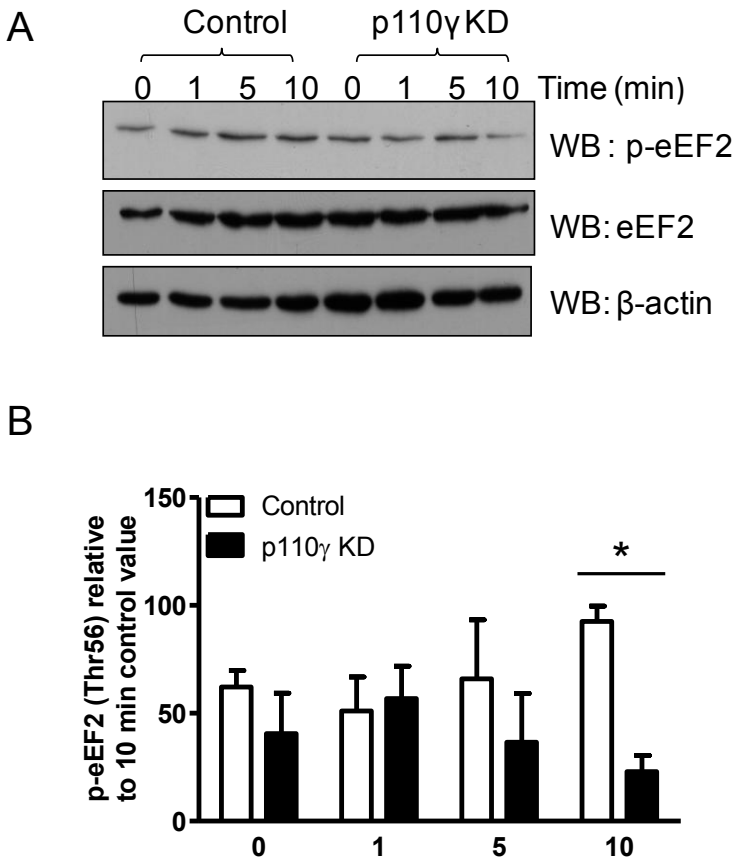

Figure 4.15: Effect of p110γ knockdown on phosphorylation of eEF2 induced by IGF-I in MDA-MB-231 cells.

(A) Cells were incubated in serum-free medium for 1 hour before being stimulated with 0.1 nM IGF-I for increasing periods of time. Cell lysates were prepared and subjected to SDS-PAGE and Western blot to detect phosphorylated eEF2. The filters were stripped and reprobed to detect total eEF2. β-actin was included as the loading control. (B) Quantitation of Western blot densitometry from panel A. eEF2 phosphorylation was quantified by densitometry, normalized to the level of β-actin and expressed as a value relative to the 10 minute control-treated values (mean ± S.E.M. of three independent experiments) as described in Materials and Methods. Asterisks indicate significantly different from the control values (2-way ANOVA with Bonferroni post-test) at *, $p < 0.05$.

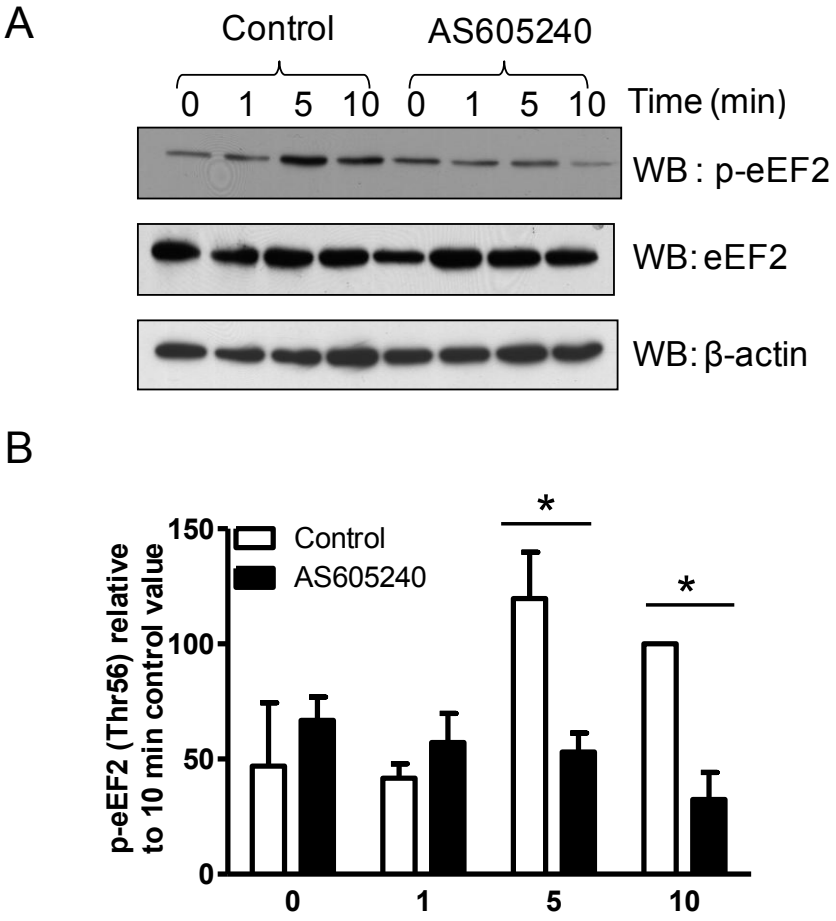

Figure 4.16: Effect of AS605240 on phosphorylation of eEF2 induced by IGF-I in MDA-MB-231 cells.

(A) Cells were pretreated with 2 μM AS605240 and incubated in serum-free media for 1 hour before being stimulated with 0.1 nM IGF-I for increasing periods of time. Cell lysates were prepared and subjected to SDS-PAGE and Western blot to detect phosphorylated eEF2. The filters were stripped and reprobed to detect total eEF2. β-actin was included as the loading control. (B) Quantitation of Western blot densitometry from panel A. eEF2 phosphorylation was quantified by densitometry, normalized to the level of β-actin and expressed as a value relative to the 10 minute control-treated values (mean ± S.E.M. of three independent experiments) as described in Materials and Methods. Asterisks indicate significantly different from the control values (2-way ANOVA with Bonferroni post-test) at *, $p < 0.05$.

