2010

GPR54 TRANSACTIVATES EGFR TO PROMOTE BREAST CANCER CELL INVASIVENESS

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GPR54 TRANSACTIVATES EGFR TO PROMOTE BREAST CANCER CELL INVASIVENESS

(Spine title: *KISS1*/GPR54 signaling in breast cancer)

(Thesis format: Integrated-article)

By

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Graduate Program in Pharmacology

Submitted in partial fulfillment of the
requirements for the degree of
Master of Science

School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

The University of Western Ontario

School of Graduate and Postdoctoral Studies

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entitled:
GPR54 TRANSACTIVATES EGFR TO PROMOTE BREAST CANCER CELL INVASIVENESS

is accepted in partial fulfillment of the
requirements for the degree of
Master of Science

Date _____

Chair of the Examination Board_____

ABSTRACT

Kisspeptins, peptide products of the Kisspeptin-1 (*KISS1*) gene are endogenous ligands for GPR54, a G protein-coupled receptor. *KISS1* encodes the metastasis suppressor of a large number of cancers. Recent studies, however, have shown that an increase in *KISS1* and *GPR54* expression in human breast tumours correlates with higher tumour grade. Whether kisspeptin signaling promotes breast cancer cell invasiveness is unknown. We found that Kisspeptin-10 (Kp-10) stimulated the invasion of human breast cancer MDA-MB-231 and Hs578T cells using Transwell chamber assays and induced the formation of invasive structures in three-dimensional Matrigel. The effect of Kp-10 on tumour cell invasion was blocked upon treatment of cells with epidermal growth factor receptor (EGFR) inhibitor AG1478. Kp-10 stimulated the transactivation of EGFR *via* Src. We aso observed by co-immunoprecipitation that the two receptors associate with each other. Taken together, our findings suggest that kisspeptin/GPR54 signaling stimulates breast cancer cell invasiveness by transactivating EGFR.

Keywords: Breast cancer, cell migration, invasion, GPCR, kisspeptin, GPR54, EGFR

Co-authorship

The contents of this chapter will be submitted for publication consideration to Molecular Endocrinology.

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Dr. Macarena Pampillo provided FRET analysis that has not been included in this thesis.

Lindsay McColl provided help with cell invasion assays conducted in this chapter.

Cynthia Pape provided technical assistance.

Dr. Gianni M. Di Guglielmo provided guidance with scratch assays conducted in this chapter.

Dr. Lynne Postovit provided guidance with gelatin zymography experiments conducted in this chapter.

Drs. Andy Babwah and Moshmi Bhattacharya provided supervisory guidance in this project.

ACKNOWLEDGMENTS

Firstly, I would like to thank Dr. Moshmi Bhattacharya for giving me the opportunity to have been a part of her laboratory and for giving me the freedom to explore new avenues of research in this project. Her continuous guidance, support, and enthusiasm throughout my studies helped in the continuous search for answers, especially during difficult times. Without her I would never have developed into the person I am today.

Secondly, I would like to share my deepest gratitude for Cindy Pape. Her work in the laboratory is so much more than that requested of a laboratory technician. Without her, progress would have been excruciatingly slow. For all of her hours spent teaching me, making DNA, contributing to trouble-shooting brainstorm sessions, and consoling me during times of failed experiments, I say thank you.

I would also like to thank Drs. Andy Babwah and Macarena Pampillo for providing their time and expertise to make sure this project progressed in the best way possible. I also would like to thank the members of the Babwah laboratory for all the times I have needed emergency DNA. A special thank you goes to my advisory committee members, Dr. Susan Meakin, Dr. Pat Watson, and Dr. John Di Guglielmo, who provided a wealth of knowledge, time, and care throughout the course of my studies.

I would like to especially express my gratitude to Dr. John Di Guglielmo, who is a truly inspiring individual, as a researcher, teacher, and mentor. I cannot express enough thanks to him for all the time he was kind enough to spend with me discussing hypothetical receptor signaling pathways. I would also like to express a big thank you to members of the Di Guglielmo lab for making me feel a part of their family. We will, someday, win a soccer game.

Thank you to my family and friends who have been supportive of me for the past few years. Without you I would never have come this far.

Lastly, I would like to thank the members of the Bhattacharya laboratory: Brittney Parlett, Lindsay McColl, Adel Aziziyeh, Tim Li, Mistre Alemayehu, and Jeffrey Law. To those of you who taught me, I thank you for your mentorship. To those of you who I have taught, I say never give up.

"If we knew what we were doing, it wouldn't be research."

- Albert Einstein.

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LIST OF ABBREVIATIONS

ADAM	A disintegrin and metalloprotease
ANG II	Angiotensin II
AP-2	Adaptor protein-2
AR	Amphiregulin
ARF	ADP-ribosylation factor
ATP	Adenosine-5'-triphosphate
cDNA	Complementary deoxyribonucleic acid
DCIS	Ductal carcinoma *in situ*
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
EPR	Epiregulin
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
G protein	Guanine-nucleotide binding protein
GFP	Green fluorescent protein
GnRH	Gonadotropin-releasing hormone
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
HB-EGF	Heparin-binding EGF-like growth factor
HIF-1	Hypoxia-inducible factor-1
HNSCC	Head and neck squamous cell carcinoma
IGF-1R	Insulin-like growth factor-1 receptor
IL-8	Interleukin-8
JAK	Janus-activated kinase
JNK	c-Jun N-terminal kinase

Kp	Kisspeptin
LCIS	Lobular carcinoma *in situ*
LH	Luteinizing hormone
LPA	Lysophosphatidic acid
mAb	monoclonal antibody
MAPK	Mitogen-activated protein kinase
MEK/MKK	Mitogen-activated protein kinase kinase
MLCK	Myosin light chain kinase
MMP	Matrix metalloprotease
mRNA	Messenger ribonucleic acid
MT1-MMP	Membrane-tethered-1 matrix metalloprotease
N-WASP	Neural Wiskott-Aldrich syndrome protein
NSCLC	Non-small cell lung cancer
NF-κB	Nuclear factor-kappa B
NRG	Neuregulin
PAR	Protease-activated receptor
PDGFR	platelet-derived endothelial growth factor receptor
PI3K	Phosphoinositol-3-kinase
PIP_2	Phosphatidylinositol bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-triphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PR	Progesterone receptor
PTEN	phosphatase and tensin homolog
PTX	Pertussis toxin
ROCK	Rho-associated kinase
ROS	Reactive oxygen species
SCC	Squamous cell carcinoma
shRNA	short-hairpin RNA
siRNA	short interfering RNA
STAT	Signal transducer and activator of transcription

TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-alpha
TIMP	Tissue inhibitor of metalloprotease
VEGF	Vascular endothelial growth factor

CHAPTER 1: INTRODUCTION

1.1. 1. Breast cancer

Breast cancer is one of the most common human neoplasms, accounting for 22% of all cancers in women worldwide, and is a highly heterogeneous group of cancers, each with distinct morphology, molecular genetics, biology, and clinical outcome (1). In Canada, breast cancer accounts for 28% of all new cancer cases and 15% of all cancer-related deaths in women[1]. Most often, breast cancer results from the malignant transformation of epithelial cells comprising either the mammary ducts (ductal carcinoma *in situ*, DCIS) or the lobules of the mammary glands (lobular carcinoma *in situ*, LCIS). These epithelial cells remain confined to the primary site of origin until they acquire the ability to invade into the surrounding tissues and to even more distant organs. Furthermore, because there are no lymphatics or blood vessels in the epithelial layer, DCIS and LCIS offer no risk for metastatic spread until malignant cells are able to cross the basal membrane (2).

DCIS is a heterogeneous collection of several morphological variants that differ in their histological appearance, cellular characteristics, and clinical behaviour, and comprises a stage of the carcinogenic continuum (Fig. 1.1). This continuum begins first with a loss of growth control by the epithelium and proliferation into the lactiferous ducts of the terminal duct/lobular unit to form micropapillary or papillary ingrowths into the lumen. This ductal hyperplasia is characterized by well-differentiated cells without significant pleomorphism, atypia, or mitosis. As these cells grow into the duct, these ingrowths produce a network within the lumen until round spaces are left interspersed throughout,

forming a cribriform pattern that is indicative of DCIS (2). As these cells begin to close the spaces in between, fully solid DCIS results, showing a greater number of anaplastic and mitotic cells. Continuing growth results in cells that are not able to reach their blood supply, becoming necrotic, leading to the classic "comedo" pattern of DCIS. Through all these stages the duct is still intact, with no invasion of the malignant cells into the surrounding tissues.

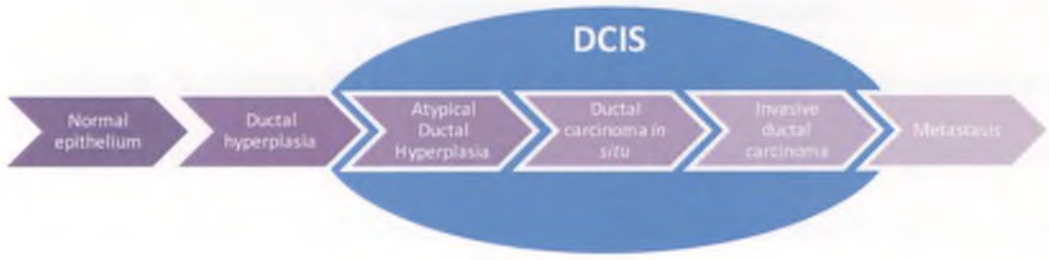

Figure 1.1. Progression of breast cancer. Normal cell growth becomes unrestricted, resuting in ductal hyperplasia. Cells begin to lose their normal morphology, progressing to an appearance indicative of ductal carcinoma *in situ* (DCIS). Progressive growth and dedifferentiation may result in cells with invasive capabilities and metastasis to distant sites.

1.1.2. Breast cancer subtypes

The primary cause of fatality in patients with breast cancer is metastasis of the tumour to distant areas, resulting in the colonization of the area and disruption of normal function (3). Conventionally, invasive human breast carcinomas have been classified morphologically into infiltrating ductal and lobular carcinoma, tubular carcinoma, mucinous carcinoma, medullary carcinoma, invasive papillary carcinoma, metaplastic carcinoma, as well as several other more rare types, including cribriform carcinoma, apocrine carcinoma, and acinic cell carcinoma (1). With the advent of transcriptional profiling techniques, a more detailed molecular description is available of breast tumours. This new classification includes five molecular subtypes: normal-like, human epidermal growth factor receptor 2 (ErbB2, HER2)-overexpressing, luminal A, luminal B, and basal-like breast tumours (4). Each subtype exhibits distinct clinical features and some, such as the HER2 and basal-like subtypes, show worse prognosis and/or resistance to therapy.

Normal-like breast cancer shows the highest expression of many genes known to be expressed by non-epithelial and adipose tissues. However, some have argued that this subtype may be due to normal tissue contamination (5). Luminal subtypes have been shown to constitute 67% of tumours, are hormone receptor-expressing breast cancers, and have expression patterns resembling the luminal epithelial component of the breast (6). Within the luminal type are two subtypes, A and B, of which A generally has higher expression of ER-related genes and lower expression of proliferative genes than B (6). The HER2 subtype

refers to a group of hormone receptor-negative breast cancers that also express high levels of HER2 (6). These cancers are more likely to be high grade, poorly differentiated, and are two-fold more likely to involve axillary lymph nodes than luminal A tumours. Basal-like breast cancers were first described as invasive breast cancers that highly express cytokeratins 5 and 17, laminin, and fatty-acid binding protein 7 (1). Further analysis included expression of EGFR, c-kit, p53, SMA, CD10, vimentin, and P-cadherin. These tumours show a markedly higher likelihood of being grade III than luminal A breast cancers and have been shown to represent 20% of breast tumours (6).

Although a significant amount of progress has been made in early detection of breast cancer and in lowering the mortality rate in patients, metastatic breast cancer remains an almost incurable disease, with treatment goals focusing on prolonging survival and providing palliative control of symptoms (7). Metastatic breast cancer remains a highly heterogeneous disease with clinical behaviour that is difficult to predict. Thus, treatment is based on the characteristics of the patient, including age, previous treatments, and co-morbidities, but also on molecular signature of the tumour (7). Most commonly, estrogen, progesterone, and HER-2 receptor expressions help determine which treatment will be most effective in combating the disease.

1.2.1. Metastasis

Metastasis is a complex process that involves a number of sequential steps, including loss of cell-cell adhesion, invasion of cells into surrounding tissue, angiogenesis, and dissemination of cancer cells throughout the vascular

and lymphatic vessels, passing of cells into distant tissues, and establishment of a new colony in that tissue (8). A tumour cell must accomplish each step in the pathway or metastases will not develop. Both pro- and anti-metastatic regulators exist, and thus implicate a number of different genes in this process. Understanding the regulation of metastasis is required to devise new methods of cancer chemotherapy, particularly those that will prevent or suppress the dissemination of cancer cells into other tissues.

Carcinomas, or cancers originating from epithelial tissues, account for the majority of human cancers (4). After their initial transformation, cancerous epithelial cells are confined to their location by the continued expression of adhesion molecules, in addition to the intact basal lamina. Growth and survival of the malignant tumour is dependent on angiogenesis, as a sufficient blood supply is required to feed the primary tumour mass (9). Without the formation of new blood vessels, a tumour's size would be limited to roughly 2 mm, unable to obtain the oxygen and nutrients required to allow continuing growth (9). Microvessel density, a measure of angiogenesis, is also a prognostic factor in predicting the survival of cancer patients (9). Tumours gain access to blood supply through the production of new blood vessels by attracting vessel production (angiogenesis) and even by mimicking blood vessels, a process in which the tumour cells themselves act like vessels (9). The molecular mechanisms underlying angiogenesis are stimulated under conditions of hypoxia. Once the tumours become too large and hypoxic conditions are established, the hypoxia inducible factor-1 (HIF-1) transcriptional complex is activated, coding genes for

angiogenesis-inducing factors, anaerobic metabolism, cell motility, and resistance to apoptosis (9).

To stimulate angiogenesis, tumour cells and the surrounding stromal cells produce soluble pro-inflammatory and pro-angiogenic cytokines, including tumour necrosis factor alpha (TNF-α), vascular endothelial growth factor (VEGF), and interleukin-8 (IL-8), which diffuse into the surrounding tissue and pre-existing vasculature (9). These products activate not only the endothelial cells, but also the tumour cells themselves to produce adhesion molecules, including integrins, matrix metalloproteases (MMPs), and plasminogen activators. With the aid of the MMPs, proliferating endothelial cells are stimulated to move to the location of the tumour, attracted by the increasing chemoattractant gradient produced by the tumour (9). This new vasculature often exhibits incomplete formation, disorganized architecture, and high permeability. The leakiness of the new vessels promotes the intravasation of tumour cells able to move from the primary tumour.

1.2.2. Epithelial-to-mesenchymal transitions

After their initial transformation, cancerous epithelial cells are confined to the primary site by the expression of adhesion molecules and the basal membrane (4). As the cancer progresses, some cells at the tumour periphery may commandeer the epithelial-to-mesenchymal program used in embryogenesis (4). Epithelial cells normally line the cavities of the body and are the major constituents of many glands. They perform a variety of functions, including

protection, secretion, absorption, filtration, and diffusion. They are composed of continuous sheets of polarized cells connected by strong cell-to-cell and cell-to-substratum adhesions. Anchorage between cells is accomplished by a variety of structures, including adherens junctions, tight junctions, gap junctions, and desmosomes (4, 10). Epithelial cells are further anchored to the basal lamina, giving the cells an apical-basolateral polarity and thus, regional organization (10). These adhesions prevent normal cells from undergoing changes in shape, polarity, and motility that are required for their invasion into the underlying stromal layer and prevent these cells from migrating away from the epithelial layer independently (4). In organotypic culture models, individual mammary cells seeded in reconstituted basement membrane will proliferate to form a hollow sphere of apico-basal polarized, growth-arrested cells, resembling the features of *in vivo* mammary gland structure (Fig. 1.2) (11, 12). Furthermore, mammary cell cultures may produce basal deposition of basement membrane components, including collagen IV and laminin V, and, in some cases, even milk proteins (12).

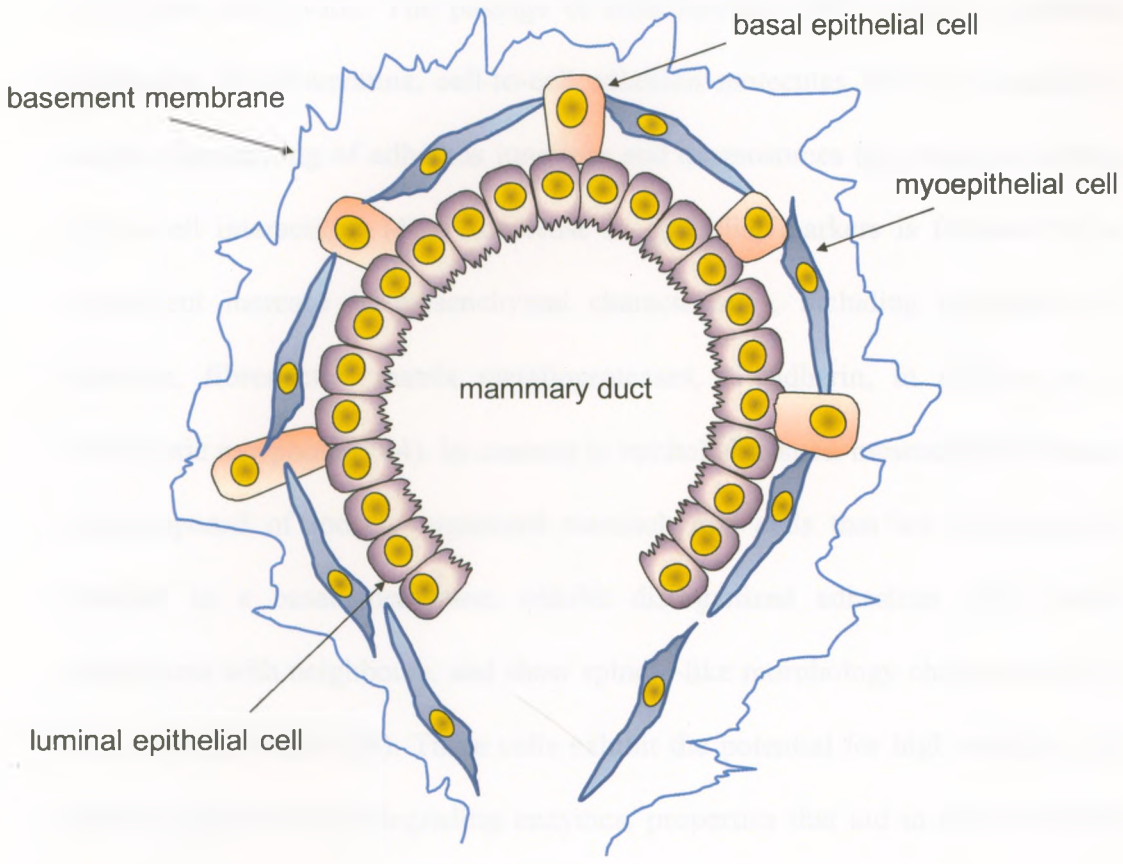

Fig. 1.2. Structure of a normal mammary acinus. Mammary epithelium possesses an apico-basal polarized architecture surrounding a hollow lumen, surrounded by an inner layer of luminal epithelial cells and an outer layer of myoepithelial and basal epithelial cells.

During EMT, epithelial cells lose normal cell-to-cell contacts and cell polarity while undergoing major cytoskeletal rearrangement, assisting their ability to migrate and invade. The passage of cells through EMT involves decreased expression of cytokeratins, cell-to-cell adhesion molecules such as E-cadherin, and the dismantling of adherens junctions and desmosomes that allow for strong cell-to-cell interactions (4). A decrease in epithelial markers is followed by a subsequent increase in mesenchymal characteristics, including expression of vimentin, fibronectin, matrix metalloproteases, N-cadherin, in addition to a fibroblastic morphology (4). In contrast to epithelial tissues, mesenchymal tissues are composed of loosely organized mesenchymal cells that are not typically attached to a basal membrane, exhibit disorganized adhesions, form weak interactions with neighbours, and show spindle-like morphology characterized by front-to-back polarity (4). These cells exhibit the potential for high motility and actively secrete matrix-degrading enzymes, properties that aid in their invasion through the basement membrane and into the surrounding tissues. However, carcinoma cells that have undergone EMT do not always exhibit every phenotypic change characteristic of EMT, suggesting that some tumour cells do not necessarily complete the entire EMT process (4).

1.2.3. Changes in the cytoskeleton necessary for migration and invasion

Tumour cell motility is the hallmark of invasion and is an initial step in metastasis. Each step of the metastatic cascade requires distinct molecular changes that result in the modification of the adhesive and migratory properties of

the disseminating tumour cells. The cytoskeleton provides the basic infrastructure required for the maintenance of cell motility. Comprised of three basic proteins, including microtubules, intermediate filaments, and actin filaments, it is the actin filaments that are responsible for dynamic cell motility (13). The polymerization of G-actin into a double-helical structure (F-actin) produces thin, flexible fibers. Actin polymerization is a dynamic process involving growth by the reversible addition of G-actin to both ends of an F-actin fiber, but elongation occurs more quickly at one end than the other (13).

Actin filaments are organized into three-dimensional networks that form lamellipodia, filopodia, and invadopodia (Fig. 1.3). Lamellipodia are flat, broad sheet-like formations observed at the leading edge of migrating cells. Long, unbranched actin filaments at the base of the lamellipodium are processed into highly branched lateral network, giving lamellipodia their typical structure. This assembly is highly regulated by the Arp2/3 complex of proteins, further controlled by actin nucleation-promoting factors, including N-WASP or the Scar/WAVE complexes recruited to and activated at the membrane by Rac1 (14). Recent studies have shown that increased expression of Arp2/3 and WAVE2 correlate with poor prognosis in breast and liver carcinomas (14). Filopodia interact with their environment to promote the continued migration of tumour cells, attaching *via* a number of adhesion molecules, including integrins and cadherins. Aimed at penetrating into the surrounding matrix, filopodia are rod-like extensions consisting of tight bundles of actin (14). Filopodia are considered as sensory structures, used to detect and assimilate signals like chemoattractants or nutrients

(14). Recently, it has been reported that filopodia can transform into lamellipodia by initiating dendritic actin nucleation, characteristic of lamellipodia formation, demonstrating that filopodia and lamellipodia are inter-convertible and dynamic structures (14). Invadopodia are intermediate-width extensions that are enriched with matrix metalloproteases, like MT1-MMP, MMP-2, and MMP-9, and are responsible for invasion into the extracellular matrix (13-15).

The directional movement of cells requires a series of steps involving: 1) protrusion of a broad lamellipodium in the desired direction of movement; 2) anchorage of membrane to the underlying substratum through focal adhesions; 3) contraction of the F-actin network producing a forward motion of the cells; and 4) disassembly of the focal adhesions located in the rear of the cell (Fig. 1.4). This cycle allows for the forward motion of cells. Actin assembly and disassembly is governed by a group of proteins, including Rac, Rho, and Cdc42 (16). Rac induces assembly of focal complexes and actin polymerization during the formation of lamellipodia, in addition to its activity in membrane ruffle formation (16-18). Activation of the RhoGTPases by chemokine and growth factor receptors and adhesion receptors leads to the assembly of actin-myosin contractile filaments into focal adhesion complexes that promote cell polarity and facilitate motion (19). Activation of RhoA, a member of the Rho family of genes, induces activation of the effector kinase ROCK, which mediates the formation of stress fibres and focal adhesions (13, 20). CDC42 is involved in the formation of filopodia, and promotes the establishment and maintenance of cell polarity, essential for directed migration (16, 21).

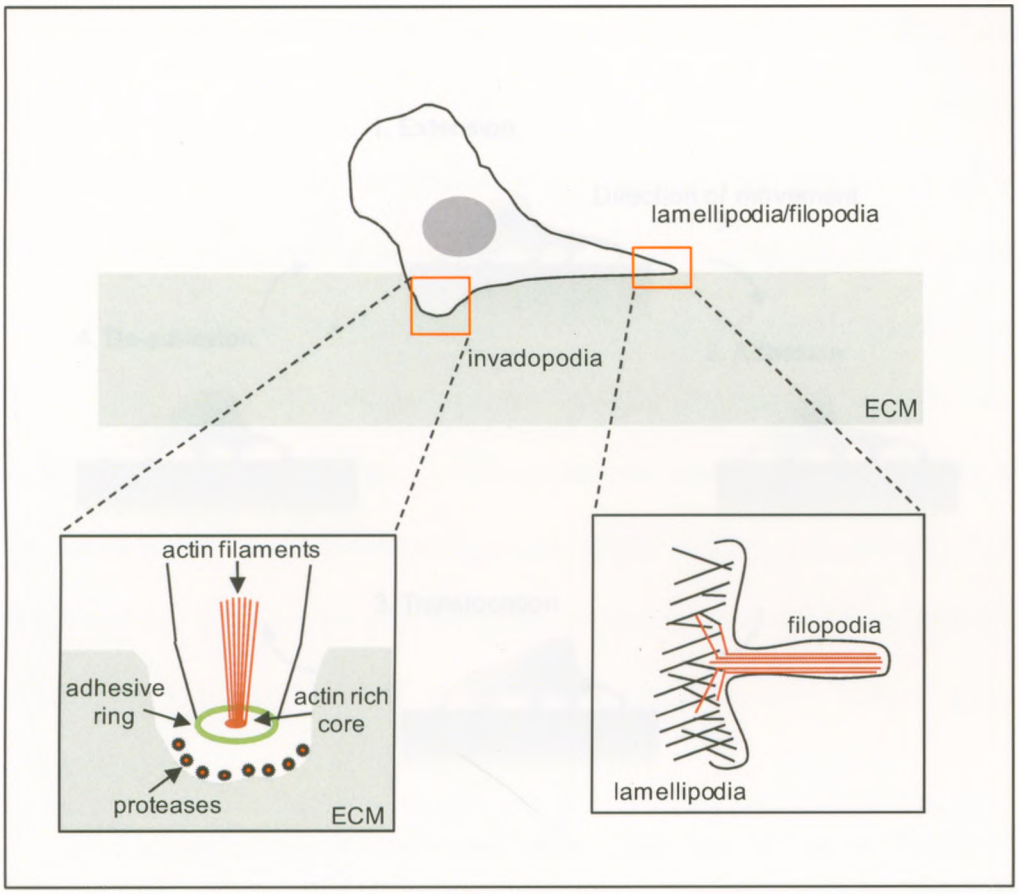

Fig. 1.3. Invasive structures created by motile cancer cells. Lamellipodia are broad, sheet-like projections that extend from the leading edge of motile cells. Filopodia, finger-like projections, are believed to be sensory structures and are used to detect chemoattractants or nutrients. Invadopodia are intermediate-width projections responsible for the invasion of cells into a three-dimensional matrix.

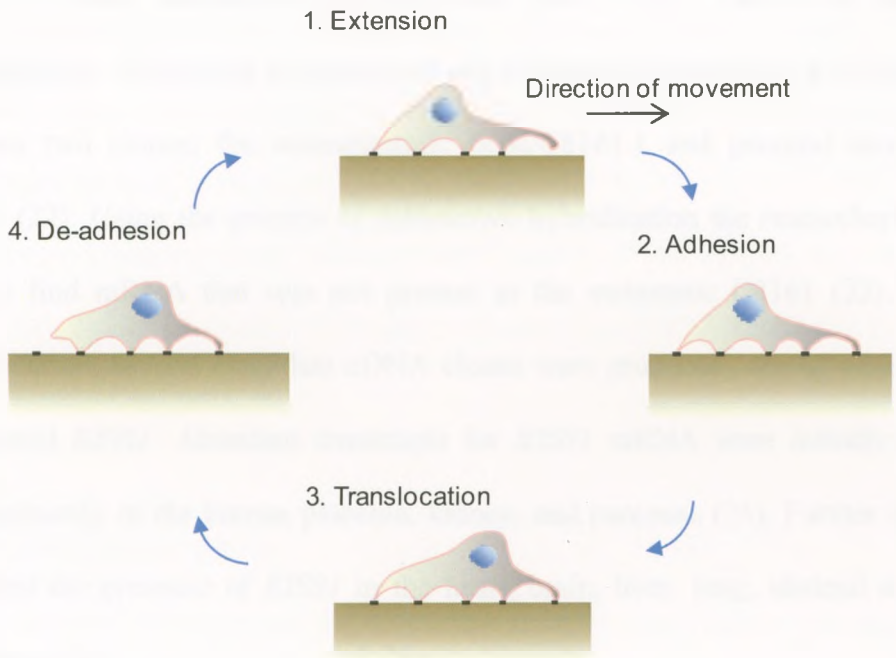

Fig. 1.4. Processes involved in the two-dimensional movement of cancer cells. Extension and adhesion at the leading edge of motile cells is followed by translocation of the cytoplasm to the front and de-adhesion of the cell from the basal layer at the back. Continuous cycling allows for the directional movement of motile cells

1.3.1. KISS1 and the kisspeptins

More than a decade ago a new metastasis-suppressor gene was discovered. Using a human amelanotic melanoma cell line, C8161, Lee *et al.* tried to investigate the differential expression of any metastasis-suppressing genes present between two clones, the nonmetastatic neo6/C8161.1 and parental metastatic C8161 (22). Using the process of subtractive hybridization the researchers were able to find mRNA that was not present in the metastatic C8161 (22). Upon amplification, several candidate cDNA clones were produced, one of which was designated *KISS1*. Abundant transcripts for *KISS1* mRNA were initially found predominantly in the human placenta, kidney, and pancreas (23). Further studies indicated the presence of *KISS1* in the heart, brain, liver, lung, skeletal muscle, and breast (24).

KISS1 produces a 145 amino acid peptide, which is then processed into 54, 14, 13, and 10 amino acid length residues before secretion by the cell (Fig. 5) (25). It was shown not only that kisspeptin-145 forms stable complexes with MMPs 2 and 9, but kisspeptin-154 could be cleaved by MT1-MMP, MT3-MMP, MT5-MMP, MMP-2, and MMP-9 to give shorter kisspeptins (26). Furthermore, the authors determined that the interaction of kisspeptin-154 occured with the propeptide domains of the MMPs, and required the N-terminal 48-amino acid fragment, including Cys^{53} of Kp-154 (26). These shorter peptides have been described and isolated in a number of species, including human, chimpanzee, bull, ovine, rat, mouse, opossum, xenopus, and zebrafish (25).

The minimal active form of the kisspeptins (Kp-10) is comprised of the last ten amino acids of the full 145-amino acid peptide (H-Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2) (27). This structure is highly conserved across a range of species, differing from the human and primate sequence only by a single amino acid in rat, mouse, platypus, sheep, and cow (25). It was revealed through saturation binding experiments that it exhibited a K_D of 1.0 ± 0.1 nM (23). In addition, it exhibited greater efficacy than Kp-54 (5.47 ± 0.03 nM), Kp-14 (7.22 ± 0.07 nM), or Kp-13 (4.62 ± 0.02 nM), with an EC_{50} of 4.13 ± 0.02 nM (23) (Fig. 1.5).

1.3.2. KISS1 transcription

Studies of transcriptional regulation of *KISS1* have shown that estradiol is able to stimulate *KISS1* expression and *KISS1* promoter activity through the activation of estrogen receptor α (ERα), an estrogen-inducible transcription factor, in hypothalamic GT1-7 cells (28). Furthermore, it was demonstrated that ERα interacts directly with transcription factors Sp1 and Sp3 to stimulate *KISS1* promoter activity (28). In breast cancer cells, *KISS1* transcription has been shown to be synergistically activated by AP-2α, a 52-kDa transcription factor that regulates genes that are important in development and metastasis, and Sp1 (29).

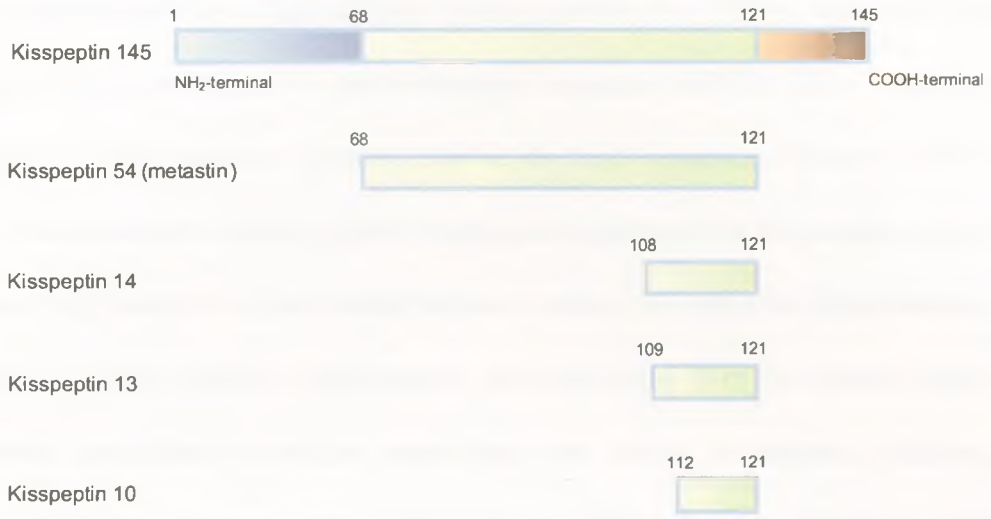

Fig. 1.5. The kisspeptins. Cleavage of kisspeptin-145 results in the production of smaller peptides, designated Kp-54 (metastin), Kp-14, Kp-13, and Kp-10.

1.4.1. Physiological roles of the kisspeptin receptor, GPR54

Although the sequence for *KISS1* has been known since its initial discovery by Lee *et al.* in 1996, it was not until 2001 that the products of *KISS1* were characterized as a high-affinity binding partner for GPR54, what was then thought of as an orphan G protein-coupled receptor (GPCR) (23). Originally thought of as a galanin receptor due to its high sequence identity, GPR54 nevertheless did not exhibit specific binding with galanin (30). Expression studies showed high levels in the rat central nervous system, including the hypothalamus, midbrain, pons, medulla, hippocampus, and amygdala (30). In human tissues, however, particularly abundant transcripts were found in placenta, pituitary, spinal cord, and pancreas, with lower levels in other tissues, including in the breast, brain, stomach, small intestine, thymus, spleen, lung, testis, kidney, and fetal liver (23, 31).

1.4.2. KISS1 and GPR54 in the hypothalamic-pituitary-gonadal axis

In 2003, Seminara *et al.* discovered that *KISS1* and GPR54 are directly involved in the hypothalamic-pituitary-gonadal axis, controlling the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus (32). Using linkage-analysis and candidate gene screening, the researchers analyzed genetic differences in families with hypogonadotropic hypogonadism, discovering a mutation in the GPR54 gene. Further studies in pubertal disorders showed that in girls with central precocious puberty, mean levels of serum kisspeptins were significantly elevated compared to normal, healthy, age-matched girls, 14.62

pmol/L and 8.35 pmol/L, respectively (33). Gpr54-knockout mice were unable to proceed through puberty or had delayed onset, with both sexes appearing to have immature sexual organs. Endocrinological analysis showed testosterone in males to be at the same concentration as that in females, but only in males with Gpr54-knockout and not in *Kiss1*-knockout (32, 34). Furthermore, males with *Kiss1* knockout and those with Gpr54-knockout both showed significantly reduced testicle size, with a greater reduction in Gpr54-knockout mice (35). Serum levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were also significantly reduced, but more so in Gpr54-knockout mice, and only in males (35). Females exhibited no significant difference in LH levels compared to wild-type mice. When mouse metastin (Kp-54) was injected subcutaneously, all genotypes and genders responded with increases in LH and FSH (except the Gpr54 knock-out mice), indicating the vital roles of *KISS1* and Gpr54 in GnRH release from the hypothalamus (35).

The *KISS1*/GPR54 pathway plays a major role in regulation of trophoblast invasion. Recent studies showed that kisspeptin repressed the invasion of trophoblasts into the maternal decidua, a process necessary for fetal development and which closely mimics the invasion of cancer cells (36). Measurements throughout pregnancy have shown a steep rise in kisspeptin levels which return to baseline levels five days post-partum (37). This seems to suggest that the placenta is producing the kisspeptins. Although the precise function of this system is currently unknown, it is thought that perhaps the kisspeptins act to down-regulate the hypothalamic-pituitary-gonadal axis (37). Studies involving *KISS1* in human

trophoblast migration have corroborated the results shown in cancer cells. In trophoblast outgrowth experiments using explanted placental tissue, treatment with Kp-10 resulted in approximately 70% diminished extravillous trophoblast outgrowth (38). Gelatin zymography experiments showed that 300 nM and 1 μM Kp-10 was able to reduce MMP-2 expression after treatment for 24 hours (38). Furthermore, expression of *KISS1* in HT-1080 fibrosarcoma cells decreased MMP-9 protein and mRNA expression (39). The effects of the kisspeptins, then, extend beyond their actions on the hypothalamic-pituitary-gonadal axis.

1.5.1. GPR54-mediated signaling

The kisspeptins are ligands for GPR54, a $G_{q/11}$-coupled GPCR. Activation of GPR54 by the kisspeptins results in the activation of phospholipase C, protein kinase C, and the mitogen-activated protein kinase cascade (MAPK), including p38, PI3K, and ERK1/2 (23) (Fig. 1.6). Treatment of Chinese hamster ovarian (CHO) cells expressing GPR54 with Kp-10 showed strong inhibition of proliferation, with no evidence indicative of cell death by apoptosis (23).

To date, one study has examined the regulation of GPR54 signaling (40). In classical GPCR signaling, binding of a ligand to the GPCR results in the activation of downstream signaling of the receptor and desensitization (Fig. 1.7) (41). Desensitization is an important physiological feedback mechanism that protects against both acute and chronic receptor stimulation. Desensitization of the receptor can occur by three distinct mechanisms: 1) heterotrimeric G proteins uncouple from the receptor as a result of receptor phosphorylation by second

messenger-dependent protein kinases or by G protein coupled receptor serine/threonine kinases (GRKs); 2) the endocytosis of cell surface receptors in endocytic vesicles; and 3) the downregulation of the receptor due to a decrease in mRNA and protein synthesis, as well as degradation in lysosomes and proteosomes (41, 42). G protein-coupled receptor kinase (GRK)-mediated phosphorylation of ligand-stimulated GPCRs results in the uncoupling of receptors from heterotrimeric G proteins, preventing further signaling (43). Receptor phosphorylation by second messenger-dependent protein kinases such as protein kinase A (PKA) and protein kinase C (PKC) can occur in a heterologous manner, in which the activated protein kinase phosphorylates GPCRs indiscriminately of ligand-binding (43).

Phosphorylation of GPCRs results in the recruitment of β-arrestins to the plasma membrane and to the stimulated receptor, where they serve to sterically inhibit the binding of heterotrimeric G proteins, further desensitizing the receptor (44). Receptor binding induces a conformational change in β-arrestin, exposing the C-terminal domain, which interacts with clathrin and the other adaptors, such as adaptor protein complex-2 (AP-2) (44). The receptor complex is then internalized through clathrin-coated pits. Once internalized, some receptors continue to signal from endosomes, until the agonist eventually dissociates from the receptor or the receptor is shut off. GPCRs are then dephosphorylated and recycled back to the cell surface in a resensitized state, or are targeted to the lysosomes where they are degraded.