CHAPTER 5

Discussion

Chapter 5: Discussion

5.1 Introduction

Activation of tyrosine kinase receptors (RTKs) and G-protein coupled receptors (GPCRs) by their ligands leads to the induction of a number of intracellular signaling cascades. While these pathways were initially thought to be distinct, operating in isolation, recent data indicate an important role for RTK-GPCR transactivation in a number of physiological and pathological cellular responses. This form of receptor transactivation has been shown to be involved in cancer biology, including regulating cell proliferation (Gschwind *et al.*, 2002; Gschwind *et al.*, 2003; Ukegawa *et al.*, 2003), migration and invasion (Akekawatchai *et al.*, 2005; Mira *et al.*, 2001; Noble *et al.*, 2003; Schafer *et al.*, 2004; Zhang *et al.*, 2004). For instance, in bladder cancer cells, LPA receptors transactivate EGFR thereby promoting cell migration and invasion (Schafer *et al.*, 2004). Some of the signaling molecules involved in this process, such MAPK/Erk (El-Shewy *et al.*, 2006; Schafer *et al.*, 2004; Zhang *et al.*, 2004), PKC (Werry *et al.*, 2005), AMP-activated protein kinase (AMPK) (Kisfalvi *et al.*, 2009) and Src (Daub *et al.*, 1997; El Zein *et al.*, 2010; Lee & Chao, 2001; Lee *et al.*, 2002a; Luttrell *et al.*, 1997; Werry *et al.*, 2005) have been identified. Previous work from our laboratory demonstrated that there is unidirectional transactivation of CXCR4 by IGF-I/IGF-1R in highly metastatic MDA-MB-231 breast cancer cell lines. This transactivation depends on constitutive formation of IGF-1R-CXCR4 heterodimers and the association allows IGF-I to transactivate CXCR4, inducing subsequent G-protein signaling, which partially mediates the chemotactic response of these cells to IGF-I. Interestingly, IGF-1R-CXCR4 transactivation is not observed in non-metastatic MCF-7 cells due to non-functional CXCR4 expression (Akekawatchai *et al.*, 2005). However, the underlying molecular mechanisms by which the IGF-1R-CXCR4 heterodimer regulates cell migration and invasion have yet to be elucidated. While PI3Ks are known major effectors downstream of both RTKs (Kundra *et al.*, 1994; Radhakrishnan *et al.*, 2008) and GPCRs (Curnock *et al.*, 2002; Procko & McColl, 2005), that regulate the chemotactic response of different cell types, they have not been investigated for a potential role in the RTK and GPCR transactivation, especially the

involvement of specific PI3K isoforms. Accordingly, the aim of this study was to investigate PI3K signaling induced by IGF-1R-CXCR4 transactivation in metastatic breast cancer cell lines to further elucidate the mechanism by which RTK-GPCR transactivation regulates cell migration.

To achieve this goal, expression of PI3K isoforms was assessed in metastatic MDA-MB-231 breast cancer cells compared to those in non-metastatic MCF-7 cells. As the catalytic subunit of PI3Kγ was found to be highly expressed in MDA-MB-231 cells compared to that in MCF-7 cells, the function of PI3Kγ was subsequently assessed in MDA-MB-231 cells. Finally, target downstream molecules of PI3Kγ were identified.

The results generated from this research project provide experimental evidence for a novel role of PI3Kγ in IGF-1R signaling and in facilitating cell migration potentially by attenuating protein synthesis via phosphorylation of eEF2 downstream of IGF-1R-CXCR4 transactivation in breast cancer cells.

5.2 MAPK/Erk signaling and breast cancer cell migration

The MAPK/Erk pathway is a well-documented signaling pathway acting downstream of receptor transactivation. For instance, IGF-1R has been shown to transactivate the S1P receptor in HEK293 cells. Blocking Giα with PTX or blocking endogenous S1P receptors with the competitive antagonist VPC23019 significantly inhibits the activation of MAPK/Erk in response to IGF-I (El-Shewy *et al.*, 2006). Thus, to investigate the potential involvement of this pathway in IGF-1R-CXCR4 transactivation, Erk phosphorylation induced by IGF-I was determined by Western blot analysis. MDA-MB-231 cells, which were used as a model for the present study, display constitutively high Erk phosphorylation, a finding that supports a previous study demonstrating the constitutive activation of Erk in MDA-231BO, a bone-homing metastatic variant of MDA-MB-231 cells (Zhang *et al.*, 2005b). Because this constitutive activation could not be further elevated in response to IGF-I it was deemed unlikely that the MAPK/Erk signaling pathway regulates transient migration in MDA-MB-231 cells following transactivation of the IGF-1R-CXCR4

complex and this avenue was not investigated further. These data indicate that the contribution of this pathway to the regulation of migration might be stimulus and/or cell type specific. However, it is possible that this high constitutive level of MAPK/Erk activation may play a role in the rapid cell growth, as inhibition of Erk activity leads to decreased cell proliferation and decreased cyclin D1 expression in these cells (Seddighzadeh *et al.*, 1999). The precise mechanisms by which the basal activity of Erk is maintained in MDA-MB-231 cells are not fully understood, although several reports demonstrated that endogenously produced uPA binding to uPA receptor is a major determinant of the high basal activity of Erk in these cell lines (Ma *et al.*, 2001).

5.3 Expression of PI3K isoforms in breast cancer cells

Several studies have demonstrated the expression of Class IA PI3K isoforms in various cancer cell lines (Benistant *et al.*, 2000; Boller *et al.*, 2008; Byun *et al.*, 2003; Carvalho *et al.*, 2010; Fenic *et al.*, 2007; Guerreiro *et al.*, 2008; Knobbe & Reifenberger, 2003; Liu *et al.*, 2008b; Ma *et al.*, 2000; Massion *et al.*, 2004; Sawyer *et al.*, 2003; Shayesteh *et al.*, 1999; Sticht *et al.*, 2005; Sujobert *et al.*, 2005; Zhang *et al.*, 2007) including breast cancer cells. Moreover, increased expression of p110β and p110δ has been observed in brain, colon and bladder tumors (Benistant *et al.*, 2000; Denley *et al.*, 2008; Denley *et al.*, 2009; Kang *et al.*, 2006; Knobbe & Reifenberger, 2003; Mizoguchi *et al.*, 2004; Zhao *et al.*, 2008). Clinical evidence suggests that expression of p110β is associated with a more aggressive profile of breast cancer and highly related to HER2-overexpression and distant metastasis (Carvalho *et al.*, 2010). PI3K mutations, especially mutations in *PI3KCA* have also been implicated in tumorigenesis. These mutants can induce oncogenic transformation in fibroblasts and mammary epithelial cells (Isakoff *et al.*, 2005; Kang *et al.*, 2005d) and contribute to tumour formation in nude mice (Zhao *et al.*, 2005). The data in the present study show that both MDA-MB-231 and MCF-7 cells express Class IA PI3K including both regulatory and catalytic subunits, as clearly indicated by RT-PCR and/or Western blot analysis. This finding concurs with a previous report showing the expression of these isoforms in various breast cancer cell lines (Sawyer *et al.*, 2003). However, according to the present data, none of Class IA PI3Ks are restricted to the metastatic cell

type with MCF-7 cells, a non-metastatic breast cancer cell line, exhibiting similar levels to that observed in the highly invasive MDA-MB-231 breast cancer cell line.