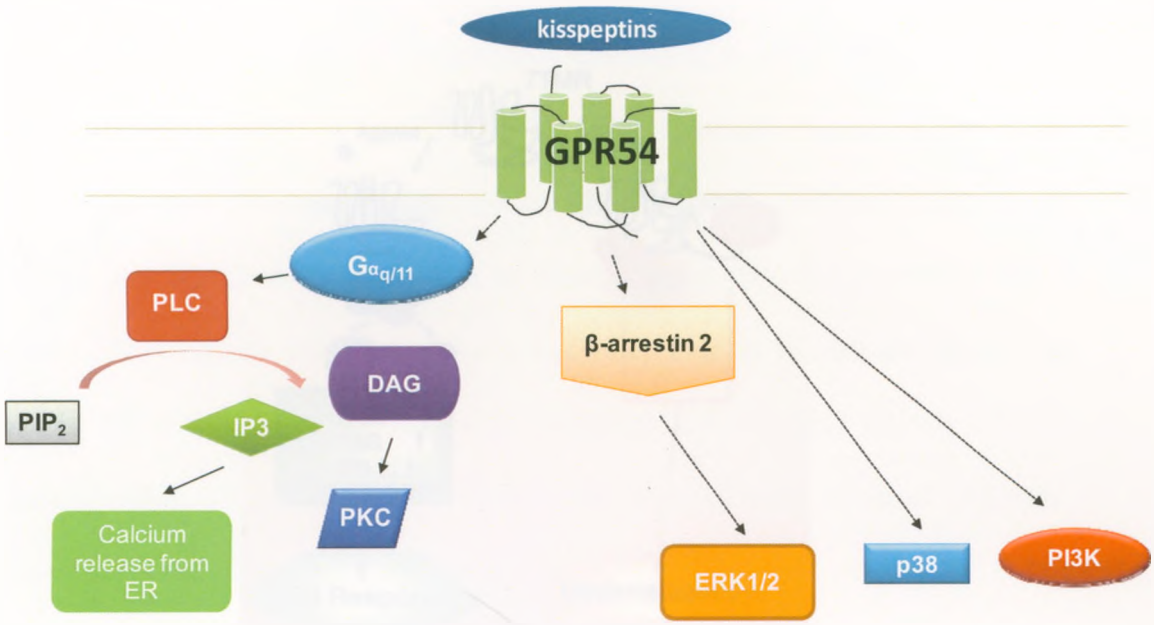

Fig. 1.6. Molecular signaling of the kisspeptins *via* GPR54. GPR54 is a $G_{q/11}$-coupled G protein-coupled receptor (GPCR), resulting in the activation of phospholipase C (PLC), protein kinase C (PKC), and members of the mitogen-activate protein kinase (MAPK) pathway, including ERK1/2, p38, and PI3K.

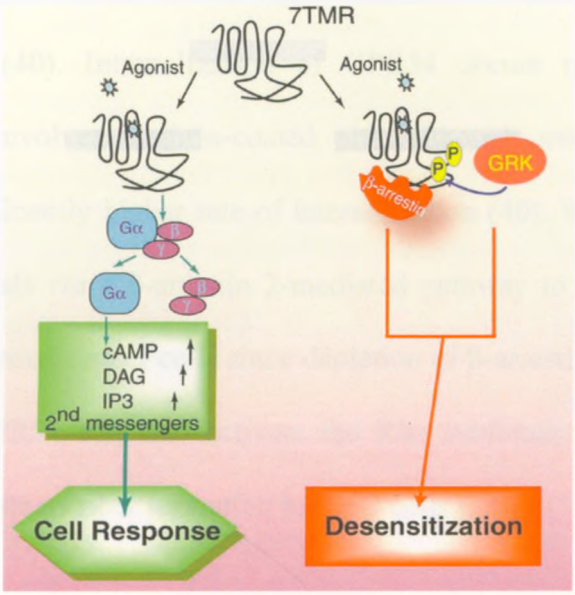

Fig. 1.7. Schematic representation of GPCR desensitization and endocytosis. Following agonist stimulation, GRKs phosphorylated agonist-activated receptors on serine and threonine residues located on the C-terminal domain or on the intracellular loops of the receptors. Phosphorylation desensitizes the receptor by facilitating the recruitment of β-arrrestin to the GPCR, uncoupling heterotrimeric G proteins from the receptor. β-arrestin then targets the receptor for clathrin-mediated endocytosis. Internalized receptors can be dephosphorylated and recycled back to the plasma membrane or targeted to the lysosomes for degradation.

We have recently shown that GPR54 is constitutively associated with G protein receptor kinase 2 (GRK2) and β-arrestins 1 and 2 (40). Mutant GPR54 lacking the C-terminus was still shown to bind GRK2, suggesting that the interaction was mediated by another intracellular loop of GPR54, possibly the second loop, which contains several basic residues that could potentially mediate this interaction (40). Internalization of GPR54 occurs rapidly under basal conditions and involves clathrin-coated pits, although treatment with Kp-10 results in a significantly higher rate of internalization (40). We previously found that GPR54 signals *via* a β-arrestin 2-mediated pathway to activate ERK1/2 in MDA-MB-231 breast cancer cells since depletion of β-arrestin 2 inhibits ERK1/2 activaty (40). GPR54 can also activate the Rho subfamily of small GTPases, resulting in actin stress fiber formation in CHO cells (23).

1.5.2. Regulation of GPR54 expression

Relatively little in known about the mechanisms that govern GPR54 expression. It was only recently that this topic was addressed. A recent study revealed that GPR54 promoter activity was repressed by a partial estrogen response element, and GPR54 expression is positively regulated by transcription factor SP1, *via* three SP1 binding sites clustered at the 5' end (45).

1.6.1. KISS1/GPR54 signaling in cancer

In several cancers, including melanoma, pancreatic cancer, anaplastic thyroid cancer and renal cell carcinoma, *KISS1*/GPR54 signaling is anti-metastatic (22, 46, 47). GPR54 activity was shown to repress matrix metalloprotease-9 (MMP-9) activity, inhibit migration, increase tissue inhibitor of metalloprotease (TIMP)-1 production, and activate focal adhesion kinase (FAK), leading to the formation of excessive focal adhesions and stress fibre formation (23). Lee *et al.* first demonstrated the powerful anti-metastatic effects of *KISS1* using the C8161 amelanotic melanoma cell line (22).

A recently study using C8161.9 metastatic melanoma cells stably transfected with metastin (Kp-54) cDNA with or without a signaling sequence revealed that kisspeptins need to be secreted in order to exert their anti-metastatic effects (48). A clinical report tried to correlate *KISS1* and GPR54 expression with prognosis in patients with clear-cell subtype ovarian carcinoma (49). In this study of 518 patients, it was discovered that both *KISS1* and *GPR54* expression are independent markers for favourable prognosis. However, when *KISS1* expression was analyzed within multiple ovarian cancer subtypes, kisspeptin-immunoreactivity status failed to achieve statistical significance, although the clear-cell subtype did approach significance (49).

Several studies have demonstrated that a reduction in *KISS1* expression correlates with poor prognosis in cancer patients. For example, Dhar *et al.* reported that in gastric carcinoma samples, reduction in *KISS1* expression was associated with increased venous and lymph vessel invasion and metastasis to

lymph nodes (50). In addition, patients with lower *KISS1* expression had significantly higher probability of disease recurrence and shorter overall survival (50). Esophageal squamous cell carcinomas scored for *KISS1* and *GPR54* showed that loss of either was a strong prognostic factor for lymph node metastasis (51). In fact, loss of *KISS1* and/or *GPR54* gene expression was detected in 86-100% of primary tumours in cases with lymph node metastasis (51). In pancreatic cancer, however, Masui *et al.* reported lower expression of *KISS1* but higher expression of *GPR54* (46). In bladder tissue, normal urothelium displayed high expression of *KISS1*, while 80% of invasive tumours showed little to no expression (52). Thus far, numerous types of cancer, including melanoma, gastric carcinoma, esophageal squamous cell carcinoma, pancreatic cancer, and bladder cancer, show that signaling of the kisspeptins and GPR54 may have anti-metastatic and tumour-suppressant effects.

1.6.2. KISS1/GPR54 in breast cancer

In contrast to the above-mentioned work, studies in breast and hepatocellular carcinoma have yielded contradictory results. A 2005 study by Martin *et al.* used human breast cancer tissues to correlate expression of *KISS1* with tumour grade and patient prognosis (3). Immunohistological staining showed high expression of *KISS1* in normal cells comprising vessels and ducts with no staining of cells in the stroma. Tumour cells, however, showed greatly increased *KISS1* expression in the cytoplasm of both tumour and surrounding cells. The researchers also observed a rise in *KISS1* and GPR54 expression in higher grade

cancers, although not statistically significant. Also, lobular cancers showed considerably higher *KISS1* expression when compared to ductal and other types of cancers, although again, significance was not reached. The researchers also tracked the clinical outcome of breast cancer patients after six years of treatment. It was discovered that patients with metastatic disease had elevated levels of *KISS1* expression (3). Furthermore, higher expression of *KISS1* was correlated with poor outcome; patients who had died of breast cancer had the highest expression of *KISS1*. In addition, when *KISS1* was introduced into human, metastatic MDA-MB-231 breast cancer cells, there was a significant increase in the invasiveness of these cells in Matrigel Transwell chamber invasion assays (3).

Similar to *KISS1/GPR54* expression studies in breast cancer, Ikeguchi *et al.* reported that expression of *KISS1* and *GPR54* correlates positively with hepatocellular carcinoma progression (53). In fact, *GPR54* mRNA was detected in 12.5% of normal livers, 26.7% of non-cancerous cirrhotic livers, and 48.3% of carcinomas. In addition, the 5-year disease-free survival rate of patients with normal expression of *KISS1* and *GPR54* was shown to be 51.8%, whereas overexpression of either gene resulted in a 25.7% survival rate (53). Thus, *KISS1/GPR54* expression and possibly signaling may be deleterious in a subset of human cancers.

1.6.3. GPR54 signaling in cancer cell lines

GPR54 has been shown to influence cell invasion, cell migration, and proliferation, in addition to its intracellular effects on the transcription of several

genes, including TIMPs and MMPs. Kp-10 has often been shown to have anti-migratory effects, including in MDA-MB-231 and MCF-7 breast carcinoma cells, and HT-1080 fibrosarcoma cells migrating towards TNF-α (54). Similar to the effects of metastin in HT-1080 cells, it was also observed that Kp-10 blocks TNF-α-induced NFκB nuclear translocation and IκB degradation (54). Furthermore, Kp-10 inhibited TNF-α-induced migration in MDA-MB-231 cells expressing a dominant active form of RhoA (RhoA G14V). Expression of a dominant-negative form of RhoA (RhoA T19N) resulted in inhibited migration towards TNF-α (54). The authors suggested, then, that TNF-α-induced cell migration required RhoA activity that was blocked by Kp-10-treatment (54).

A recent study examining the progression of non small-cell lung cancer (NSCLC) and matrix metalloprotease (MMP)-9 expression showed that a negative correlation exists between disease stage and *KISS1* expression (55). Furthermore, *KISS1* and MMP-9 expression exhibit a negative correlation (55). *In vitro* data showed more direct effects of *KISS1* on MMP-9. Expression of *KISS1* in HT-1080 resulted in decreased MMP-9 protein expression and showed decreased cell invasion in Matrigel-coated Transwell assays when compared to non-transfected cells (39). Studies in renal cell carcinoma cell lines demonstrated that treatment of KU19-20 cells with metastin resulted in reduced levels of MMP-2 in conditioned medium in comparison to untreated cells, upregulation of TIMP-1 (tissue inhibitor of metalloprotease-1), significantly inhibited wound closure and inhibited invasion through Matrigel-coated Transwell inserts (56). The pancreatic cell line PANC-1 treated with metastin also showed significantly inhibited

migration, but metastin had no significant effect on invasion across Matrigel-coated Transwell inserts (46). In CHO cells expressing GPR54, treatment with metastin resulted in a dose-dependent inhibition of migration towards 10% fetal bovine serum and significantly reduced wound closure (57).

A wide variation of results exists obtained from both *in vivo* and *in vitro* studies concerning *KISS1* and GPR54 signaling in cancer. Generally, *KISS1*/GPR54 signaling in numerous cancer cell lines is thought to produce anti-migratory and anti-invasive effects. In breast cancer it is unclear if kisspeptin/GPR54 signaling potentiates cell migration and invasion, processes required for metastasis. Gaining a better understanding of *KISS1*/GPR54 roles in breast cancer migration and invasion, therefore, is the focus of this study. The epidermal growth factor receptor is commonly observed to play roles in the migration and invasion of cancer, and has previously been shown not only to engage in indirect downstream cross-talk with GPCRs, but also directly interact to promote their signaling.

1.7.1. The epidermal growth factor receptor

The human epidermal growth factor receptor (HER, ErbB) family comprises four closely related receptors: ErbB1/EGFR/HER1 (from here designated as EGFR), ErB2/EGFR-2/HER2 (from here designated as ErbB2), HER3, and HER4 (58). Each member of the ErbB family has a similar structure consisting of a large extracellular domain, a single transmembrane-spanning domain, an intracellular juxtamembrane region, a tyrosine kinase domain, and a

C-terminal regulatory region (59). The extracellular region of the ErbBs comprises up to four subdomains: L1 and L2, comprised of a leucine-rich repeat domain, and CR1 and CR2, cysteine-rich domains (58).

1.7.2. ErbB ligands

The ligands for ErbB receptors can be divided into three main categories: a) epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), and amphiregulin (AR), which bind specifically to EGFR; b) betacellulin (BTC), epigen (EPN), epiregulin (EPR), and heparin-binding EGF-like growth factor (HB-EGF), which show dual specificity for both EGFR and ErbB4 (59); c) the neuregulins (NRG) that bind ErbB3 and ErbB4 (NRG-1 and NRG-2) or only ErbB4 (NRG-3 and NRG-4) (60). Each ErbB ligand contains an EGF-like core domain of approximately 60 amino acids, which is minimally required to elicit activity (59). ErbB2 has no known ligands and although the intracellular tyrosine kinase domain of ErbB receptors is highly conserved, ErbB3 contains substitutions of critical amino acids and lacks kinase activity (60). ErbB3 does, however, contain binding sites that when phosphorylated dock phosphatidylinositol-3-kinase (PI3K) (61). These residues are not present on the C-terminal domain of EGFR, and thus ErbB3 may co-operate with EGFR in promoting downstream signaling that EGFR alone is not able to participate in (61).

Binding of ligands to the extracellular domain of ErbB receptors induces homo- or heterodimer formation, resulting in the activation of the intrinsic

tyrosine kinase domain. The ability of ErbB ligands to induce homo- and hetero-dimerization expands ErbB receptor signaling potential (62). Heterodimerization appears to follow a hierarchical approach, with ErbB2 being the preferred binding partner for the other ErbB family members (63). Studies have shown that six possible heterotypic interactions exist within the ErbB family and three particularly strong combinations stand out: ErbB2-ErbB3 > ErbB2-ErbB4 > ErbB1-ErbB4 (63).

1.7.3. Downstream signaling of ErbB receptors

Downstream signaling of ErbB receptors is a multi-layered and cross-connected signaling network and includes the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/Akt, and signal transducers and activators of transcription (STAT) signaling cascades (58). The Ras/Raf/MAPK pathway is a major downstream signaling route of the ErbB family. Ligand binding to EGFR causes the phosphorylation of tyrosine residues that then act as binding sites for adaptor proteins Grb2 and the Ras guanine nucleotide exchange factor, Sos, that bind either directly (*via* Src homology 2 domains) or indirectly through Shc adaptor protein to specific intracellular EGFR docking sites (58, 64). This protein-receptor interaction results in the activation of Sos and activation of Ras (Ras-GTP) (58). Ras-GTP then activates downstream pathway cascades consisting of Raf, MEK, and ERK. Activated MAPKs phosphorylate and regulate specific intranuclear transcription factors, and may even activate their own upstream regulators or regulatory enzymes, such as phospholipase A_2 (58, 65).

The PI3K/Akt pathway serves to regulate cell growth, cell survival, apoptosis, resistance to chemotherapy, tumour invasion, and migration (58). EGFR-dependent activation of Akt occurs through heterodimer formation with ErbB3. PI3K docking sites are missing on EGFR, but are highly prevalent on ErbB3 (58). PI3K anchors to phosphorylated residues *via* its SH2 domain and catalyzes the phosphorylation of phosphatidylinositol 4,5-diphosphate to the second messenger phosphatidylinositol 3,4,5-triphosphate and activates the protein serine/threonine kinase Akt (58).

EGFR is able to regulate STAT pathways through Janus kinase (JAK)-dependent or independent pathways (66, 67). The STAT family of proteins are cytoplasmic transcription factors that exist as inactive monomers. When phosphorylated on tyrosine residues, the monomers dimerize by reciprocal SH2 phosphotyrosine interaction, then enter the nucleus to regulate transcription of specific target genes (68-70). In normal cells, STAT activation is transient, but in cancer, constitutive activity is often seen, especially with STAT3 and STAT5 (71, 72). In a number of tumour-derived cell lines, the STATs are required to maintain a transformed phenotype (72, 73). Excessive activation of JAK kinase through dysregulated tyrosine kinase activity and increased levels of tyrosine phosphorylation seems to be the most common method to constitutively activate STAT (72). EGFR expression levels have been shown to positively correlate with STAT3 activation in a variety of human malignancies, including head and neck squamous cell carcinoma (HNSCC) and breast cancer (71, 74).

Gene-targeting studies in mice have helped deduce the physiological importance of ErbB family signaling. Mice deficient for ErbB2, ErbB4, and NRG die at approximately E10.5 (embryonic day 10.5) due to defects in cardiac trabeculae formation, leading to an enlarged heart with an irregular heartbeat and poor circulation, and irregular peripheral nervous system development (75-77). Mice lacking ErbB3 showed thinning of the myocardium at E13.5 and cardiac valves that appeared to be underdeveloped and unable to support normal cardiac function (78). In addition, ErbB3-deficient mice showed abnormal cerebellar development, with little differentiation of the cerebellar plate and the neuroepithelial layer lining the fourth ventricle (78). Whereas the ErbB3-knockout mice had comparatively slight defects, EGFR-null mice died before implantation, at mid-gestation, or within 2-3 weeks after birth, depending on genetic background (79). These mice also displayed respiratory failure, necrotizing enterocolitis-like disorder, and general defective or delayed epithelial development (79).

1.7.4. EGFR and cancer

The role of growth factor-driven signaling in pathogenesis has been long established. Multiple studies have shown that overexpression of either EGFR or ErbB2 leads to *in vitro* transformation of mouse NIH-3T3 cells (80, 81). Overexpression of ErbB ligand TGF-α has also been shown to transform Rat-1 and NRK fibroblasts, but NIH-3T3 cells overexpressing TGF-α also required concomitant expression of EGFR (82, 83). *In vivo* studies, however, generally

show that ErbB2 has a higher transforming ability than EGFR. Studies have shown that ErbB2 to be an amplifier for the signaling of other ErbB family members. Transgenic mice expressing *neu* (the mouse analog of ErbB2) also showed an upregulation in EGFR expression (84). In another model, expression of *neu* resulted in increased expression of ErbB3, and, more interestingly, showed constitutive activation (85). Co-expression of *neu* and TGF-α resulted in an incidence of 95% in palpable mammary tumours by 250 days, whereas either gene alone produced tumours only after a long latency, with only a 6% and 35% incidence in TGF-α and *neu*-expressing mice, respectively (85).

Evidence confirms that the transforming abilities of the ErbB receptors are enhanced when different receptors are co-expressed. In the rodent fibroblast cell line NIH 3T3, overexpression of EGFR transforms cells only when EGF was added to the culture medium, and expression of ErbB2 alone was not sufficient to transform cells (86). However, co-expression of EGFR and ErbB2 caused transformation, producing colonies in soft agar, and forming tumours in nude mice (86).

Taken together, studies in ErbB-induced transformations suggest that co-expression of different ErbB receptors is necessary for full transformation, both *in vitro* and *in vivo*. Studies involving human primary carcinomas have strengthened this point and have brought to attention the complexities involved in targeting ErbB in cancer progression. High expression of ErbB receptors in the majority of human carcinomas has previously been observed, with positive EGFR or ErbB3 expression ranging from 50% to 70% of breast carcinomas, and ErbB2 in 30% of

breast carcinomas (87). Due to the high incidence of increased ErbB receptor expression among human carcinomas, co-expression of multiple ErbB types will occur in the majority of tumours.

A study of invasive breast carcinoma found that 38% of patients showed ErbB2 expression, and of these 35% also expressed EGFR (88). Nearly all of the EGFR-positive tumours (87%) co-expressed ErbB2 (88). In addition, 5% of patients also expressed an activated form of ErbB2, designated P-ErbB2 (88). Patients who expressed ErbB2, P-ErbB2, and EGFR showed the shortest relapse-free survival and disease-free survival. In contrast, tumours that were negative for all three markers, or expressed only EGFR or non-phosphorylated ErbB2 showed better clinical outcome (88). In agreement with this study, co-expression of EGFR, ErbB2, and ErbB3 was found to have a negative synergistic effect on clinical outcome, independent of tumour size or lymph node status (89). In addition, EGFR and ErbB2 showed significant correlation with tumour grade. Similarly, best clinical outcome was seen in patients who were negative for all four members of the ErbB family, with the shortest disease-free survival for patients expressing two or more receptors (89).

Although the importance of co-expression of the ErbB receptors in human carcinoma is well established, we cannot overlook the effects of ErbB ligands on disease progression. A number of studies have reported on the effects of ErbB ligands in human carcinomas. In numerous cancer cell lines, including from breast, ovarian, gastric, and bladder, knockdown of HB-EGF, amphiregulin, or TGF-α using siRNA resulted in significant apoptosis (90). Combinations of ErbB

ligand-neutralizing antibodies have shown synergistic activity on growth inhibition of the head and neck squamous cell carcinoma cell (HNSCC) line LICR-HN-5 (91).

Genetic alterations of the ErbB receptors in human cancer have been well studied. Gene amplifications and mutations have all been shown to occur and have important consequences for patients. Of these mutations, type III EGFR variant (EGFRvIII) represents more than 50% of the genomic alterations observed in numerous tumors, including intraductal and infiltrating ductal breast cancer (92). This variant carries an in-frame deletion of 801 base-pairs in the extracellular domain, resulting in a new codon at a novel site in the transcript that translates to a glycine residue at the fusion point (93). It has been noted that NIH 3T3 cells expressing this variant show weak, but ligand-independent, transforming activity. EGFRvIII has also been shown to constitutively interact with the adaptor proteins, Shc and Grb2, resulting in a two-fold increase in Ras activity and a two-fold increase in DNA synthesis when compared to parental U87MG glioblastoma cells (94). Interestingly, NIH-3T3 cells expressing EGFRvIII were three times as resistant to paclitaxel (a microtubule stabilizer) toxicity as parental cells and showed suppressed paclitaxel-induced tubulin polymerization (95). Introduction of EGFRvIII into the human breast carcinoma cell line MCF-7 resulted in enhanced growth in anchorage-independent assays and enhanced tumorigenicity in nude mice (96).

1.7.5. EGFR and cell migration

The effects of EGFR activation on cancer cell migration and invasion, processes required for metastasis, has been well studied. The downstream mechanisms vary depending on cell line, ErbB complement, and ErbB downstream-signaling pathway component expression. In 3-dimensional Matrigel invasion assays, treatment of invasive human breast carcinoma cell line MDA-MB-231 with EGF resulted in a significant increase in cell invasion (97). The authors suggested that EGFR activation promotes the formation of invadopodia through the activation and localization of the Arp2/3 complex, N-WASP, and cortactin to the cell membrane (97). Treatment with AG1478, an EGFR kinase inhibitor, abolished the morphological changes associated with invadopodia formation in MDA-MB-231 cells.

EGF can stimulate BT474 breast carcinoma cell invasion *via* c-Src and FAK (98). In addition, treatment with EGF showed a marked increase in FAK phosphorylation, suggesting that FAK plays a role in the EGF-induced invasion of these cells (98). Indeed, it has been noted that treatment with EGF can result in the disruption of focal adhesions, allowing the cell to break free of cell-substratum contacts (99). In addition, it was noted that inhibition of MEK activity also resulted in a prolonged EGF-induced contraction, suggesting that the ERK pathway partially regulates cell de-adhesion (100).

Other studies have shown that EGFR can directly activate proteins involved in cytoskeletal rearrangement. In human breast cancer MDA-MB-231 cells, EGFR activation causes the activation of the activation of the small GTPase

Arf6 (101). Arf6 then recruits proteins involved in the formation of membrane protrusions, including cortactin and paxillin, resulting in membrane invagination and the production of invadopodia (101, 102). Treatment of cells with EGF resulted in the phosphorylation of the myosin light chain both by Rho kinase and myosin light chain kinase (MLCK) (103). Activation of myosin II is necessary for force generation during cell motility. In addition, EGFR has been shown to activate the small GTPase Rac, necessary for the formation of lamellipodia and cell spreading on fibronectin (104).

EGF-treatment can also induce upregulation of integrin expression. The integrins are a major family of cell surface receptors that have been recognized as the predominant family of cell adhesion receptor that mediate attachment to the extracellular matrix and provide traction to migrating cells (105, 106). EGF-treatment of cervical adenocarcinoma cells (CAC-1) resulted in the upregulated expression of α2β1-integrin (105). An anti-α2β1-integrin antibody prevented EGF-induced motility of CAC-1 cells on both collagen IV and fibronectin (105).

Stimulation of cells overexpressing EGFR results in the temporal activation of certain downstream pathways that positively regulate cell migration. In breast cancer cell lines MCF-7 overexpressing EGFR (EGFR/MCF-7), as well as in MDA-MB-468 which naturally have elevated EGFR expression, a role for PKC-δ and MLCK in the regulation of persistent cell migration was shown (from 4 to 12 hours), while early stages of migration (up to 4 hours) were mediated by MAPK signaling (107).

Since EGFR signaling regulates tumour progression, this receptor is a critical target for chemotherapy. To date, two major classes of EGFR-targeted therapies have been developed: anti-ErbB monoclonal antibodies and ErbB-specific tyrosine kinase inhibitors. The purpose of the monoclonal antibodies is to bind to the extracellular domain of EGFR, preventing ligand binding and ligand-receptor internalization (58). Tyrosine kinase inhibitors aim to block the binding of adenosine triphosphate to the intracellular tyrosine kinase domain of EGFR, blocking tyrosine kinase activity and abrogating downstream signaling.

1.7.6. Targeting breast cancer

As more information becomes available with the aid of scientific research, more physicians are choosing to employ combination therapy to optimize clinical outcomes. However, to date it is not fully known whether or not sequential treatment with a second agent after disease progression or a combination of multiple agents given concurrently is better for patient outcome (7). Evidence suggests, however, that once a patient has been diagnosed with metastatic breast cancer, a finite life expectancy may be expected, and some patients may already have acquired resistance due to the use of chemotherapeutics for non-metastatic disease (7). Ideal combinations usually include two or more chemotherapeutics possessing complementary action, with no cross-resistance and safety profiles that do not overlap. In addition, these treatments should show synergistic or additive effects, resulting in outcomes more advantageous than any medication alone (7).

Currently the Federal Drug Administration (FDA) has approved a variety of combination therapies for the treatment of metastatic breast cancer. Primary or acquired resistance often precludes the use of anthracyclines (DNA intercalating agents) or taxanes (microtubule-stabilizing agents), and the inclusion of high doses of anthracyclines increases the risk of cardiotoxicity (7). Therefore, many standard combinations use two non-anthrocycline cytotoxic agents, including an alkylating agent, an antimetabolite, or a vinca alkaloid (7). Newer medications are now included in the treatment of tumours resistant to taxanes or anthracyclines, or to patients unable to tolerate such medications. The FDA has approved the use of drugs like ixabepilone, nano-particle albumin-bound paclitaxel, trastuzumab, lapatinib, and bevacizumab in a defined group of patients with metastatic breast cancer (7).

Treatment for HER2– patients has only recently been developed. Approved for first-line therapy against metastatic breast cancer is the vascular endothelial growth factor (VEGF)-targeting antibody bevacizumab in combination with paclitaxel. At present, bevacizumab is the most advanced anti-VEGF agent in development, showing promising phase III results in metastatic breast cancer (108). Results indicate that bevacizumab combination with either paclitaxel or docetaxel showed significantly longer progression-free survival. Most importantly, it was noted that bevacizumab treatment was effective across a broad range of patient subgroups, including the elderly, those with prior adjuvant chemotherapy, and those with ER/PR/HER2– status (108). A review of current treatments against cancer may be seen in Fig. 1.8.

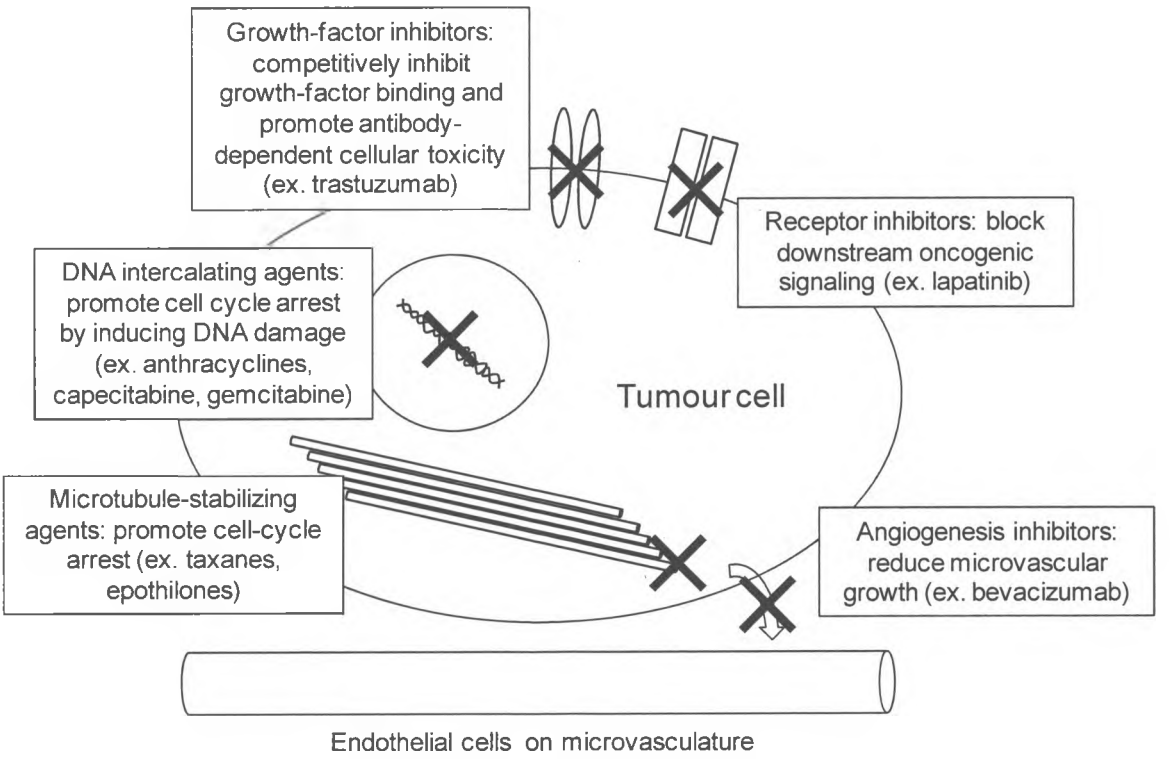

Fig. 1.8. Currently used drug treatments for cancer therapy. Classical chemotherapy is still a main component of cancer therapy, and includes agents that promote cell death by inducing DNA damage and agents that cause cell-cycle arrest by inhibiting components of the cytoskeleton. More advanced therapies aim to target specific mechanisms in order to mitigate the off-target side effects commonly observed in classical chemotherapy. These agents include those that inhibit signaling of growth factor receptors and those that inhibit angiogenesis.

1.7.7. Targeting EGFR for cancer therapy

Two main treatment options exist for targeting EGFR and its interacting ErbB family members: monoclonal antibodies and tyrosine kinase inhibitors. Among the monoclonal antibodies (mAb), cetuximab (IMC-225, Erbitux™, Bristol-Meyers Squibb, Merck, Imclone Systems), a human-murine hybrid immunoglobulin has been the best studied, and in 2004 was the first mAb to be approved by the FDA for the treatment of irinotecan-refractory metastatic colorectal cancer (mCRC) (109). Cetuximab has a higher affinity for EGFR than do either EGF or TGF-α, and treatment resulted in antibody-dependent cell-mediated cytotoxicity, inhibition of EGFR activation, and marked tumour growth inhibition *in vivo* (110). Cetuximab is not only effective against wild-type EGFR, but also targets mutant forms that have been found to naturally occur in humans. In non-small cell lung cancer expressing the point mutation L858R, deletion mutant delL747-T753ins, and T790M, binding of the mutant EGFR was shown, and resulted in an inhibition of MAPK activity (111). Interestingly, while cetuximab did not induce any significant degradation of wild-type EGFR, it did result in profound degradation of mutated receptors as early as 24 hours post-treatment. *In vivo* studies showed growth inhibition of all wild-type and mutant-EGFR harbouring NSCLC cell lines (111). Taken together, these results showed that patients with EGFR mutations may benefit from cetuximab treatment, and may even respond better than patients with wild-type EGFR.

Apprehension surrounding the induction of an immunological response to cetuximab prompted the creation of a fully human mAb. Panitumumab (Vectibix,

Amgen, Thousand Oaks, CA, USA) works in a similar manner to cetuximab, binding the extracellular domain of EGFR. Because of its fully human architecture, it offers a lower rate of allergic reactions or anaphylaxis (112). However, it was noted that panitumumab response was limited to patients with wild-type K-ras, as no patient harbouring a K-ras mutation responded to panitumumab treatment (113). The authors suggested that the Ras/Raf/MAPK signaling pathway is primarily responsible for the clinical activity of panitumumab in metastatic colorectal carcinoma (113).

The humanized anti-ErbB2 monoclonal antibody trastuzumab (Herceptin™, Genentech) is commonly used in breast cancer either as a mono- or combination therapy. In patients expressing ErbB2, the use of the monoclonal antibody trastuzumab has been evaluated against a combination of trastuzumab and an anthracycline (7). However, severe cardiotoxicity was observed (a risk in any anthracycline-involved treatment), and thus the use of combined trastuzumab with anthracycline-based therapy is no longer recommended (7). Notable however, is the use of trastuzumab with liposomal anthracyclines that have improved safety profiles for future development in metastatic breast cancer. Trastuzumab in combination with either paclitaxel or docetaxel is approved for first-line therapy in ErbB2-positive breast cancer patients.

The mechanisms by which trastuzumab act are still not completely clear. Unlike the antibodies against EGFR, the efficacy of trastuzumab appears to be mediated by several pleomorphic processes, as ErbB2 has no known ligand (114). Some reports show that treatment with trastuzumab results in the inhibition of

PI3K and MAPK signaling, promoting cell cycle arrest and apoptosis (114). Other studies have shown that trastuzumab activates antibody-dependent cellular cytotoxicity through its FC (fragment crystallisable) region, recruiting Natural killer cells *via* their FcRγIII receptor (115, 116).

1.7.8. Resistance to EGFR inhibition

Resistance to antibody therapy can occur in several ways. In colorectal cancer, it has been found that almost all patients harbouring a K-Ras mutation are resistant to monoclonal antibody therapy with panitumumab or cetuximab (117). A loss of PTEN (phosphatase and tensin homolog, a tumour suppressor), furthermore, was shown not only to predict objective tumour response, but also worse overall survival in patients with metastatic colorectal carcinoma treated with panitumumab and cetuximab (117). A similar consequence is seen in breast tumours, where PTEN loss results in poor outcome and contributes to trastuzumab resistance (118, 119). In head and neck squamous cell carcinoma, EGFRvIII, which lacks the extracellular ligand-binding domain and has constitutive activity, was found in 42% of patients (120). HNSCC cell lines expressing EGFRvIII showed resistance to cetuximab-induced cell death, prompting the idea that HNSCC patients harbouring this mutation will receive little benefit from monoclonal antibody treatment but would benefit from a genetic screen looking at their EGFR compliment (120).

Changes in receptor ligands have also been shown to contribute to EGFR antibody treatment resistance. It was noted that trastuzumab treatment resulted in

the upregulation of TGF-α expression in a neoplastic tissue biopsy (119). Increased TGF-α impaired trastuzumab-induced ErbB2 downregulation and led to resistance to anti-proliferative effects (119). Additionally, it has been postulated that because trastuzumab binds an epitope in ErbB2 that is not involved in ErbB2 heterodimerization with other ErbB receptors, increased levels of those receptors could help overcome trastuzumab effects (121).

Increased EGFR/ErbB2 cross-talk has found to play important roles in tumour behaviour, especially in trastuzumab-resistant cells. Dual-kinase inhibitors have recently been developed that target both EGFR and ErbB2 in an effort to prevent EGFR-mediated activation of ErB2 (121). The use of dual kinase inhibitors, in addition to HER2-targetting, has shown great promise in the treatment of advanced and HER2-overexpressing breast cancer following treatment with other agents, including taxanes, anthracyclines, and trastuzumab. These inhibitors block the intracellular signaling of the epidermal growth factor receptor (EGFR), known to promote invasion and worsen clinical behaviour, in addition to their effects on HER2. Lapatinib, the first dual kinase inhibitor approved by the FDA for metastatic breast cancer, is currently indicated in combination with capecitabine in advanced or metastatic HER2-overexpressing breast cancer (7). Lapatinib has been shown to readily prevent the phosphorylation of ErbB2 and spontaneous ErbB2 fragments, and clearly reduces tumour growth (122).

Resistance to tyrosine kinase inhibitors commonly involves mutations in both EGFR and ErbB2. Acquired resistance to tyrosine kinase inhibitors may

occur when patients who initially responded to tyrosine kinase inhibitors and develop EGFR mutations, or where treatment with tyrosine kinase inhibitors selects for already existing mutants. Understanding of pharmacogenetics and genetic screening of patients will help clinicians in tailoring a treatment regimen that will produce the best possible outcomes in the least time, hopefully avoiding acquired resistance.

1.8.1. GPCR/epidermal growth factor receptor signaling cross-talk

Signaling cascades were classically considered to be discrete pathways that linked the activation of a receptor to downstream signaling partners and physiological outcomes in a linear manner. Recent insight into how receptors function has shed new light on the true complexity of receptor networks. With the discovery that receptor families integrate signaling, there is a realization that the cell is vastly more complicated than we have previously considered.

In the early 1990s, it was noticed that stimulation of some cells with GPCR agonists resulted in the activation of the Ras/MAPK pathway leading to mitogenesis, although the underlying mechanisms were not known (123). It was not until 1996 that Daub *et al.* reported that the epidermal growth factor receptor (EGFR) was responsible for transduction of these GPCR-originating signals (124). This landmark work showed that EGFR could be activated in Rat-1 fibroblasts in response to endothelin-1, lysophosphatidic acid (LPA), thrombin and thrombin-receptor peptide. Studies that followed revealed that other receptor

tyrosine kinases could become tyrosine phosphorylated in response to EGFR activation in certain cells, a phenomenon known as transactivation (124).

Receptor tyrosine kinases, such as EGFR, platelet-derived epidermal growth factor receptor (PDGFR), and insulin-like growth factor-1 receptor (IGF-1R), transmit signals that regulate cell proliferation and differentiation, promote cell migration, survival, and alter metabolism (125). They play critical roles in a wide range of physiological processes, including embryonic development, growth of an organism, angiogenesis, synaptic plasticity, and oncogenesis.

1.8.2. Physiological roles of EGFR transactivation

A number of receptor tyrosine kinases are now known to mediate GPCR signaling in normal physiological processes, in addition to pathological conditions. Angiotensin II (ANG II), for example, is a multifunctional peptide for the ANG II type I and II receptors (AT_1R and AT_2R, respectively) that are involved in the renin-angiotensin system controlling blood pressure, and have important roles in the survival of fetal vascular smooth muscle cells (126). ANG II induces the activation of EGFR, which then serves as a scaffold for the assembly of signaling proteins, including the MAPKs and Akt (126). Pathologically, it was noted that ANG II signaling through EGFR results in hypertension and left ventricular hypertrophy, in addition to inducing renal deterioration (127, 128).

The transactivation of EGFR by the β_2-adrenergic receptor has recently been shown to be intimately involved in cardiac function (129, 130). EGFR is

itself critical for normal cardiac function and formation; the loss of EGFR or HB-EGF in mice results in enlarged hearts, hypertrophic cardiomyocytes, abnormal cardiac valves, dilation of cardiac chambers, and decreased systolic function (131).