While expression of Class IA PI3Ks has been a subject of extensive investigation in human breast cancer cells, with all three catalytic subunit isoforms being shown to be expressed in a range of cell lines classified as both metastatic and non-metastatic (Sawyer *et al.*, 2003), emerging data suggest that like the Class IA PI3Ks, Class IB PI3Ks may also be involved in cancer biology. As an example, it has been shown that p110γ is specifically overexpressed in human pancreatic intraepithelial neoplasia and ductal carcinoma, which correlates with increased levels of PIP_3 and phosphorylated Akt (Edling *et al.*, 2010; El Haibi *et al.*, 2010). Furthermore, overexpression of p110γ was shown to transform chicken embryo fibroblasts (Denley *et al.*, 2008; Kang *et al.*, 2006), suggesting that overexpression alone may render p110 catalytic subunits transforming. The p110γ subunit has also been shown to correlate with the progression of different types of carcinoma (Edling *et al.*, 2010; Gonzalez-Garcia *et al.*, 2010; Monterrubio *et al.*, 2009; Sasaki *et al.*, 2000a) as human colorectal HCT8/S11 tumour cells transfected with constitutively active, membrane-targeted PI3Kγ display an increased cell invasion capacity compared to those transfected with catalytically inactive PI3Kγ (Barbier *et al.*, 2001). The p101 regulatory subunit has also been reported to have oncogenic potential. It has been shown that p101 is a common site of retroviral insertion in a mouse model of T-cell lymphomas and overexpression of p101 enhances the activity of p110γ and confers significant protection against ultraviolet light-induced apoptosis in human leukemia cell lines (Johnson *et al.*, 2007). Data in the current study suggest that two regulatory subunits of PI3Kγ, p101 and p84 are expressed at a similar level in both MDA-MB-231 and MCF-7 cells. In contrast, high levels of p110γ expression were only observed in MDA-MB-231 cells.

5.4 PI3K signaling and breast cancer cell migration

Activation of the Class IA PI3Ks, PI3Kα, β and δ following ligation of IGF-1R by IGF-I is well documented (Doepfner *et al.*, 2007a; Doepfner *et al.*, 2007b; Guerreiro *et al.*, 2008; Hers, 2007; Sun *et al.*, 2006). However, the two major PI3K isoforms known to be activated downstream of GPCRs that play a role in cell migration in response to GPCR

ligands are PI3Kδ (Puri *et al.*, 2004; Saudemont *et al.*, 2009) and PI3Kγ (Hirsch *et al.*, 2000; Liu *et al.*, 2007; Oak *et al.*, 2007; Procko & McColl, 2005; Ward *et al.*, 2003). More specifically, they have been implicated in migration of leukemia (Hoellenriegel *et al.*, 2011) and prostate cancer cells (El Haibi *et al.*, 2010). The role of these two PI3K isoforms on breast cancer cell migration downstream of IGF-1R-CXCR4 transactivation are discussed below.

5.4.1 Lack of involvement of PI3Kδ in IGF-I-induced MDA-MB-231 breast cancer cell migration

PI3Kδ has been shown to be an important downstream effector of RTKs and GPCRs regulating cell migration and invasion. Specifically, a study shows that PI3Kδ is a major class IA PI3K isoform mediating breast cancer cell migration in response to EGF (Sawyer *et al.*, 2003). Conversely, according to the data presented in this thesis, inhibition of PI3Kδ using p110δ-specific inhibitor failed to alter the chemotactic response to IGF-I in MDA-MB-231 cells, suggesting that PI3Kδ is not involved in IGF-I-induced cell migration. Thus, it is likely that the involvement of PI3Kδ in tumour cell migration is stimulus-dependent. This stimulus-dependent phenomenon has been reported previously where it was demonstrated that PI3Kδ is required for neutrophil chemotaxis to CXCL1 but not to C5a or fLMP (Pinho *et al.*, 2007). Alternatively, there may be functional redundancy in class IA isoforms downstream of IGF-I activation in MDA-MB-231 cells and therefore inhibition of PI3Kδ alone may not be sufficient to block class IA PI3K signaling. For instance, it has been demonstrated that inhibition of p110α alone does not affect insulin signaling while combined inhibition of p110α/p110β or p110α/p110δ does (Chaussade *et al.*, 2007). Finally, it is also possible that PI3Kδ regulates cell migration over a time scale other than that tested in the present study. It has been demonstrated that the production of PIP_3 in response to chemoattractants is biphasic, with the first 30-second response being entirely dependent on PI3Kγ activity, whereas the delayed, 5-minute phase is entirely dependent on p110δ (Boulven *et al.*, 2006). Another *in vivo* study also demonstrates that the leukocyte emigration response to CXC chemokines, including CXCL1 and CXCL2 is entirely dependent on PI3Kγ or PI3Kδ in a non-overlapping manner, with PI3Kγ being important in early CXC chemokine response while PI3Kδ replaces and maintains the later

phase of chemokine-induced neutrophil recruitment into inflamed tissues (Liu *et al.*, 2007). However, whether it is the case for tumour cells remains unclear.