The activation of receptor tyrosine kinases by GPCRs also plays critical roles in the central nervous system. The transactivation of receptor tyrosine kinases by GPCRs further plays important roles in gene transcription in the hypothalamic-pituitary-gonadal axis, specifically in pituitary derived LβT2 gonadotropes (132). It was observed that stimulation of GT1-7 immortalized neuronal cells with gonadotropin-releasing hormone (GnRH) resulted in the transactivation of EGFR, the induction of its downstream partners, including ERK1/2, activation of c-*fos*, and luteinizing hormone gene expression (132). Interestingly, no nuclear accumulation of ERK1/2 was visible, and thus it was unclear how transcriptional activation could occur. The importance of EGFR signaling was realized when it was discovered that GnRH signaling through EGFR causes the marked stimulation of RSK-1, a cytoplasmic serine/threonine kinase that once activated by ERK1/2, translocates to the nucleus to alter gene transcription (132).

1.8.3. EGFR transactivation in pathological processes

GPCR-mediated receptor tyrosine kinase transactivation also regulates pathological processes. The groundbreaking 2002 work by Gschwind *et al.* demonstrated for the first time that transactivation of EGFR by the bioactive lipid

molecule lysophosphatidic acid (LPA) not only results in increased proliferation of HNSCC cells, but also enhanced the migration and motility of these cells (133). LPA also causes the transactivation of EGFR in ovarian cancer cells and activation of ERK1/2 (134). In kidney cancer cell lines (Caki2, ACHN, HK2, A498, and A704) and bladder cancer cell lines (SCABER, HT1372, TccSup, and 5637), LPA stimulated cell migration and invasion, an effect that was abrogated in the presence of EGFR kinase inhibitor, AG1478 (135).

Several other GPCRs have been shown to interact with EGFR in cancer cells. A recent study revealed using FRET that the $G_{i/o}$-coupled somatostatin receptors (SSTR), SSTR1 and SSTR5 exist as pre-coupled heterodimers with EGFR under basal conditions in human breast cancer MCF-7 and MDA-MB-231 cells (136). Upon treatment with EGF, SSTR1/EGFR heterodimers dissociated in MCF-7 cells, while an increase was shown in MDA-MB-231. In MCF-7 cells, treatment with SST did not change the association between either SSTR1 or SSTR5 and EGFR (136). Most interestingly, SSTR5/EGFR heterodimers dissociated with SST treatment, but remained unaltered with EGF (136). The authors also determined that dissociation of the receptors is necessary in promoting the activation of ERK1/2 in response to either SST or EGF (136). Combined treatment with SST and EGF resulted in delayed ERK1/2 activation, suggesting that regulation of EGFR signaling can be altered at the cell surface directly by other receptors (136). It is clear, then, that the details of the mechanism by which GPCRs crosstalk with EGFR require further investigation.

For a summary of the functions of EGFR transactivation, please see Table 1 below.

1.9.1 Mechanisms of EGFR transactivation by GPCRs

1.9.2. The ADAMs

The mechanisms that govern transactivation of receptor tyrosine kinases by GPCRs have been the subject of some debate. The knowledge that GPCRs could contribute to mitogenic signals has been known for quite some time. Lysophosphatidic acid, thrombin, bradykinin, endothelin, angiotensin II, acetylcholine, dopamine, and adrenergic agonists were shown to elicit mitogenic responses and could promote proliferative signaling (137). The idea that GPCRs could activate the Ras-Raf-MEK-ERK1/2 pathway was widely known, but how they were producing activation of this cascade was not clear. Involvement of Src, adaptor proteins like Shc, and non-receptor tyrosine kinases such as Fyn, Lyn, and Yes, Ca^{2+}-dependent protein tyrosine kinase Pyk2, and PI3K were all considered as players in this mechanism. A revelation occurred in 1999 when a new model was proposed explaining receptor tyrosine kinase transactivation: the "triple membrane passing pathway" (138). In this model, agonist stimulation of GPCRs results in the activation of matrix metalloproteases of the ADAM (a disintegrin and metalloprotease) family, resulting in the production of EGF-like ligands (HB-EGF, TGF-α, amphiregulin, neuregulin) (138). Subsequent release of the mature growth factor activated EGFR and its downstream signaling cascades.

The ADAMs belong to the metzincin subfamily of zinc proteases, are membrane-bound, and shed the ectodomains of membrane-bound growth factor precursors. There are 19 ADAM genes in humans, but several appear to have multiple splice forms and show differences in their cellular expression patterns (137). The pro-domain of catalytically active ADAMs is believed to function as an intramolecular chaperone, and once an ADAM is properly folded, the chaperone keeps the enzyme inactive until its removal by a furin-type pro-protein convertase or even by autocatalysis (139).

Which MMP is used in triple membrane passing appears to be dependent on cell type and the GPCR involved. For examples, the transactivation of EGFR by the platelet-activating factor receptor (PAFR) specifically involved ADAM10-mediated cleavage of HB-EGF in human epithelial cell line HM3 (140). Existing data has also shown that one agonist may release a particular EGFR-ligand *via* different ADAMs. For example, LPA can generate HB-EGF by ADAM17 in CaKi2 and A498 cells, but use ADAM10 in ACHN cells (137). What has also become clear is that a particular agonist may release different EGFR-ligands through activation of the same ADAM. In CaKi2 cells, LPA stimulates the release of HB-EGF, but in squamous cell carcinoma (SCC) cells, amphiregulin is shed, in both cases by ADAM17 (137, 141). In addition, one agonist can stimulate different ADAMs to process the same EGFR-ligand. LPA, for example, stimulates the release of amphiregulin *via* ADAM17 in SCC cells but uses ADAM15 in 5637 bladder carcinoma cells (135, 137, 141).

The mechanisms involved in GPCR-mediated activation of ADAMs appear to be highly complex and diverse. Multiple GPCRs are able to activate ADAM-dependent EGFR transactivation, thus implicating the involvement of two or more distinct heterotrimeric G proteins and associated subunits in this reaction. Using the α_{2A}-adrenergic receptor (α_{2A}AR) in COS-7 cells as a model of GPCR-mediated transactivation of EGFR and ERK1/2 activation, Pierce *et al.* were able to show that upon stimulation of cells with carbachol, the G$\beta\gamma$ subunit release from G_i was required for ERK1/2 activation, an effect that was abrogated in the presence of pertussis toxin (which inhibits G_i *via* ADP-ribosylation) (142). In addition, they determined that the dissociation of G$\beta\gamma$ is required for the activation of c-Src, an activation that was required in HB-EGF shedding and EGFR transactivation (142). The authors thus proposed a model whereby dissociated G$\beta\gamma$ was directly required for c-Src activation. However, the authors noted that the direct and EGFR-mediated routes to ERK1/2 activation diverged downstream of c-Src (142). In contrast, McCole *et al.* noted that carbachol stimulation of colonic epithelial cells requires TGF-α release by a then-unknown MMP, but TGF-α shedding did not require Src activity (143). Based on previous work and data from this publication, the authors did suggest, however, a mechanism of transactivation involving Ca^{2+} (143). Carbachol-induced increases in intracellular calcium concentrations would activate calmodulin, which then activates Ca^{2+}-dependent tyrosine kinase Pyk-2. Pyk-2 then associates with Src and may activate ERK1/2 directly or potentiate EGFR signaling through the phosphorylation of specific tyrosine residues (143).

Studies involving G_q-coupled GPCRs have shown interesting results. Using the angiotensin-1 receptor (AT_1R), Mifune *et al* were able to show that inhibition of G_q markedly inhibited intracellular calcium elevation, HB-EGF shedding, and EGFR transactivation (144). Furthermore, the authors noted that inhibition of intracellular Ca^{2+} elevation completely blocked HB-EGF shedding and that treatment of COS-7 cells expressing AT_1R with the selective phospholipase C (PLC) activator m-3M3FBS stimulated EGFR transactivation (144). The data suggested that G_q-dependent PLC-β activation is required for metalloprotease activation involved in EGFR transactivation through AT_1R (144). Most interestingly, shedding of HB-EGF was also dependent on the production reactive oxygen species (ROS), previously shown to be dependent on AT_1-mediated activation of NADPH oxidase *via* PKC (145).

1.9.3. The PKCs

The importance of PKCs in ADAM activation has been highlighted in several experimental systems. In *ADAM17*$^{-/-}$ mouse embryonic fibroblasts, stimulation with phorbol 12-myristate 13-acetate (PMA), a potent PKC activator, showed significantly less shedding of TGF-α, amphiregulin, epiregulin, and HB-EGF shedding when compared to wild-type cells, suggesting a critical role for PKC in ADAM17 activation (146). C9 rat liver epithelial cells treated with ANG II showed increased EGFR phosphorylation, in what was determined to be a PKC-mediated MMP-involved mechanism (147). Likewise, stimulation of the α_1-adrenergic receptor by phenylephrine caused the activation of EGFR in GT1-7

neuronal cells (148). The authors found that this transactivation required MMP activation by PKC, as PKC inhibition abrogated ERK1/2 activation in response to phenylephrine (148).

1.9.4. Src kinase

Src involvement with PKC-mediated metalloprotease activation has been noted to occur downstream of activation of several GPCRs. Activation of the Gonadotropin-releasing hormone receptor (GnRHR) by GnRH activates ERK1/2 in a partly EGFR-mediated process in HEK293 and in GT1-7 cells (132). Activation of GnRHR in GT1-7 cells employs a mechanism that involves PKC signaling to Src, Src-mediated activation of MMPs, EGF-ligand signaling to EGFR, and finally activation of ERK1/2 (132).

Phosphorylation of ADAM by a protein kinase appears to be a common occurrence. Diaz-Rodriguez *et al.* were able to show that ERK1/2 phosphorylates the intracellular domain of ADAM17 on threonine^{735} (149). Interestingly, it was shown that in COS-7 cells, treatment with sorbitol or hydrogen peroxide resulted in EGFR activation, in what was shown to be a p38-mediated mechanism, but dependent on MMPs for HB-EGF shedding, implying p38 as an upstream component of HB-EGF shedding by MMPs (150). However, the most commonly reported mechanism involves Src kinase directly involved in the shedding of EGFR ligands. Using various α_{2A}-adrenergic receptor-based models of EGFR transactivation, it was determined that inhibition of Src in a cell designated "donor" (for being the cell "donating" the shed HB-EGF) but not in the acceptor

cell (the cell using the shed EGFR ligand from the donor cell) abrogated shedding of HB-EGF and prevented ERK1/2 activation in the acceptor cell (142).

1.9.5. β-arrestins

Interactions of GPCRs with scaffolding proteins have also been shown to play vital roles in transactivation. Studies using β1-adrenergic receptor (β1-AR) have shown that recruitment of the GPCR scaffolding protein β-arrestin is essential not only for the induction of β1-AR-induced EGFR transactivation, but also for the interaction between β1-AR and EGFR (151). It was also shown that β-arrestin is required in dobutamine (β1-AR agonist)-induced internalization of the β1-AR/EGFR complex and pERK1/2 trafficking in the cell (151). However, stimulation of EGFR with EGF does not recruit β-arrestin, and EGFR internalizes alone (151). Buchanan *et al.* were able to show in colorectal carcinoma cell lines LS-147T and HCA-7 that treatment with prostaglandin E_2 caused EGFR activation *via* a β-arrestin 1-dependent activation of c-Src (152). In addition, using a phosphorylated mutant of β-arrestin 1 (P91G-P121E β-arrestin 1) that is unable to bind c-Src, they showed that PGE_2-stimulated migration and invasion of LS-147T cells was significantly reduced (152). β-arrestin 2 has been implicated to regulate ANG II-mediated transactivation of EGFR in vascular smooth muscle cells, signaling *via* c-Src (130). The authors noted that Src inhibition causes abrogation of EGFR activation, suggesting that Src is located upstream of EGFR activation (130). The general mechanisms known to date by which GPCRs can transactivate EGFR are summarized in Table 1.1 and Figure 1.9.

Cell type	Ligand (Receptor)	Mechanism	Functional Purpose
MDA-MB-231	S1P (S1PR), LPA (LPA_1-R), thrombin (PAR-1)	TACE and ADAM15-mediated shedding of HB-EGF	Promote migration
T47D and MDA-MB-468	Vasoactive intestinal peptide (VPAC1 and VPAC2 receptors)	Src-activated ADAM-mediated shedding of HB-EGF	Promotes activation of HER2
MCF-7	S1P (S1PR), LPA (LPA_1-R) thrombin (PAR-1)	TACE and ADAM15-mediated shedding of HB-EGF	?
Primary breast epithelial cells	Bradykinin (Bradykinin receptors 1 and 2)	PKC-δ	Induced proliferation, ERK1/2 activation, c-Fos expression
Kidney cancer cells lines (Caki2, ACHN, HK2, A498, A704)	Angiotensin (Angiotensin II-R), bombesin (Bomb-R), bradykinin, carbachol (mAchRs), ET-1 (ET_A), LPA (LPA_1-R), thrombin (PAR-1)	ADAM 10, 15, 17-mediated shedding of amphiregulin, HB-EGF, TGFα	Promote migration and invasion
Bladder cancer cell lines (SCABER, HT1376, TccSuo, 5637)	Angiotensin (Angiotensin II-R), bombesin (Bomb-R), bradykinin, carbachol, ET-1 (ET_A), LPA (LPA_1-R), thrombin (PAR-1)	ADAM 10, 15, 17-mediated shedding of amphiregulin, HB-EGF, TGFα	Promote migration and invasion
Pancreatic (PC3, N15C6)	SDF-1 (CXCR4)	Src-activated ADAM 10- and 17-mediated shedding of amphiregulin	Promote proliferation
HCEC	LPA (LPA_1-R)	ERK-induced activation of ADAM17-mediated HB-EGF shedding	Enhanced wound healing of cornea

Table 1.1. Functional consequences of EGFR transactivation and common mechanisms involved.

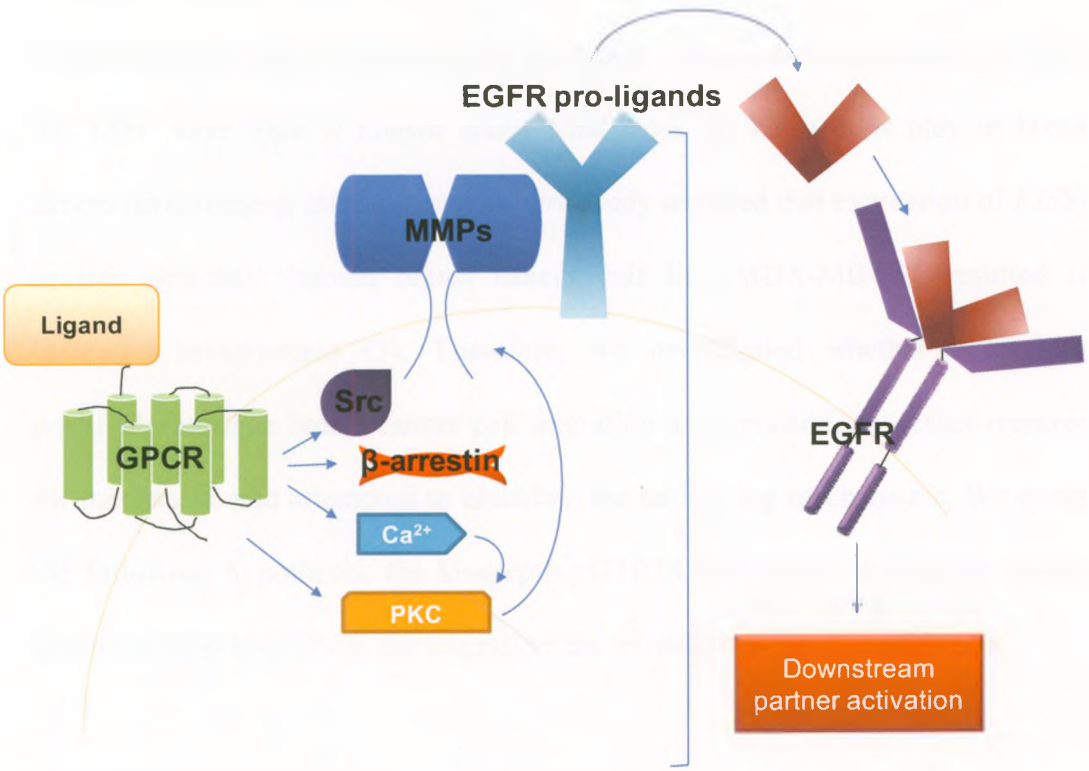

Fig. 1.9. General mechanisms involved in the transactivation of EGFR by GPCRs. Mechanisms include 1) shedding of EGFR pro-ligands from the surface of cells by β-arrestin/Src-activated matrix metalloproteases, 2) involvement of Ca2+ and 3) PKC-mediated activation of the matrix metalloproteases.

1.10. Rationale and Hypothesis

Kisspeptin signaling *via* GPR54 mediates a plethora of cellular processes in both non-malignant and tumourigenic cells. In many cancers, including pancreatic, thyroid, renal, and melanoma, expression of *KISS1/GPR54* results in anti-metastatic effects and negatively correlates with tumours progression (22, 46, 47, 153). Very little is known about what roles the kisspeptins play in breast cancer invasiveness, although one *in vitro* study revealed that expression of *KISS1* in the metastatic human breast cancer cell line MDA-MB-231 resulted in increased invasiveness (3). Therefore, we investigated whether kisspeptins positively regulate breast cancer cell migration and invasion, processes required for metastasis, and attempted to elucidate the underlying mechanisms. We tested the following hypothesis: the kisspeptins/GPR54 activate the epidermal growth factor receptor to promote the migration and invasion of breast cancer cells.

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[1] http://www.cancer.ca/canada-wide/about%20cancer/cancer%20statistics/stats%20at%20a%20glance/breast%20cancer.aspx

CHAPTER 2: GPR54 TRANSACTIVATES EGFR TO PROMOTE BREAST CANCER CELL INVASIVENESS

2.1 Introduction

In 1996 a new metastasis-suppressor gene was discovered (1). Named *KISS1*, it produces a 145 amino acid peptide, which is then processed into 54, 14, 13, and 10 amino acid length residues – known as kisspeptins – before secreted by the cell (2). The kisspeptins are ligands for GPR54, a $G_{q/11}$-coupled G protein-coupled receptor (GPCR) (3). Activation of GPR54 by the kisspeptins results in the activation of phospholipase C, protein kinase C, and the mitogen-activated kinase cascade (MAPK), including p38, PI3K, and ERK1/2 (4).

In numerous cancers such as melanoma, pancreatic cancer, and gastric carcinoma, *KISS1*/GPR54 signaling is anti-metastatic. GPR54 activity was shown to repress matrix metalloprotease-9 (MMP-9) activity, inhibit migration, increase tissue inhibitor of matrix metalloprotease-1 (TIMP-1) production, and activate focal adhesion kinase (FAK), leading to the formation of excessive focal adhesions and stress fibres (3, 4).

GPR54's role in breast cancer has been difficult to discern. Recent studies have shown that the expression of *KISS1* and *GPR54* correlates with breast tumour grade and poor patient prognosis (5). Indeed, patients who died of breast cancer had the highest expression of *KISS1* (5). Furthermore, 16-fold higher *GPR54* mRNA expression was found in invasive tumours compared to normal mammary tissue. A 2007 study by Marot *et al.* demonstrated *KISS1* mRNA was higher in benign tumours and ductal carcinomas (11-fold higher in the latter) compared to normal mammary tissue (6). This study showed that high *KISS1* and high *GPR54* mRNA levels positively correlated with shorter relapse-free survival

(6). However, whether kisspeptins actually promoted breast cancer invasiveness and the underlying mechanisms that may be involved are unknown.

The epidermal growth factor receptor (EGFR), a member of the ErbB family of receptor tyrosine kinases, is an important therapeutic target for several epithelial tumors, including breast cancer. EGFR is overexpressed in human breast tumours and the EGFR signaling pathway is implicated in the control of cell survival, proliferation, angiogenesis and metastasis (7). Upon stimulation, EGFR is known to autophosphorylate tyrosine residues on the C-terminal domain of its dimerized binding partner. These residues then serve as docking sites for a number of proteins, including Grb2/Sos, c-cbl, c-Src, phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ (PLCγ), leading to activation of the MAPK pathway and AKT/protein kinase B. The internalization of EGFR has further been shown to be required for mitogenic signaling *via* the activation of MAPKs, resulting in transcriptional changes leading to increased migration, invasion, and metastasis (8, 9).

EGFR can be transactivated by GPCRs in a variety of cells types including tumor cells (10-13). Defined as the phosphorylation of ERK1/2 in response to an unrelated agonist, transactivation appears to require EGFR kinase activity, the proteolytic release of EGFR ligands, and a cascade of secondary messengers, including a rise in cytosolic Ca^{2+} concentration, activation of protein kinase C (PKC), and tyrosine kinases such as Src and PYK2 (14, 15). Recently, it has been shown that the GPCR scaffolding protein β-arrestin 2 promotes EGFR transactivation *via* interactions with Src in vascular smooth muscle cells (14).

In this study we investigated for the first time whether kisspeptins directly stimulate breast cancer cell invasiveness and the underlying mechanisms involved. Our results illustrate that kisspeptin increases breast cancer cell motility and invasion, and this involves β-arrestin 2. Moreover, we establish for the first time that GPR54 complexes with EGFR, and that GPR54 transactivates EGFR, through a mechanism that involves Src.

2.2. Materials and Methods

Materials and DNA Constructs

Kp-10 was purchased from Phoenix Pharmaceuticals (Burlingame, CA). The following were purchased from Sigma Aldrich (St. Louis, MO): EGF, Protein G-sepharose beads, anti-FLAG mouse and rabbit antibodies. FLAG-GPR54 was obtained from Dr. Andy Babwah.

Cell Culture

Cell lines were purchased from ATCC (Manassas, VA) and cultured at 37 °C with 5% CO_2. Human invasive breast carcinoma MDA-MB-231 and human carcinosarcoma Hs578T cells were cultured in RPMI 1640 (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma). Human embryonic kidney (HEK)-293 cells were cultured in Eagle's minimum essential medium (MEM; Invitrogen, Burlington, Ontario, Canada), supplemented with 10% (v/v) FBS (Sigma) and 50 μg/mL gentamicin. HEK cells were transiently transfected with FLAG-GPR54 and EGFR-GFP using

a modified calcium phosphate method (16). Fresh medium was added to the cells 18 hours after transfections and the cells were allowed to recover for 8 hours before being reseeded onto either coverslips for receptor internalization assays, 35 mm glass-bottomed culture plates for confocal microscopy, or 100 mm culture plates for co-immunoprecipitation experiments. Expression of receptors was determined in each experiment using immunofluorescence.

Stable transfections and gene knockdowns

MDA-MB-231 cells (5 x 10^6) were transfected with FLAG-GPR54 constructs (MDA-MB-231 FLAG-GPR54) (25 μg) by electroporation (250V, 950 μF) using the Gene Pulser Xcell (Bio-Rad, Mississauga, Ontario, Canada) according to the manufacturer's instructions. A heterogenous population of stable transfectants was selected by using media containing 750 μg/mL G418 (Invitrogen) and expression verified by immunostaining or Western blot for FLAG-GPR54. Gene knockdown of β-arrestin 2 in MDA-MB-231 cells was achieved using shRNA constructs (OriGene Technologies, sequence ATGGAAGAGGCTGATGACACTGTGGCACC) as we have previously described (17). A heterogeneous population of stable transfectants was obtained *via* puromycin (1 μg/mL) selection. Knockdown of β-arrestin 2 was verified by Western blot analysis.

Cell migration and invasion assays

Transwell filters (8 μm pores) were placed into a 24-well plate containing either serum-free media or media supplemented with 10% FBS. For cell invasion assays, the tops of filters were coated with 50 μL of diluted phenol-red free Matrigel (9.4 mg/mL stock, BD Biosciences) in serum-free RPMI 1640. Cells were serum-starved for 4 hours and 4.0 x 10^4 cells were plated in the upper chamber in either serum-free media or serum-free media supplemented with 10 nM or 100 nM Kp-10 and incubated for 20 hours. For studies involving EGFR inhibitor AG1478, cells were pre-treated with AG1478 (500 nM) for 30 min as previously described, and seeded in serum-free RPMI with or without Kp-10 (10 nM) in addition to AG1478 (500 nM) or vehicle control (DMSO) (18). Cells were then fixed with a 20% acetone:80% methanol solution and nuclei stained with 0.1% Hoechst 33258 (Invitrogen) and counted on the bottom of the filters. Two replicates were conducted for each condition, and 10 random fields in each replicate were chosen and counted using an Olympus IX-71 inverted microscope. Results are presented as a ratio of cells that migrated relative to cells that migrated or invaded in control conditions (cells seeded in serum-free media and migrating towards 10% (v/v) FBS in RPMI). Results are from at least three independent experiments. For three-dimensional cell invasion assays, cells were seeded in a 1:1 dilution of phenol red-free Matrigel and culture medium at 2.5 × 10^6 cells/mL on Matrigel-coated 35 mm glass-bottomed culture dishes (Mattek, Ashland, MA). Cultures were overlaid with culture medium and grown for up to 5 days in the presence or absence of 10 nM Kp-10. Cell colonies were scored blindly as being

either stellate or spheroidal after growth in Matrigel. A colony was deemed stellate if one or more projections from the central sphere of cells were observed. Overlaying medium was supplemented with 750 μg/mL of G418 for MDA-MB-231 cells stably transfected with FLAG-GPR54. Images were taken on an IX-81 microscope (Olympus, Center Valley, PA) using InVivo Analyzer Suite (Media Cybernetics).

Zymographic analysis of secreted metalloproteases.

Zymographic analysis was performed as previously described (19). Briefly, samples were collected and 40μl of conditioned media was resolved in an SDS/polyacrylamide gel containing 1 mg/mL gelatin. The gels were then washed to remove the SDS and incubated overnight at 37 °C in a solution of 50 mM Tris pH 7.0 and 5 mM $CaCl_2$ to allow for enzymatic digestion of the gelatin. After incubation, gels were stained using Coomassie blue, destained, and visualized using the VersaDoc imaging system (Bio-Rad).

Scratch assays for cell motility

MDA-MB-231 cells were seeded into a 12-well dish in RPMI 1640 supplemented with 10% FBS and allowed to grow to confluency. Cells were then serum-starved in RPMI for 4 hours and scratched with sterile pipette tips followed by treatment with 10 nM or 100 nM Kp-10 in RPMI supplemented with 10% FBS. Cells were allowed to migrate into the scratch for 12 hours and visualized every hour using an IX-81 microscope (Olympus). Distance travelled (in μm) was

then measured over the course of the 12 hours, using two duplicates for each condition and five fields per duplicate, using ImagePro software (Media Cybernetics) and graphed versus time.

EGFR immunoprecipitation

MDA-MB-231 or Hs578T cells were serum-starved for 24 hours and then stimulated with various ligands at the indicated concentrations for the indicated times. Cell fractions were solubilised in lysis buffer (20 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 2 mM Na_3VO_4, 1 mM NaF, 10% glycerol, 1% Triton X-100). Lysates (850 μg of total protein) were used for immunoprecipitation studies. EGFR was immunoprecipitated using a polyclonal anti-EGFR antibody (1:100, Upstate Millipore) and protein G-sepharose beads (Sigma) over-night at 4°C. Immunoprecipitated proteins were separated by SDS-PAGE and phosphorylation of EGFR was examined using a mouse monoclonal anti-phosphotyrosine antibody (PY-20, 1:1000, Santa Cruz) and visualized by enhanced chemiluminescence. Western blots were then reprobed with rabbit polyclonal anti-EGFR antibody (1:5000, Upstate Millipore) to assess total EGFR. EGFR expression from cell lysates (50 μg of total protein) was examined using a rabbit polyclonal EGFR antibody. To determine the mechanism of EGFR transactivation, cells were grown to 80% confluency and serum-starved for 24 hours before treating with Src inhibitor PP2 (8 μM, Millipore) as previously described (20), or vehicle control (DMSO) for 20 minutes prior to stimulation

with 10 nM Kp-10 for the indicated time points. EGFR was immunoprecipitated as described above.

Confocal microscopy

MDA-MB-231 FLAG-GPR54 transfected with EGFR-GFP were serum-starved for 4 hours. Cells were plated on cover-slips and FLAG-GPR54 receptors were labelled with a monoclonal anti-FLAG antibody (1:500, Sigma) for 45 min at 4°C, warmed to 37°C and treated with Kp-10 or EGF (as indicated in the figure legends). Cell were subsequently fixed and permeabilized with 4% paraformaldehyde followed by 0.2% Triton X-100 and FLAG receptors were labelled by anti-mouse AlexaFluor-546 (1:1200, Invitrogen). Nuclei were stained with 0.1% Hoechst 33258 (Invitrogen). Cells were visualized using a Zeiss LSM-510 META laser scanning microscope (Zeiss, Oberkochen, Germany).

Co-immunoprecipitation and immunoblot

HEK 293 cells were transfected with FLAG-GPR54 and EGFR-EGFP using modified calcium phosphate method (16). Forty-eight hours following transfection, cells were serum-starved for 4 hours and then stimulated with Kp-10 for the indicated times. Cell fractions were solubilised in lysis buffer (20 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100). Cell lysates (500 µg of total protein) were used for co-immunoprecipitation studies. FLAG-GPR54 was immunoprecipitated using a mouse monoclonal anti-FLAG antibody and protein G-Sepharose beads (Sigma) over-night at 4°C. Immunoprecipitated

proteins were separated by SDS-PAGE and EGFR-EGFP expression examined using a mouse monoclonal anti-GFP antibody (1:2000, Upstate Millipore) and visualized by enhanced chemiluminescence, following the manufacturer's protocol.

Statistical analysis. One-way analysis of variance (ANOVA) with a Dunnett's post-hoc test or Student's t-test was performed using GraphPad Prism 4 (GraphPad Software, Inc.). Differences with $P < 0.05$ were considered statistically significant.

2.3. Results

Kp-10 promotes the migration and invasion of breast cancer cells

We first sought to determine whether Kp-10 stimulates migration of breast cancer cells as a chemoattractant using standard Transwell chamber migration assays. MDA-MB-231 cells stably expressing FLAG-GPR54 were seeded in serum-free medium and were allowed to migrate towards 10 or 100 nM Kp-10 in serum-free medium. A significant increase in their migration compared to untreated cells migrating towards serum-free media was not observed (Fig. 2.1). To see if Kp-10 could stimulate cell migration without a chemoattractant, MDA-MB-231 cells stably expressing FLAG-GPR54 were seeded in serum-free media or serum-free media containing 10 or 100 nM Kp-10 and allowed to migrate for 20 hours towards serum-free media (Fig. 2.1). Kp-10 did not significantly increase migration compared to untreated cell migrating towards serum-free

media. We then sought to determine whether Kp-10-treatment would enhance the migration of MDA-MB-231 cells stably expressing FLAG-GPR54 towards a chemoattractant. Using Transwell chamber migration assays, Kp-10-treatment of MDA-MB-231 FLAG-GPR54 significantly enhanced cell migration towards media supplemented with 10% serum (Fig. 2.2). To determine whether Kp-10 could enhance migration and invasion of parental MDA-MB-231 towards a chemoattractant, parental MDA-MB-231 cells were seeded in either serum-free media or serum-free media containing various concentrations of Kp-10 and allowed to invade across a Matrigel-coated Transwell membrane for 20 hours. Dose-response studies indicated an increase in invasion of MDA-MB-231 cells when treated with various concentrations of Kp-10 (Fig. 2.3) with a maximal response obtained using 100 nM Kp-10, followed by a decrease at 500 nM. All further experiments were done using either 10 nM or 100 nM Kp-10 as reported in other studies (21-23).

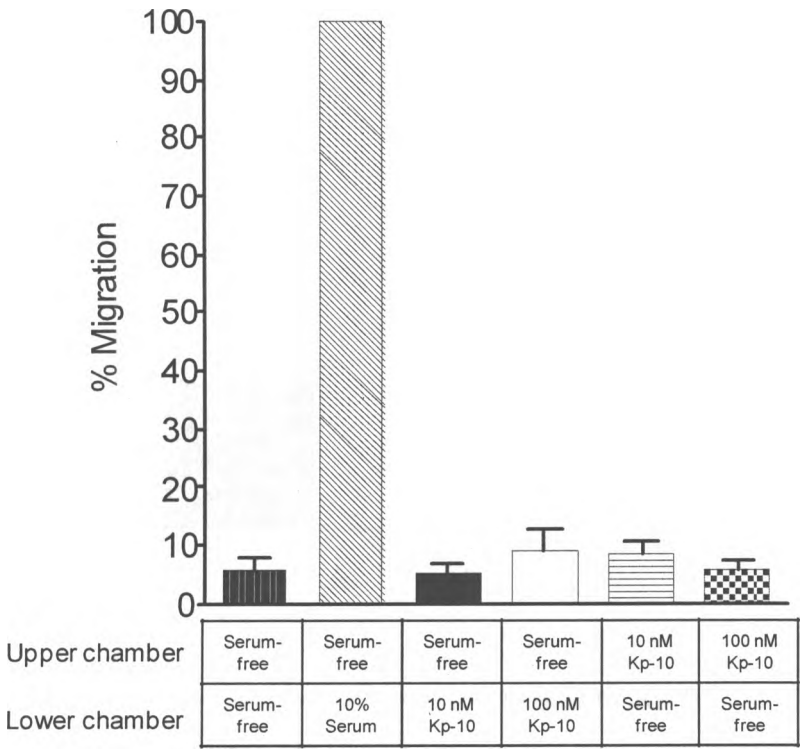


Fig. 2.1. Kp-10 does not act as a chemoattractant.

Cell migration was measured by Transwell chamber assays. MDA-MB-231 FLAG-GPR54 cells (40,000) seeded in serum-free media were allowed to migrate towards 10 or 100 nM Kp-10 in serum-free media. A significant increase in their migration compared to untreated cells migrating towards serum-free media was not observed ($P > 0.05$). Cells were seeded in serum-free media or serum-free media containing 10 or 100 nM Kp-10 and allowed to migrate for 20 hours towards serum-free media. Kp-10 did not significantly increase migration compared to untreated cell migrating towards serum-free media. Columns represent mean percentage of cells migrated as compared to control cells migrating towards 10% serum medium (100%) ± SEM from at least three independent experiments.

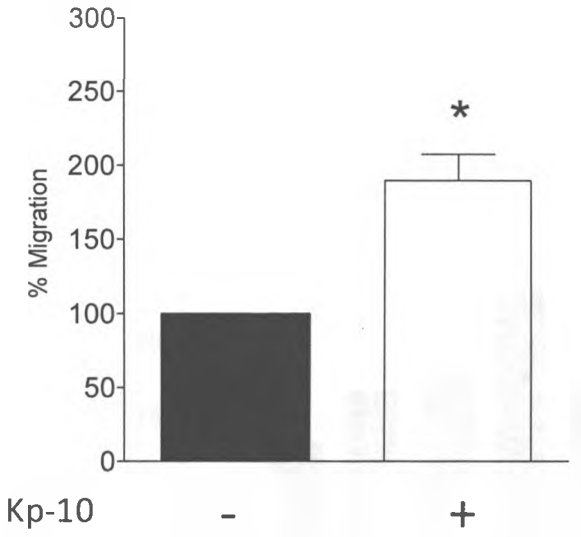


Fig. 2.2. Migration of MDA-MB-231 FLAG-GPR54 is enhanced by Kp-10.

Cell migration was measured by Transwell chamber assays. Cells were seeded in serum-free media or serum-free media containing 10 nM Kp-10 and allowed to invade for 20 hours towards media with 10% serum. Kp-10 treatment significantly increased the number of MDA-MB-231 FLAG-GPR54 cells migrating towards 10% serum. *, $P < 0.05$ when compared to untreated MDA-MB-231 FLAG-GPR54 cells invading towards 10% serum. Columns represent mean percentage of cells migrated as compared to control cells migrating towards media with 10% serum ± SEM from at least three independent experiments.

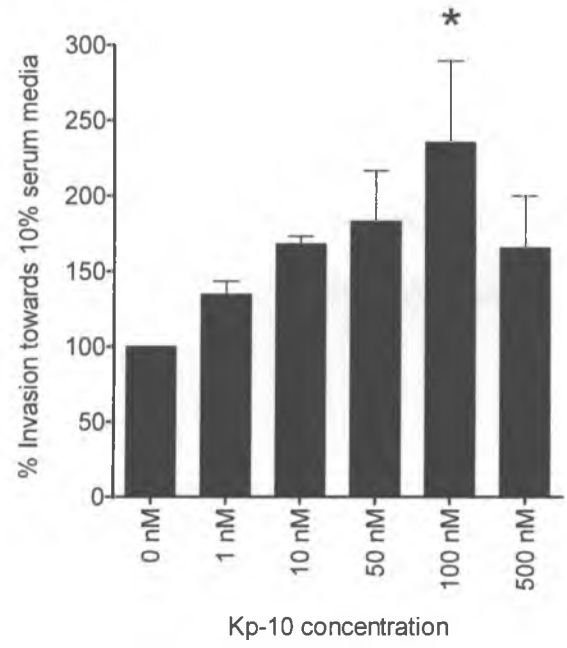


Fig. 2.3. Kp-10 enhances MDA-MB-231 invasion in a dose-dependent manner.

Cell invasion was measured by Matrigel-coated Transwell chamber assays. MDA-MB-231 cells (40,000) were seeded in serum-free medium or serum-free medium containing various concentrations of Kp-10 and allowed to invade for 20 hours towards media with 10% serum. Kp-10 treatment significantly increased the number of MDA-MB-231 cells invading towards media with 10% serum. *, $P < 0.05$. Bars represent the mean number of cells invaded as compared to control cells invading towards media with 10% serum ± SEM from at least three independent experiments.

Kp-10 promotes breast cancer cell invasion in three-dimensional cultures

Both normal and malignant breast cells can be cultured in reconstituted extracellular matrix as a three-dimensional (3D) model, mimicking the *in vivo* micro-environment (17). Treatment of MDA-MB-231 (Fig. 2.4-ii), MDA-MB-231 FLAG-GPR54 (Fig. 2.5-ii), and Hs578T cells (Fig. 2.6-ii) with 10 nM Kp-10 resulted in an increase in the formation of stellate structures, when compared to untreated cells, with a significant increase observed at day 2 and day 3 in MDA-MB-231, day 3 in MDA-MB-231 FLAG-GPR54, and day 3 and day 4 in Hs578T cells.

Kp-10 stimulates MMP-9 secretion and activity

In order to determine how Kp-10 signaling could be promoting invasion, we employed the use of zymographic analysis of matrix metalloprotease secretion. We found using a gelatin-containing gel that treatment of MDA-MB-231 cells with either 10 or 100 nM of Kp-10 increases the secretion of MMP-9 (Fig. 2.7), revealing for the first time that Kp-10 signaling *via* GPR54 in breast cancer cells stimulates the secretion of MMP-9.

Kp-10 promotes motility of MDA-MB-231 cells

In order to visualize whether or not Kp-10 stimulates motility of breast cancer cells in real-time, we performed scratch assays as described (24). We observed that Kp-10 treatments (10 nM or 100 nM) significantly enhanced the distance travelled by the MDA-MB-231 cells over time, when compared to cells

seeded only in 10% serum (Fig. 2.8). Taken together, these results suggest that Kp-10 stimulates not only breast cancer cell invasion, but also enhances their motility.