The independence of PI3Kδ in IGF-I-induced migration points to the possibility that other PI3K isoforms may play a key role in breast cancer cell migration. It is not uncommon that specific PI3K isoforms perform distinct roles, depending on the cell type studied (Camps *et al.*, 2005; Geng *et al.*, 2004; Hayakawa *et al.*, 2006; Jackson *et al.*, 2005; Knight *et al.*, 2004; Knight *et al.*, 2006; Pomel *et al.*, 2006; Sadhu *et al.*, 2003a; Sadhu *et al.*, 2003b). For instance, overexpression of PI3Kα leads to increased cell growth in small cell lung carcinoma, an outcome which is not observed in those cells overexpressing PI3Kβ or PI3Kδ (Arcaro *et al.*, 2002). Moreover, individual PI3K isoforms may even perform opposing roles. For example, it has been demonstrated that transfection of breast epithelial cells with p110α or p110β increased the migration speed whereas transfection with p110δ restricts cell migration (De Laurentiis *et al.*, 2011). The possible molecular mechanisms that underlie the differential functions of PI3K isoforms in chemotaxis within the same or different cell types have been discussed in several studies. Firstly, PI3K isoforms may generate distinct pools of PIP_3. For example, a recent study has shown that in response to insulin stimulation or other stimuli, an acute flux of PIP_3 is largely produced by PI3Kα and is efficiently coupled to phosphorylated-Akt whereas PI3Kβ generates basal PIP_3 with little effect on Akt phosphorylation (Knight *et al.*, 2006). Secondly, in addition to lipid kinase activity, PI3Ks possess protein kinase activity with distinct substrate specificities being displayed by different p110 isoforms (Bondev *et al.*, 1999; Carpenter *et al.*, 1993; Dhand *et al.*, 1994; Vanhaesebroeck *et al.*, 1999; Vasudevan *et al.*, 2011), thereby triggering specific signaling cascades. Finally, different subcellular localization of PI3K isoforms may also account for their distinct effects. It is known that PI3Ks are present in the cytosol in resting cells (Kelly *et al.*, 1992). The cytosolic PI3Ks are translocated to plasma membrane after stimulation (Klingmuller *et al.*, 1997) to exert their effects, such as regulating cell survival, proliferation and migration whereas nuclear PI3Ks are more linked to cell differentiation (Neri *et al.*, 1999a; Neri *et al.*, 1999b) and DNA repair (Kumar *et al.*, 2010).

5.4.2 Effect of PI3Kγ on breast cancer cell migration

PI3Kγ is generally activated by GPCRs, including chemokine receptors, such as CXCR4 and acts predominantly in signal transduction cascades that drive cell migration (Barbier *et al.*, 2001; Del Prete *et al.*, 2004; Heit *et al.*, 2008; Hirsch *et al.*, 2000; Lee *et al.*, 2002b; Liu *et al.*, 2007; Naccache *et al.*, 2000; Oak *et al.*, 2007; Procko & McColl, 2005; Sasaki *et al.*, 2000b; Ward & Marelli-Berg, 2009). While the vast majority of data in the literature support a role for PI3Kγ in leukocyte migration, there are some reports demonstrating that activation of PI3Kγ by chemokines also plays an important role in migration and invasion of tumour cells, such as melanoma (Monterrubio *et al.*, 2009) and prostate cancer cells (El Haibi *et al.*, 2010). The data in the present study clearly demonstrate that IGF-I stimulation of highly metastatic breast cancer cells leads to the membrane translocation of p110γ, an indicator of PI3K activation (Lopez-Ilasaca *et al.*, 1997; Stephens *et al.*, 1994) and inhibition of PI3Kγ results in decreased phosphorylated Akt and impaired cell migration to IGF-I. These data indicate that PI3Kγ can be activated by IGF-I, thereby regulating cell migration through phosphorylation of Akt. IGF-I-induced PI3Kγ activation involves the activation of CXCR4 since Akt phosphorylation induced by IGF-I was significantly reduced in CXCR4 knockdown cells. Moreover, blocking of PI3Kγ with AS605240 failed to alter the chemotactic response to IGF-I in CXCR4 knockdown cells. This finding is further supported by the observation that IGF-I-induced Akt phosphorylation in non-metastatic MCF-7 breast cancer cells, which have been shown to lack functional CXCR4 expression (Akekawatchai *et al.*, 2005), was not affected by treatment with AS605240. Collectively, these data provide experimental evidence that functional CXCR4 is essential for PI3Kγ/Akt activation downstream of IGF-1R. Together with the previous report demonstrating that IGF-1R-CXCR4 transactivation-dependent MDA-MB-231 cell migration is dependent on $G_{\beta\gamma}$ mobilisation and is sensitive to PTX (Akekawatchai *et al.*, 2005) which inhibits $G_{i\alpha 2}$-dependent events (Fields & Casey, 1997), it is likely that upon transactivation of CXCR4 by IGF-I, p110γ is activated and translocated to the plasma membrane by $G_{\beta\gamma}$ (Lopez-Ilasaca *et al.*, 1997; Stephens *et al.*, 1994) and through interaction with two regulatory subunits, p101 and p84, which are thought to regulate the activation of p110γ (Wymann *et al.*, 2003). Alternatively, p110γ may bind directly to GTP-Ras (Pacold *et al.*, 2000) which may activate its catalytic activity

(Suire *et al.*, 2002). This proposal is supported by several studies suggesting that activation of p110γ is dependent on both $G_{\beta\gamma}$ and GTP-Ras (Suire *et al.*, 2006; Wymann *et al.*, 2003).

While Akt phosphorylation is known to be one of the earliest detectable events activated downstream of PI3K, several studies indicate that Akt activity may be differentially regulated by PI3K isoforms within the same or different cell types. For instance, inhibition of p110α by siRNA has been shown to decrease IGF-I-induced Akt activity and cell proliferation whereas siRNA knockdown of p110β leads to an increased Akt activity in myoblasts (Matheny & Adamo, 2010). The results presented in this thesis demonstrate that activity of Akt induced by IGF-I was significantly decreased after blocking PI3Kγ, indicating PI3Kγ is the primary PI3K isoform involved in Akt activation in MDA-MB-231 cells. The involvement of PI3Kγ in Akt activation has also been reported in pancreatic cancer (Edling *et al.*, 2010). However, as shown in this thesis, it is not the case in MCF-7 cells, as Akt activity in response to IGF-I was not affected after PI3Kγ inhibition, indicating a predominant role of non-PI3Kγ isoforms in these cells. It is apparent that class IA PI3Ks may play a role, as downregulation of p110β or p110δ by RNAi has been shown to impair IGF-I-induced Akt activation in acute myeloid leukemia cells (Doepfner *et al.*, 2007b). On the other hand, the observation of Akt activation in both metastatic and non-metastatic breast cancer cells also challenges the notion that phosphorylated Akt acts as a "biomarker" for cancer prognosis. In this regard, involvement of isoform-specific PI3Ks, especially PI3Kγ, may be more relevant.

In addition to regulating activation of Akt, PI3Ks have also been shown to be upstream of Erk1/2, based on a report demonstrating that inhibition of PI3K leads to decreased PAK1/Erk activity and impaired cell migration in response to LPA in breast cancer cells (Du *et al.*, 2010). However, the data in the present study show the expression level of phosphorylated Erk is not affected by p110γ knockdown, suggesting that PI3Kγ is not directly or indirectly involved in Erk signaling in MDA-MB-231 cells.

Breast cancer cell migration is clearly complex and involved multiple signaling pathways. Data presented in this thesis suggest that inhibition of PI3Kγ does not completely abolish the chemotactic activity to IGF-I. It is likely that the residual cell migration in response to IGF-I results from the activation of tyrosine kinase-dependent pathways through IGF-1R. As discussed before, class IA PI3K are generally activated by RTKs through phosphorylation of tyrosine kinase residues (Kodaki *et al.*, 1994; Rodriguez-Viciana *et al.*, 1994). The SH2 domain of the regulatory subunit, p85 binds to phospho-tyrosine residues of the activated RTKs (Songyang *et al.*, 1993), leading to the generation of PIP_3 which regulates the activity of a subset of proteins, including Akt, thereby mediating cell migration (De Laurentiis *et al.*, 2011; Guerreiro *et al.*, 2008; Sawyer *et al.*, 2003). It has been shown that IGF-I induces p85 phosphorylation in human chondrosarcoma cells and that transfection of cells with a dominant-negative mutant of p85, decreases the chemotatic response of the cells to IGF-I (Wu *et al.*, 2011), indicating a role of class IA PI3K in cell migration. Although the data in the present study suggest that PI3Kδ is not involved in IGF-I-induced cell migration, other class IA PI3K isoforms may play a role in this process. However, further studies are required to confirm this. Apart from class IA PI3Ks, other signaling transduction pathways triggered by the activation of tyrosine kinase may also contribute to IGF-I-mediated cell migration, such as the Rho kinase (ROCK) and p38 pathway (Zhang *et al.*, 2005b).

5.5 Downstream effectors of PI3Kγ identified by 2D Fluorescence Difference Gel Electrophoresis (DIGE) and mass spectrometry analysis

Although evidence for the functional involvement of PI3Kγ in breast cancer cell migration to IGF-I was obtained in this study, downstream effectors of PI3Kγ remained to be identified, an essential objective for the full understanding of molecular events occurring in cancer cells. In this study, 2D DIGE and mass spectrometry analysis were conducted to identify specific targets of PI3Kγ downstream of IGF-1R-CXCR4 transactivation in MDA-MB-231 cells, by comparing the cytosolic proteome of p110γ knockdown cells to that of control cells. Data in the present study show that the expression levels of several proteins are altered in p110γ knockdown cells compared to those in control cells. As outlined in

Chapter 4, the proteins identified were eukaryotic elongation factor 2 (eEF2), pyruvate kinase isozymes M1/M2 (KPYM), phosphoglycerate kinase 1(PGK1), alpha-enolase (ENOA), L-lactate dehydrogenase A chain (LDHA), and purine nucleoside phosphorylase (PNPH). Of note, differential expression of KPYM and PGK1 was also observed in the absence of IGF-I stimulation, suggesting a role for PI3Kγ in regulation of the level of total protein rather than post-translational modification. Some of these proteins have been implicated in cancer biology, including eEF2, KPYM and PGK1, and are further discussed below.

5.5.1 Eukaryotic elongation factor 2 (eEF2)

eEF2 is a critical enzyme regulating protein synthesis via mediating the ribosomal translocation from the A to the P-site in eukaryotic tissues, the reaction that induces movement of mRNA along the ribosome during translation (Ryazanov *et al.*, 1991). Phosphorylation of eEF2 by eEF2 kinase prevents binding to the ribsome and delays the elongation step, leading to termination of translation and therefore, of protein synthesis (Carlberg *et al.*, 1990).

eEF2 is not merely a translation factor. It is also involved in cell cycle regulation via eEF2 kinase. For instance, it has been shown that eEF2 inactivation by eEF2 kinase induced by PKA in Jurkat cells leads to G1 arrest (Gutzkow *et al.*, 2003). On the other hand, the entry into S phase requires the activation of eEF2 kinase which is mediated by a rise in intracellular cAMP and Ca^{2+} levels. Inhibition of eEF2 kinase delays entry into S phase (White-Gilbertson *et al.*, 2009). eEF2 is also involved in the G2/M phase. Blocking of eEF2 by phosphorylation or siRNA knockdown leads to G2/M accumulation in prostate (White *et al.*, 2007) and gastric cancer cells (Nakamura *et al.*, 2009). eEF2 is also implicated in cell differentiation (Baek *et al.*, 1994; Gutzkow *et al.*, 2003; Kim *et al.*, 1992; Kim *et al.*, 1993; Nilsson & Nygard, 1995; Patel *et al.*, 2002). A recent study shows that eEF2 phosphorylation is reduced shortly after the induction of differentiation in L6 rat skeletal myoblasts (Woo & Kim, 2006).