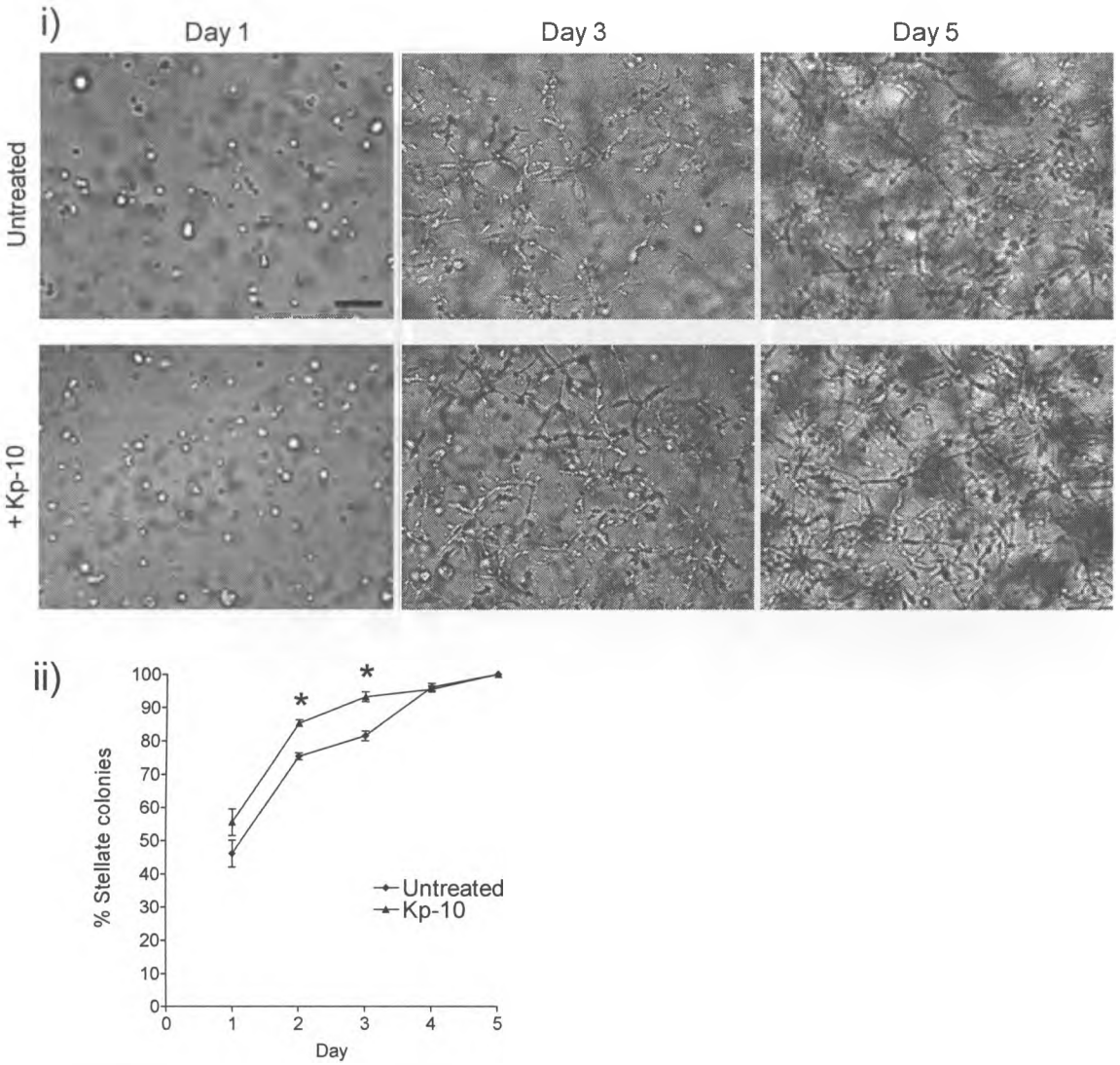


Fig. 2.4. Invasiveness of MDA-MB-231 cells is enhanced by Kp-10.

(i) MDA-MB-231 cells invade into the surrounding matrix in a three-dimensional Matrigel culture assay. 20,000 cells were seeded in a mixture of Matrigel and media with 10% serum. Cells were allowed to grow for five days in either 10% serum medium or 10% serum medium supplemented with 10 nM Kp-10. Treatment of cells with Kp-10 results in a significant increase in stellate structure formation on days 2 and 3 when compared to untreated cells. *, $P < 0.05$ compared to untreated MDA-MB-231 cells invading in Matrigel from 3 independent experiments. Scale bar, 100 μm.

(ii) Colony shape was scored blindly as being either stellate or spheroidal after growth in Matrigel. Line represents the mean percentage of stellate colonies versus total colonies counted that day ± SEM from at least three independent experiments

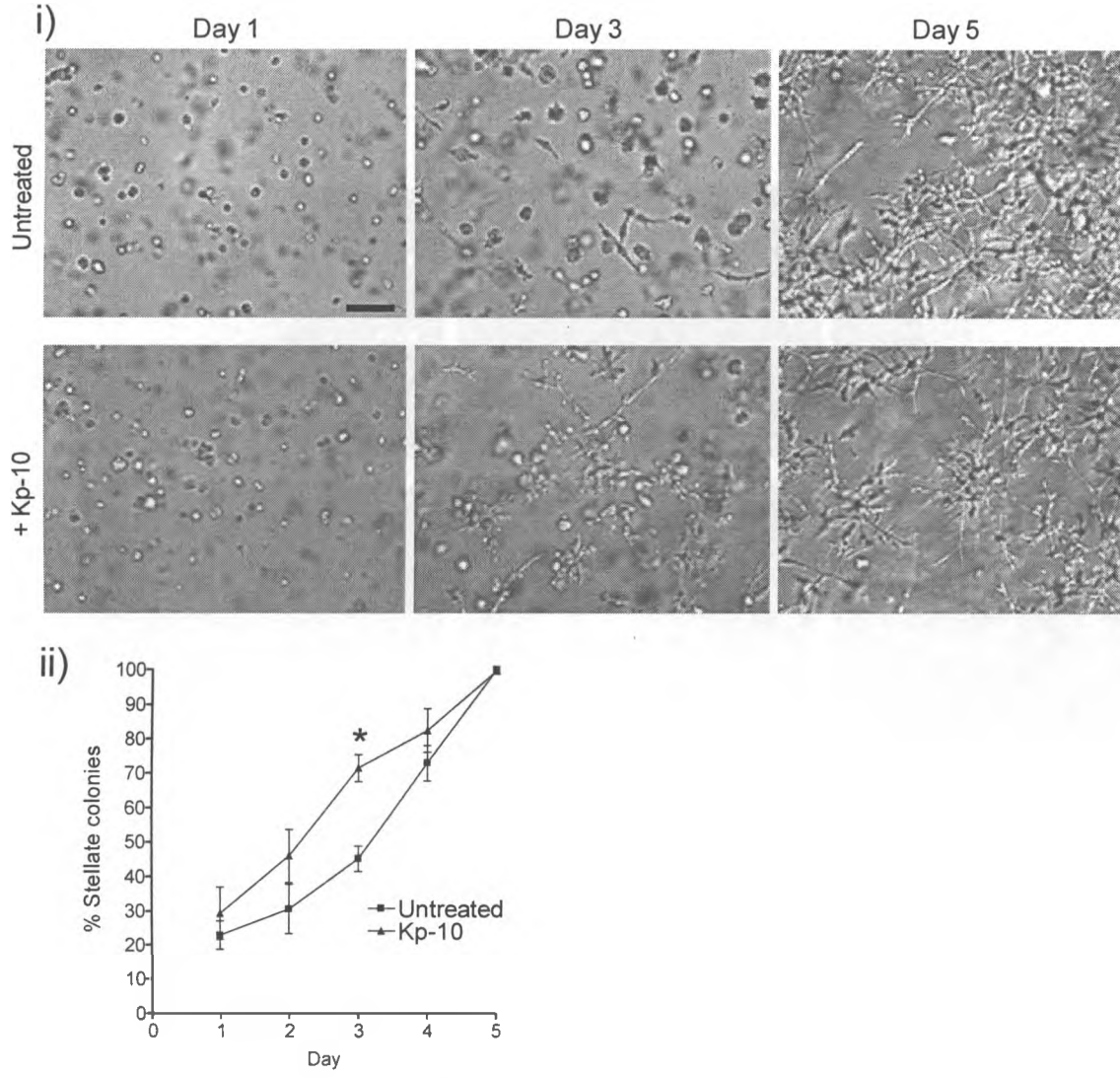


Fig. 2.5. Invasiveness of MDA-MB-231 FLAG-GPR54 cells is enhanced by Kp-10.

(i) MDA-MB-231 FLAG-GPR54 cells invade into the surrounding matrix in a three-dimensional Matrigel culture assay. 20,000 cells were seeded in a mixture of Matrigel and media with 10% serum. Cells were allowed to grow for five days in either 10% serum medium or 10% serum medium supplemented with 10 nM Kp-10. Treatment of cells with Kp-10 results in a significant increase in stellate structure formation on day 3 when compared to untreated cells. *, $P < 0.05$ compared to untreated MDA-MB-231 FLAG-GPR54 cells invading in Matrigel from 3 independent experiments. Scale bar, 100 μm.

(ii) Colony shape was scored as being either stellate or spheroidal after growth in Matrigel. Line represents the mean percentage of stellate colonies versus total colonies counted that day ± SEM from at least three independent experiments

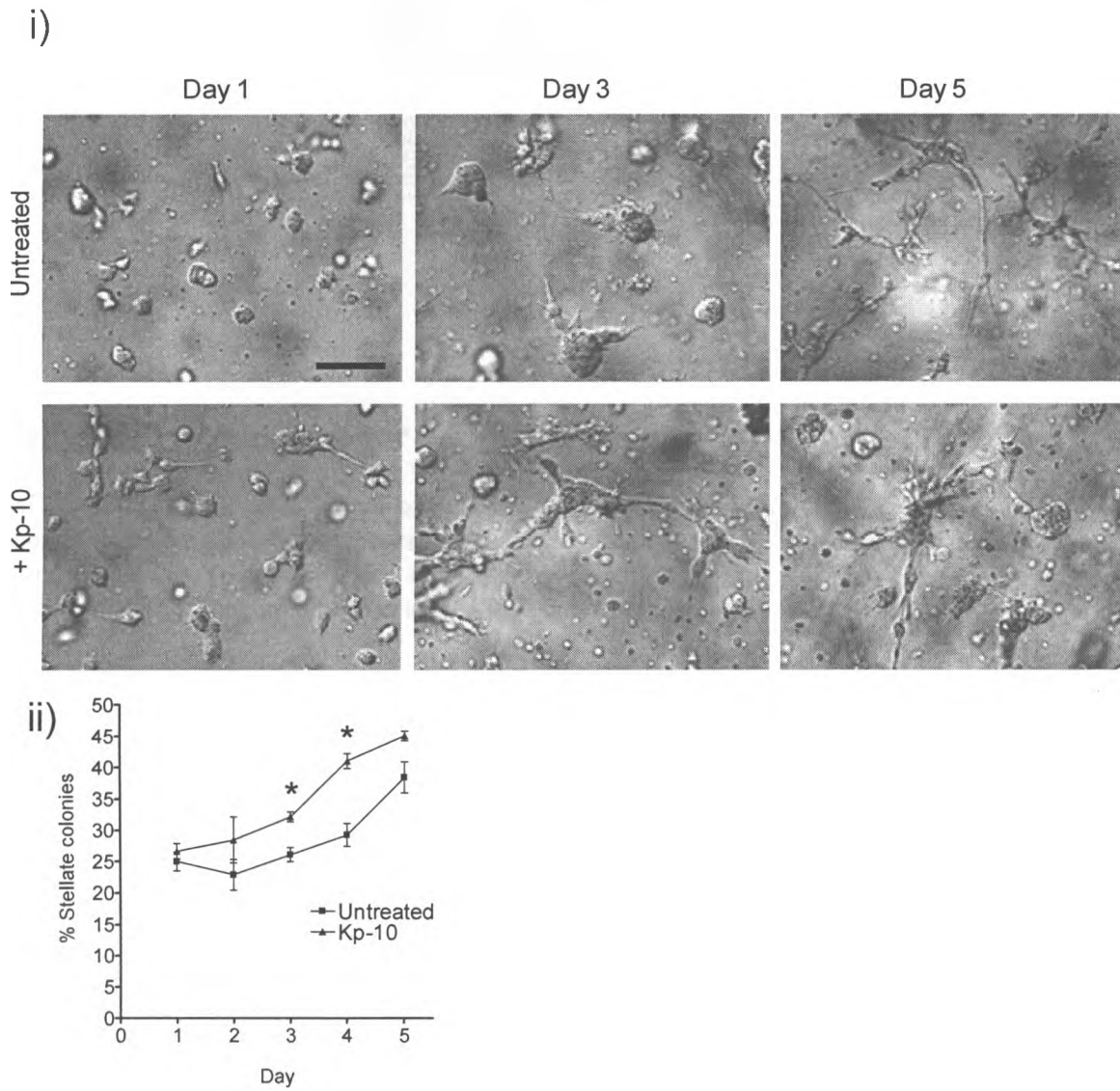


Fig. 2.6. Invasiveness of Hs578T cells is enhanced by Kp-10.

(i) Hs578T cells invade into the surrounding matrix in a three-dimensional Matrigel culture assay. Cells (20,000) were seeded in a mixture of Matrigel and 10% serum medium. Cells were allowed to grow for five days in either 10% serum medium or 10% serum medium supplemented with 10 nM Kp-10. Treatment of cells with Kp-10 results in a significant increase in stellate structure formation on days 3 and 4 when compared to untreated cells. *, $P < 0.05$ compared to untreated Hs578T cells invading in Matrigel from 3 independent experiments. Scale bar, 40 μm.

(ii) Colony shape was scored blindly as being either stellate or spheroidal after growth in Matrigel. Line represents the mean percentage of stellate colonies versus total colonies counted that day ± SEM from at least three independent experiments.

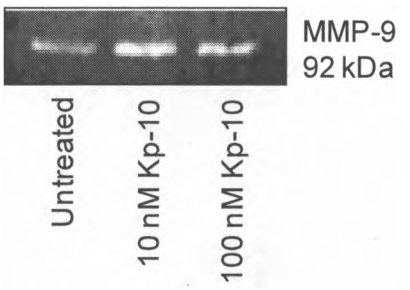


Fig. 2.7. Kp-10 upregulates MMP-9 production in MDA-MB-231 cells.

MDA-MB-231 cells were grown to 50% confluency and serum-starved for 4 hours and subsequently treated for 48 hours with 10 or 100 nM Kp-10 in serum-free media. Conditioned media was then collected and subjected to gelatin zymography. Representative zymograph from at least 3 independent experiments.

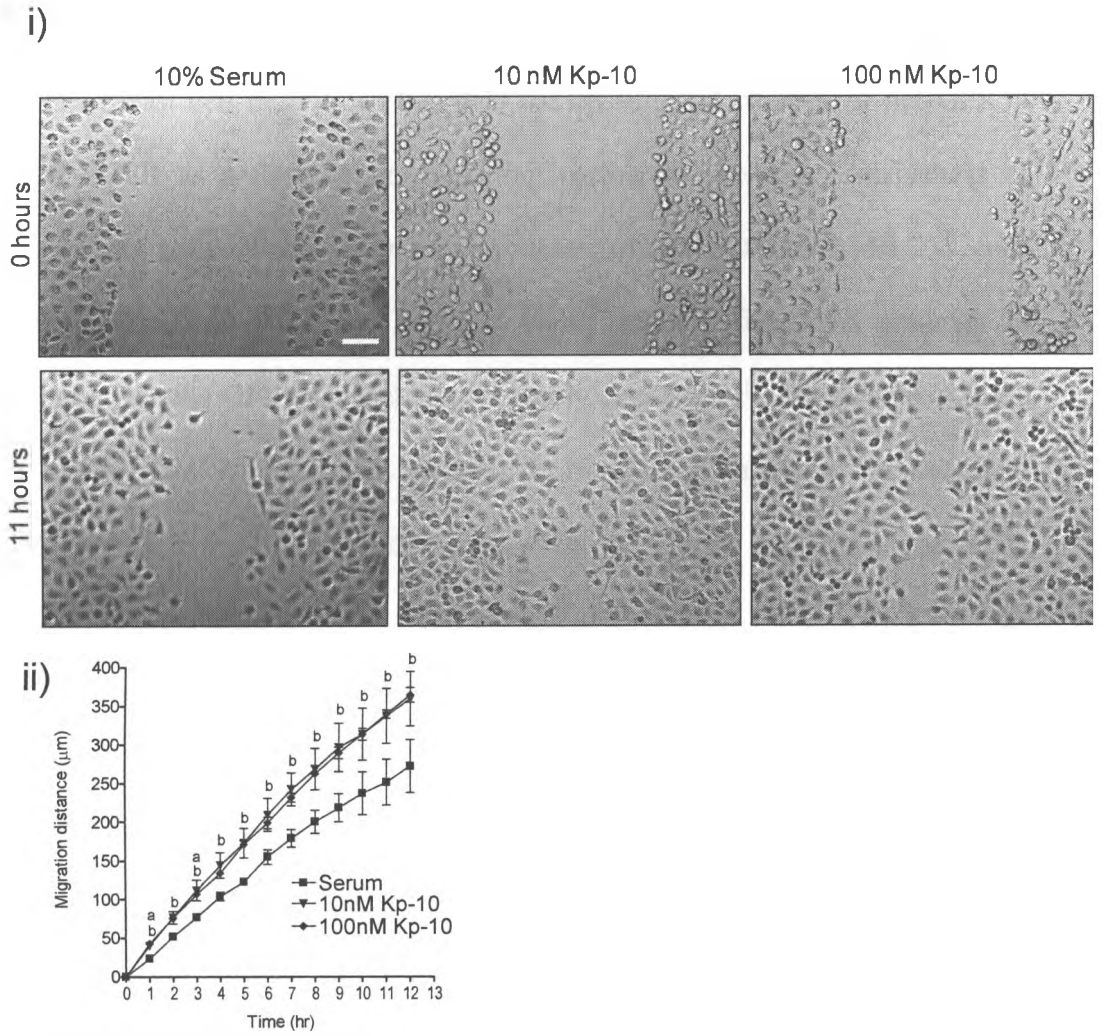


Fig. 2.8. Kp-10 promotes scratch closure in MDA-MB-231 cells.

i) MDA-MB-231 cells were grown to confluency and serum-starved for 4 hours before scratching. Cells were then plated in media with 10% serum, media with 10% serum supplemented with 10 nM Kp-10 or media with 10% serum supplemented with 100 nM Kp-10. Scratch closure was visualized every hour using an Olympus IX81 microscope and measured using ImagePro software. Scale bar, 100 µm.

ii) Both 10 and 100 nM Kp-10 treatments enhanced the distance closed by MDA-MB-231 cells. *a*, $P < 0.05$ for 10 nM Kp-10 when compared to untreated control cells seeded in serum; *b*, $P < 0.05$ for 100 nM Kp-10 when compared to untreated control cells seeded in serum. Bars represent mean distance travelled in µm ± SEM from at least three independent experiments.

Kp-10-stimulated breast cancer cell invasion is attenuated by EGFR tyrosine kinase inhibitor

Since our data revealed that Kp-10 does not act as a chemoattractant to stimulate breast cell migration (Fig. 2.1) we investigated whether or not GPR54 utilizes EGFR as a downstream signaling partner to promote invasiveness of breast cancer cells. We have previously demonstrated that MDA-MB-231 cells express endogenous GPR54 (5, 21). However, since a functional antibody to detect endogenous GPR54 is lacking, we stably expressed FLAG-GPR54 in these cells to easily detect the tagged receptor for biochemical analysis. Pre-treatment of MDA-MB-231 FLAG-GPR54 cells with an EGFR antagonist, AG1478, significantly decreased invasion when compared to untreated cells (Fig. 2.9), even in the presence of Kp-10. We next sought to determine whether Kp-10-treatment alters cell invasion towards EGF (Fig. 2.10). Treatment of MDA-MB-231 FLAG-GPR54 cells with Kp-10 did not enhance their invasion towards EGF. As expected, pre-treatment of these cells with AG1478 showed decreased invasion towards EGF (Fig. 2.10). Addition of Kp-10 to AG1478-treated cells appeared to reverse some of the block in invasion observed with AG1478 alone (Fig. 2.10). We also observed that Kp-10 (10 nM) increased the invasion of Hs578T cells towards medium supplemented with 10% serum, in the presence or absence of AG1478 (Fig. 2.11). Of note, we did observe less EGFR expression in Hs578T cells in contrast to MDA-MB-231 cells (Fig. 2.12).

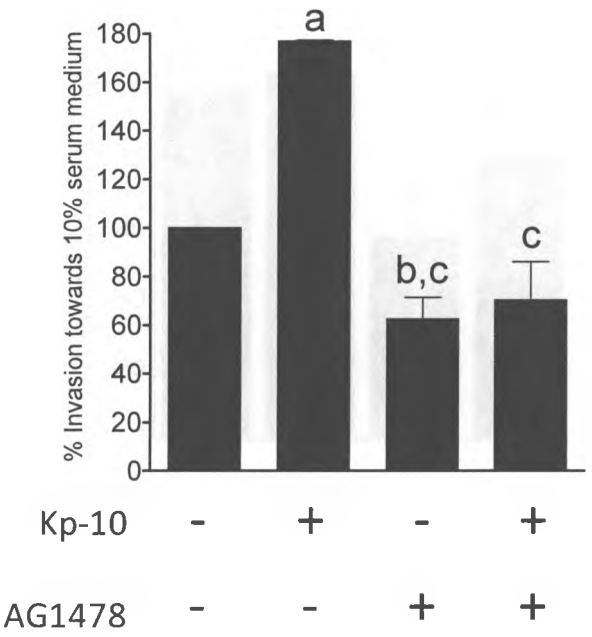


Fig. 2.9. Invasion of MDA-MB-231 FLAG-GPR54 is enhanced by Kp-10 and involves EGFR.