Of particular relevance to the present study, eEF2 and eEF2 kinase have also been implicated in tumourigenesis. The expression of eEF2 and eEF2 kinase has been demonstrated to be upregulated in a variety of cancer cell lines, including breast cancer cells (Arora *et al.*, 2005; Cheng *et al.*, 1995; Nakamura *et al.*, 2009; Parmer *et al.*, 1999; White-Gilbertson *et al.*, 2009). Forced expression of eEF2 promotes cell growth *in vitro* and enhances tumorigenecity *in vivo* in gastrointestinal cancers in mice (Nakamura *et al.*, 2009). Moreover, it has been shown that functional inhibition of eEF2 kinase by siRNA knockdown or pharmacological inhibition reverses the anti-proliferation and anti-migration effects induced by Resveratrol in vascular endothelial cells (Khan *et al.*, 2010). Recently, eEF2 has been implicated in autophagy, a cellular process for large-scale degradation of proteins and organelles to maintain the cells in an energy-saving manner for survival (Kuma *et al.*, 2004; Meijer & Codogno, 2004) of several cancers, such as human glioblastoma and breast cancer cells (Cheng *et al.*, 2010; Wu *et al.*, 2006; Wu *et al.*, 2009). Knockdown of eEF2 kinase leads to inhibition of autophagy while overexpression increases autophagy (Wu *et al.*, 2006; Wu *et al.*, 2009). This blunted autophagy results in increased apoptosis (Wu *et al.*, 2009) and impeded cell growth (Cheng *et al.*, 2010), suggesting eEF2 may promote cancer cell survival through autophagy. Since eEF2 is an important regulator in tumourigenesis, drugs targeting eEF2 have been developed accordingly, some of which have been successfully used to treat several malignancies (White-Gilbertson *et al.*, 2009). As an example, introduction of denileukin diftitox (Ontak), a novel recombinant fusion protein linked to human IL-2, leads to inhibition of protein synthesis by ADP ribosylation of eEF2, resulting in eventual cell death in hematological tumours (Duvic & Talpur, 2008).

In addition to the studies indicating a critical role of eEF2 in cellular biology, several reports have demonstrated the various molecular mechanisms by which eEF2 is regulated. It has been shown that AMPK and protein kinase A (PKA) act as negative regulators of eEF2 by activating eEF2 kinase, whereas p38MAPK, Erk and PI3K are believed to deactivate eEF2 kinase which upregulates protein synthesis (Bolster *et al.*, 2002; Browne & Proud, 2004; Gutzkow *et al.*, 2003; Krause *et al.*, 2002; Proud, 2007; Wang *et al.*, 2001; White-Gilbertson *et al.*, 2009). As a particular example, it has been shown that pan

inhibition of PI3K results in increased phosphorylation of eEF2, which is involved in myoblast differentiation (Woo & Kim, 2006). However, precisely how PI3Ks regulate eEF2 activity in cancer cell migration, especially the effect of specific PI3K isoforms has not been fully addressed.

The 2D-DIGE data generated in this study show for the first time that eEF2 is one of the downstream effectors of PI3Kγ following IGF-1R-CXCR4 transactivation in MDA-MB-231 cells. The characteristics of the expression pattern of this protein as well as the short stimulation time used in the experiment indicated that the difference observed may be due to alteration of a posttranslational modification. Based on the shift from acidic to basic site of the gel, it is speculated that the protein is less phosphorylated in the p110γ knockdown cells when compared to the control cells. This finding was further validated by Western blot analysis showing that phosphorylation of eEF2 induced by IGF-I was significantly attenuated after p110γ knockdown or pharmacological inhibition of PI3Kγ function, which would theoretically lead to increased protein synthesis. On the surface, this result is in contrast to that previously discussed (Woo & Kim, 2006), in which inhibition of PI3Ks with LY294002 results in increased phosphorylation of eEF2. It is possible that isoform specific PI3Ks may perform distinct roles as has been reported in a number of studies (Camps *et al.*, 2005; Geng *et al.*, 2004; Hayakawa *et al.*, 2006; Jackson *et al.*, 2005; Knight *et al.*, 2004; Knight *et al.*, 2006; Pomel *et al.*, 2006; Sadhu *et al.*, 2003a; Sadhu *et al.*, 2003b). Alternatively, regulation of eEF2 by PI3Ks may be cell type- or stimulus-dependent.

The novel data in the present study showing that in response to IGF-I, PI3Kγ promotes metastatic breast cancer cell migration while inhibiting eEF2 signaling points to the existence of potential regulatory mechanisms that may be switched on or off during the metastatic progression of breast cancer. Experimental evidence shows that cell motility and proliferation are inversely correlated because migratory cells tend to have lower proliferation rates (Giese *et al.*, 2003). This phenomenon is known as the "migration-proliferation dichotomy" or the "Go or Grow" mechanism (Giese *et al.*, 1996). The

existence of this important phenomenon is supported by a number of studies, indicating that migration suppresses cell proliferation and visa versa (Evdokimova *et al.*, 2009; Giese *et al.*, 1996; Godlewski *et al.*, 2010; Merzak *et al.*, 1994). There is evidence that migratory and proliferative processes share common signaling pathways, indicating a unique intracellular mechanism regulating both functions (Giese *et al.*, 2003). However, the detailed underlying mechanisms regulating the migration-proliferation dichotomy remain unknown although several proteins have been implicated in this regulation (Evdokimova *et al.*, 2009; Ghosh *et al.*, 2010; Godlewski *et al.*, 2010). Because of the known key role of PI3Kγ in cell migration, the data in the present study support the view that reduced proliferation is an integral part of migration and more specifically that in metastatic breast cancer cells the initiation of both processes might be facilitated by PI3Kγ.

Regulation of eEF2 activity by PI3Kγ may result from multiple molecular mechanisms. Firstly, the mTOR pathway has been shown to be involved in PI3K/eEF2 signaling. mTOR is a 289-kDa enzyme that belongs to the PI3K-related kinase family (Martelli *et al.*, 2009). The best understood roles of mTOR in mammalian cells are related to the control of mRNA translation and protein synthesis (Albanell *et al.*, 2007) via phosphorylation of components of the protein synthesis machinery, including p70S6K and 4E-BP1, targeting eEF2 and eIF4E, respectively (Engelman *et al.*, 2006; Martelli *et al.*, 2009; White-Gilbertson *et al.*, 2009). There is a report demonstrating that inhibition of PI3K using a pan PI3K-inhibitor decreases activity of mTOR and increases phosphorylation of eEF2 in response to IGF-I in myoblasts (Woo & Kim, 2006). In contrast, data presented in this thesis suggest that inhibition of PI3Kγ using p110γ knockdown or an isoform-specific inhibitor leads to decreased eEF2 phosphorylation in response to IGF-I in MDA-MB-231 cells. This finding further points to the possibility that eEF2 phosphorylation may be differentially regulated by individual PI3K isoforms and/or in a cell-type dependent manner. However, more studies are required to test this proposal. Secondly, PI3Kγ might be able to increase the level of phospho-eEF2 directly. This is supported by the previous studies demonstrating that in addition to the lipid kinase activity signaling through Akt/PKB (Bondeva *et al.*, 1998), PI3Ks also possess protein kinase activity (Dhand *et al.*, 1994; Vanhaesebroeck *et al.*, 1997a). As an example, 293 cells that were transfected with

p110α hybrids, although unable to support lipid-dependent PI3K signaling, such as activation of Akt/PKB and p70S6K, retain the capability to associate with and phosphorylate IRS-1 (Pirola *et al.*, 2001). Nonetheless, more evidence would be required to confirm this proposed mechanism, because the current study indicates the phosphorylation site of eEF2 is Thr^{56} whereas most of the reports demonstrate that PI3Ks catalyze phosphorylation of serine residues (Dhand *et al.*, 1994; Pirola *et al.*, 2001; Vasudevan *et al.*, 2011). Thirdly, PI3Kγ may reduce the rate of eEF2 dephosphorylation through inhibiting the activity of a protein phosphatase. A serine-threonine protein phosphatase 2A (PP2A) has been shown to be impaired in some human cancers (Ruediger *et al.*, 2001) and inhibition of PP2A may attenuate the reduction of phospho-eEF2 (Everett *et al.*, 2001; Nairn & Palfrey, 1987). Of note, an important endogenously occurring regulator of PP2A (Li & Damuni, 1998), the inhibitor of protein phosphatase 2A (I2-PP2A), also known as SET has recently been identified as a protein-kinase substrate of PI3Kγ (Vasudevan *et al.*, 2011). Taken together with the data in the current study, it is therefore possible that PI3Kγ acts as a protein kinase to phosphorylate I2-PP2A (SET), which subsequently inhibits PP2A activity, resulting in a decreased rate of eEF2 dephosphorylation and therefore inhibition of protein synthesis (Figure 5.1).

5.5.2 Pyruvate kinase isozymes M1/M2 (KPYM)

Pyruvate kinase isozymes M1/M2 (KPYM), is a glycolytic enzyme that has been implicated in tumour biology. It is expressed in different tissues and all cells, including tumour cells (Al-Ghoul *et al.*, 2008; Brinck *et al.*, 1994; Corcoran *et al.*, 1976; MacDonald & Chang, 1985; Reinacher & Eigenbrodt, 1981; Schering *et al.*, 1982; Steinberg *et al.*, 1999; Tolle *et al.*, 1976). Elevated levels of dimeric form of KPYM2, also known as KPM2 are preferentially expressed in tumour cells in comparison with KPYM1/KPM1 (Christofk *et al.*, 2008a; Mazurek *et al.*, 2005). Expression of KPM2 is crucial for cell growth (Christofk *et al.*, 2008a; Mazurek *et al.*, 2005). Knockdown of KPM2 leads to decreased cell proliferation in H1299 lung cancer cells (Christofk *et al.*, 2008b). Although the tyrosine kinase signaling pathway has been shown to be involved in the regulation of KPM1/M2, the detailed signaling events regulating KPM1/M2 activity have not been fully understood.

The data in the present study show that protein abundance of KPM1/M2 was increased in p110γ knockdown cells compared to the control cells under both resting conditions and following IGF-I-stimulation with a fold change of 1.2 and 1.3 respectively. While the biological consequences of this are unresolved, previous data show that reduced expression of KPM2 resulted in decreased cell proliferation (Christofk *et al.*, 2008b). This finding correlates well with the data presented in this thesis showing that PI3Kγ may facilitate breast cancer cell migration in response to IGF-I by deactivating protein synthesis and cell proliferation, as discussed in the context of phosphorylation of eEF2. However, confirmation of the 2D-DIGE results on KPM1/M2 by Western blot analysis is still required.

Precisely how PI3Kγ would regulate KPM1/M2 expression is not known. A previous study using a novel proteomic screening for phosphotyrosine-binding proteins showed that KPM2 regulates cell proliferation through direct binding to tyrosine-phosphorylated peptides (Christofk *et al.*, 2008b). Thus, it is possible that tyrosine phosphorylated IGF-1R may act as a platform linking PI3Kγ to KPM1/M2. Of note, in the present study increased protein abundance of KPM1/M2 was also observed in p110γ knockdown cells under resting conditions, which is possibly due to constitutively phosphorylated tyrosine residues on IGF-1R. The constitutive activation of IGF-1R has been reported in acute myelogenous leukemia due to IGF-I autocrine production in these cells (Chapuis *et al.*, 2010).

5.5.3 Phosphoglycerate kinase 1 (PGK1)

Phosphoglycerate kinase 1 (PGK1) is another glycolytic enzymes employed by tumour cells to generate ATP (Daly *et al.*, 2004). It has previously been implicated in several malignancies, such as prostate (Wang *et al.*, 2007), ovarian (Duan *et al.*, 2002), pancreatic (Hwang *et al.*, 2006) and breast cancer (Zhang *et al.*, 2005a). A proteomic study suggested that expression of PGK1 may be linked with increased proliferation of the HER-2/neu-overexpressing breast cancer cells (Zhang *et al.*, 2005a). The detailed mechanism by which PGK1 is regulated has not been fully understood although it has been shown that overexpression of CXCR4 resulted in downregulation of PGK1 expression in prostate cancer cell lines (Wang *et al.*, 2007).

Proteomic data in the present study showed that protein abundance of PGK1 was increased in p110γ knockdown cells compared to the control cells under both resting conditions and following IGF-I-stimulation with a fold change of 1.5 and 1.2 respectively, which still requires further validation by Western blot analysis. Nonetheless, the downregulation of PGK1 by PI3Kγ observed in this study may lead to decreased cell proliferation, therefore facilitating cell migration, as hypothesized above for effects on both eEF2 and KPM1/M2. However, as was the case with KPM1/M2, increased expression of PGK1 was also observed in p110γ knockdown cells without IGF-I stimulation suggesting other unknown mechanisms may be involved in this IGF-I-independent regulation of PGK1.

5.6 Concluding remarks and future studies

In this study, three novel observations with respect to PI3K signal transduction pathway downstream of IGF-I-induced activation of IGF-1R-CXCR4 heterodimers in invasive MDA-MB-231 cells were made. Firstly, while other PI3K subunits are expressed in both MDA-MB-231 and MCF-7 cells at a similar level, high levels of class IB PI3K catalytic subunit, p110γ were only observed in highly metastatic MDA-MB-231 cells. Secondly, PI3Kγ is the major PI3K isoform regulating IGF-I-induced cell migration of MDA-MB-231 cells and it does this in a CXCR4-dependent manner. Thirdly, several novel targets downstream of PI3Kγ were identified using 2D-DIGE and mass spectrometry analysis, including eEF2, KPM1/M2 and PGK1. IGF-1R-CXCR4 transactivation leads to PI3Kγ-dependent phosphorylation of eEF2. Collectively, these findings indicate that PI3Kγ mediates cell migration in response to IGF-I-induced transactivation of CXCR4 and suggest a novel role for PI3Kγ in facilitating cell migration by attenuating protein synthesis via phosphorylation of eEF2 and potentially by suppression of synthesis of KPM1/M2 and PGK1 (Figure 5.1 shown only for eEF2).

While the findings of this study have provided an insight into the mechanism of activation of the novel IGF-1R-CXCR4 heterodimer, there still are important questions that remain to be answered. Firstly, mechanisms by which eEF2 is regulated by PI3Kγ must be verified. The potential involvement of eEF2 kinase, of which eEF2 is the only known substrate, as well as of PP2A, target of the PI3Kγ protein kinase substrate, I2-PP2A (SET) needs to be

investigated using siRNA knockdown and pharmacological inhibition (Arora *et al.*, 2003; Mailhes *et al.*, 2003). It is also important to extensively investigate other metastatic cell lines for a similar role of PI3Kγ in regulation of the eEF2. Also it will be valuable to examine whether the regulation of protein synthesis is a general mechanism for all migrating cells, including leukocytes or is limited to cancer cells. Given the limitation of DIGE, identification of differentially-expressed proteins between control and p110γ knockdown cells in this study was restricted to cytoplasmic proteins. Thus, it would be important to examine where these proteins are localized in the cell to elucidate whether there is only a change in subcellular localization. Furthermore, because only a small number of proteins whose expression and function may be modulated by IGF-I were identified by 2D-DIGE and because other proteins in the membrane and nucleus which might be regulated by PI3Kγ may have been missed in this study, additional proteomics analysis should be conducted. This may take the form of more detailed DIGE analysis on well-defined subcellular fractions, or also, employing a different approach such as isotope-coded protein labeling which would improve proteome coverage. Finally, the IGF-1R-CXCR4 heterodimer appears to be confined to metastatic cells (Akekawatchai *et al.*, 2005; Holland *et al.*, 2006). Thus it potentially represents an important therapeutic target in the treatment of metastatic cancer. The results of the present study have provided novel insight into the biology of this heterodimer by implicating PI3Kγ as critical effector system operating downstream of the heterodimer. However, further research will undoubtedly uncover other insights. It is hoped that the understanding of the molecular mechanisms underlying IGF-1R-CXCR4 transactivation including PI3K signaling transduction pathways in the progression of breast cancer metastasis and invasion may lead to development of more effective diagnostic and therapeutic strategies.

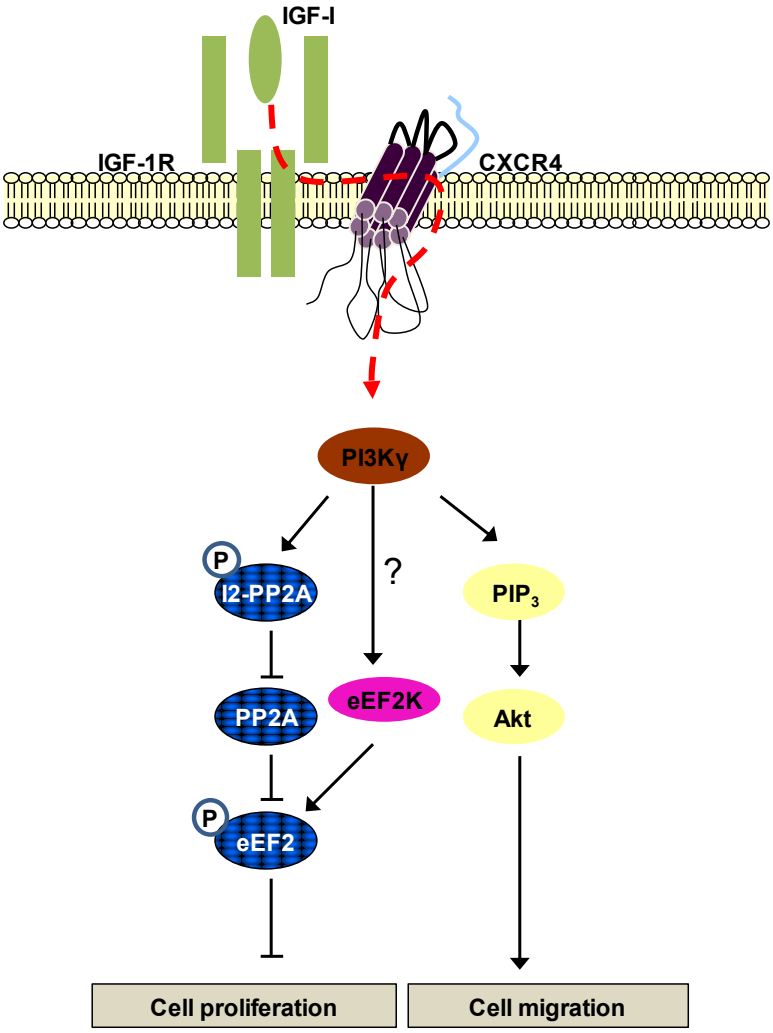

Figure 5.1: A hypothetical model for a novel role for PI3Kγ in cell migration at a level of IGF-1R-CXCR4 transactivation.

The figure depicts the PI3K signal transduction pathway induced by IGF-I stimulation in MDA-MB-231 cells downstream of IGF-1R-CXCR4 transactivation. IGF-I stimulation leads to activation of PI3K and PI3Kγ is the major isoform that contributes to subsequent activation of Akt, thereby regulating cell migration. At the same time, PI3Kγ negatively regulates eEF2 activity, indicating that PI3Kγ facilitates cell migration by attenuating protein synthesis and cell proliferation. Abbreviations: insulin-like growth factor-I (IGF-I); insulin-like growth factor-I receptor (IGF-1R); phosphatidylinositol 3-kinase (PI3K); inhibitors of protein phosphatase 2A (I2-PP2A); protein phosphatase 2A (PP2A); eukaryotic elongation factor 2 (eEF2); phospha- tidylinositol 3-phosphate (PIP_3).

CHAPTER 6

References

Chapter 6: References

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