Cell invasion was measured by Matrigel-coated Transwell chamber assays. MDA-MB-231 FLAG-GPR54 cells (40,000) were seeded in serum-free media or serum-free media containing 10 nM Kp-10 and allowed to invade for 20 hours towards media with 10% serum. Kp-10 (10 nM) treatment significantly increased the number of MDA-MB-231 FLAG-GPR54 cells invading towards media with 10% serum. Treatment with AG1478 (500 nM) significantly decreased invasion of MDA-MB-231 FLAG-GPR54 cells *(b, $P < 0.05$)*. Kp-10 treatment of AG1478-pretreated cells did not significantly change invasiveness when compared to AG1478-preatreated cells. *a, $P < 0.05$* compared to untreated MDA-MB-231 FLAG-GPR54 cells invading towards media with 10% serum. *b, $P < 0.05$* compared to cells invading towards media with 10% serum. *c, $P < 0.05$* compared to Kp-10-treated MDA-MB-231 FLAG-GPR54 invading towards media with 10% serum. Columns represent the mean percentage of cells invaded as compared to control cells invading towards media with 10% serum ± SEM from at least three independent experiments.

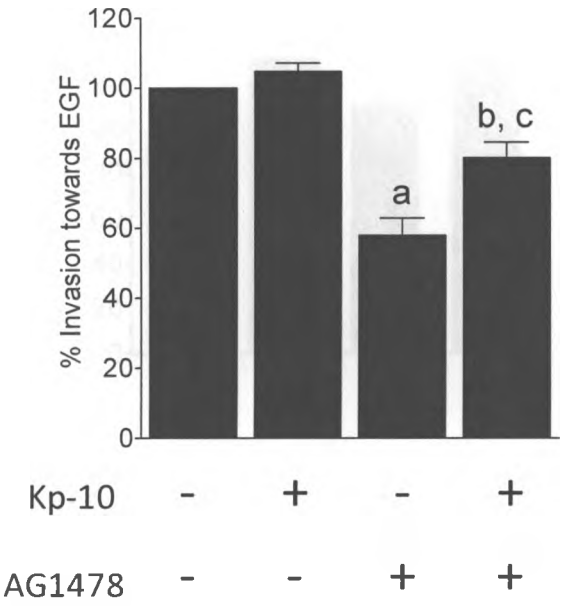


Fig. 2.10. Kp-10 promotes invasion towards EGF even in the presence of AG1478.

Cell invasion was measured by Matrigel-coated Transwell chamber assays. MDA-MB-231 FLAG-GPR54 cells (40,000) were seeded in serum-free media or serum-free media containing 10 nM Kp-10 and allowed to invade for 20 hours towards serum-free medium supplemented with EGF (100 ng/mL). Treatment with Kp-10 (10 nM) does not significantly change invasion towards EGF compared to cells without Kp-10 treatment ($P > 0.05$). Pretreatment of cells with AG1478 (500 nM) significantly reduced invasion towards EGF when compared to untreated cells (*a*, $P < 0.05$). However, addition of Kp-10 (10 nM) to AG1478-treated cells significantly increases their invasion compared to cells treated with AG1478 alone (*b*, $P < 0.05$). In the presence of both AG1478 and Kp-10, invasion was blocked significantly compared to untreated cells (*c*, $P < 0.05$). Columns represent the mean percentage of cells invaded as compared to control cells invading towards 100 ng/mL EGF in serum-free medium ± SEM from at least three independent experiments.

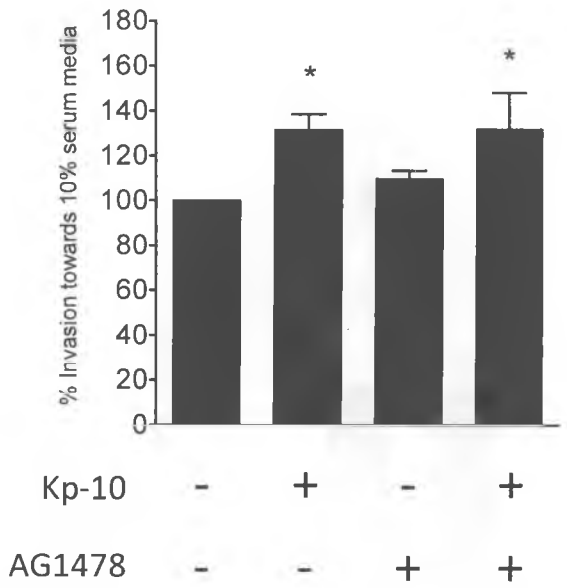


Fig. 2.11. Kp-10-mediated invasion of Hs578T cells does not require EGFR kinase activity.

Cell invasion was measured by Matrigel-coated Transwell chamber assays. Hs578T cells (40,000) were seeded in serum-free media or serum-free media containing 10 nM Kp-10 and allowed to invade for 20 hours towards medium with 10% serum. Kp-10 treatment significantly increased the number of Hs578T cells invading towards 10% serum medium (*, $P < 0.05$). Treatment with AG1478 (500 nM) did not significantly decrease invasion of Hs578T cells ($P > 0.05$). Kp-10 treatment of AG1478-pretreated cells produced significantly enhanced invasion that was not different from cells treated only with Kp-10 (*, $P < 0.05$). Columns represent the mean percentage of cells invaded as compared to control cells invading towards media with 10% serum ± SEM from at least three independent experiments.

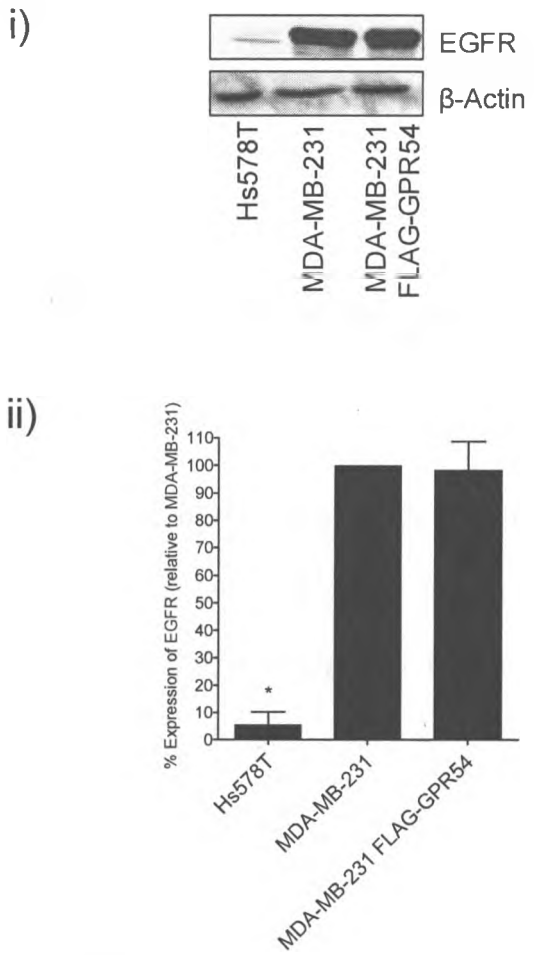


Fig. 2.12. EGFR protein expression in breast cancer cells.

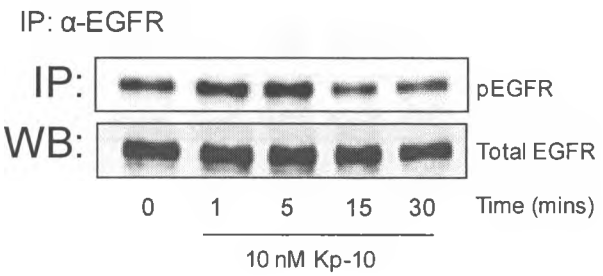
i) Hs578T cells express significantly less endogenous EGFR than MDA-MB-231 (*, $P < 0.05$). Cells were grown to confluency, solubilised in lysis buffer, and 100 µg of protein was resolved on SDS-PAGE. Samples were probed for endogenous EGFR and standardized to β-actin. MDA-MB-231 FLAG-GPR54 cells do not express significantly different quantities of EGFR in comparison to parental MDA-MB-231 cells.

ii) Densitometric analysis of Western blots for EGFR protein expression using VersaDoc gel documentation system. Columns represent mean expression of EGFR as compared to parental MDA-MB-231 cells ± SD from three independent experiments.

GPR54 transactivates EGFR in breast cancer cells via Src

To assess whether GPR54 crosstalks with EGFR and can transactivate this tyrosine kinase receptor, lysates from Kp-10-treated cells were examined for phosphorylated endogenous EGFR by immunoblotting as described (25, 26). We observed that treatment of MDA-MB-231 cells with 10 nM Kp-10 resulted in an increase in the phosphorylation of EGFR, reaching peak activation at 5 and 30 min post-treatment (Fig. 2.13). Treatment of Hs578T cells with 10 nM Kp-10 also resulted in increased phosphorylation of EGFR, reaching a peak between 5 and 15 min post-treatment (Fig. 2.14). Our results show for the first time that Kp-10 signaling *via* endogenous GPR54 leads to the transactivation of endogenous EGFR in breast cancer cells. To date, reports have generally implicated the role of Src-family of tyrosine kinases in GPCR-induced transactivation of EGFR (27). To determine the mechanism(s) by which Kp-10 signaling *via* GPR54 leads to EGFR transactivation in breast cancer cells, we determined the effect of blocking Src activity using an inhibitor, PP2. We found that EGFR transactivation by Kp-10 was markedly reduced in MDA-MB-231 cells treated with PP2, implicating a role for Src (Fig. 2.15).

i)



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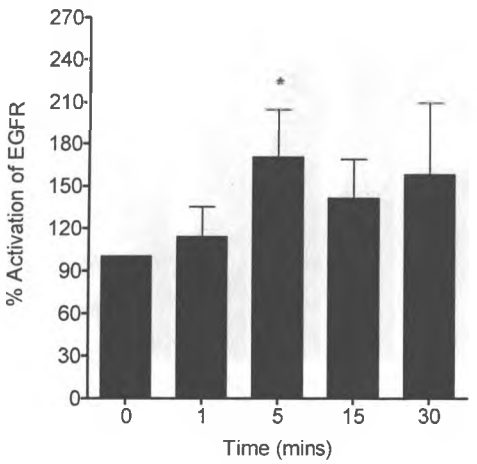

Fig. 2.13. Treatment of MDA-MB-231 cells with Kp-10 results in the transactivation of EGFR.

(i) MDA-MB-231 cells were serum-starved for 24 hours and subsequently treated with 10 nM Kp-10 for the indicated time points before they were solubilised in lysis buffer. Endogenous EGFR was immunoprecipitated using a polyclonal anti-rabbit antibody conjugated to protein G-sepharose.

(ii) Densitometric analysis of Western blots for EGFR protein expression using VersaDoc gel documentation system. Treatment of MDA-MB-231 cells with 10 nM Kp-10 resulted in a significant increase in EGFR phosphorylation (*, $P < 0.05$). Basal levels of phosphorylated EGFR are regarded as 100%. Columns represent mean percentage change of phosphorylated EGFR as compared to basal level ± SD from at least three independent experiments.

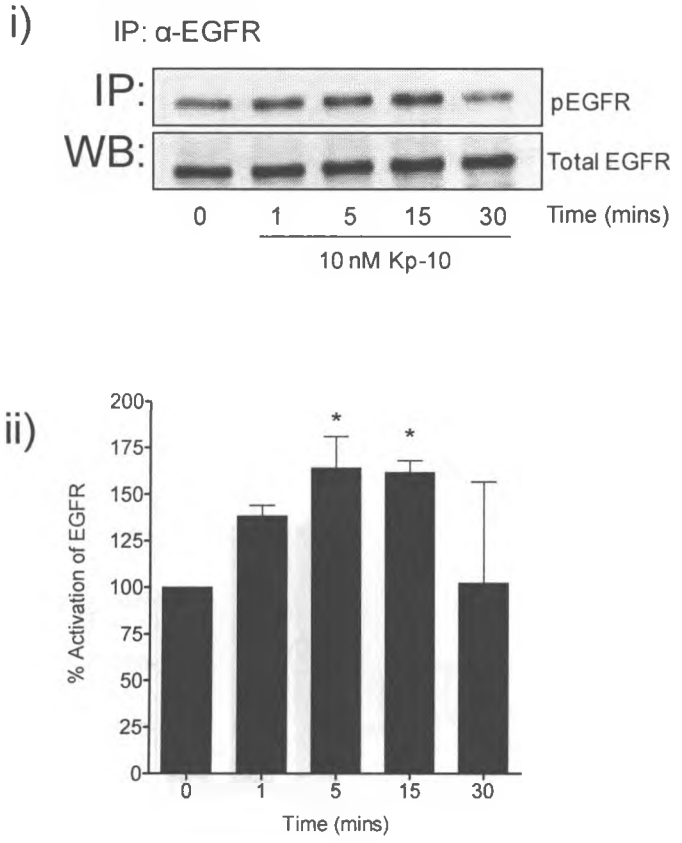

Fig. 2.14. Treatment of Hs578T cells with Kp-10 results in the transactivation of EGFR.

(i) Hs578T cells were serum-starved for 24 hours and subsequently treated with 10 nM Kp-10 for the indicated time points before they were solubilised in lysis buffer. EGFR was immunoprecipitated using a polyclonal anti-rabbit antibody conjugated to protein G-sepharose.

(ii) Densitometric analysis of Western blots for EGFR protein expression using VersaDoc gel documentation system. Treatment of Hs578T cells with 10 nM Kp-10 resulted in a significant increase in phosphorylated EGFR (*, $P < 0.05$). Basal levels of phosphorylated EGFR are regarded as 100%. Columns represent mean percentage change of phosphorylated EGFR as compared to basal level ± SD from at least three independent experiments.

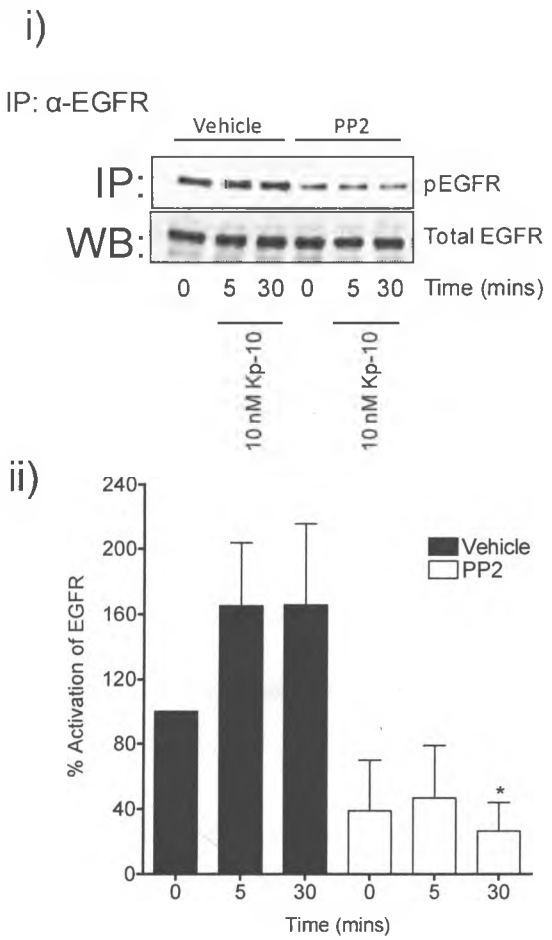

Fig. 2.15. Src is involved in Kp-10-mediated transactivation of EGFR.

(i) MDA-MB-231 cells were serum-starved for 24 hours and pre-treated with PP2 (8 μM) for 20 min and subsequently treated with 10 nM Kp-10 for the indicated time points before they were solubilised in lysis buffer. EGFR was immunoprecipitated using a polyclonal anti-rabbit antibody conjugated to protein G-sepharose.

(ii) Densitometric analysis of Western blots for EGFR protein expression using VersaDoc gel documentation system. EGFR phosphorylation was significantly inhibited by PP2 at 30 min post-Kp-10 treatment (*, $P < 0.05$). Columns represent mean percentage change of phosphorylated EGFR as compared to basal level ± SD from at least three independent experiments.

β-arrestin 2 is required for GPR54-mediated enhancement of invasion

We have recently demonstrated that the GPCR adaptor protein, β-arrestin, regulates MDA-MB-231 cell invasiveness (17). In addition, we found that GPR54, which is endogenously expressed in MDA-MB-231 cells, was unable to stimulate ERK1/2 activity, upon depletion of β-arrestin 2 (21). This suggests that β-arrestin 2 is required for GPR54 signaling to ERK1/2. To define a molecular mechanism by which Kp-10-induces breast cancer cell invasiveness, we investigated the effect of depleting β-arrestin 2 on this process. Knock-down of β-arrestin 2 in MDA-MB-231 cells significantly reduced invasion in the presence or absence of Kp-10, compared with the scrambled shRNA control (Fig. 2.16-i).

Kp-10 or EGF stimulates the internalization of GPR54 in breast cancer cells

Our data so far indicates that GPR54 transactivates EGFR to regulate breast cancer cell invasion. To better understand the nature of the crosstalk between these two receptors, we sought to determine whether or not Kp-10 or EGF can stimulate GPR54 endocytosis in MDA-MB-231 FLAG-GPR54-expressing cells. FLAG-GPR54 was localized at the cell surface in unstimulated cells (Fig. 2.17-i). Treatment with 10nM Kp-10 or 10ng/mL EGF resulted in FLAG-GPR54 receptor internalization within 5 min of stimulation to a peri-nuclear region (Fig. 2.17-ii and -iii, respectively). This localization is also visible at 15 min after stimulation (Fig. 2.17-iv and -v). This demonstrated that EGF, like Kp-10, induces the internalization of GPR54 in breast cancer cells.

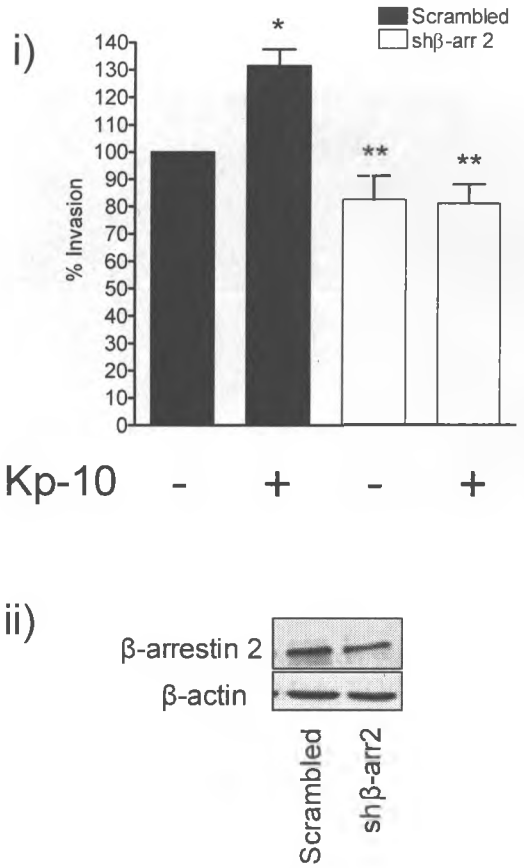

Fig. 2.16. Invasion of Kp-10-stimulated MDA-MB-231 cells requires β-arrestin 2.

(i) MDA-MB-231 cells expressing a scrambled control vector (Scrambled) and β-arrestin 2-depleted MDA-MB-231 cells (shβ-arr 2) were plated in serum-free medium or serum-free medium containing 10 nM Kp-10 and allowed to invade for 20 hours towards media with 10% serum. Kp-10 treatment significantly increased the number of MDA-MB-231 cells transfected with scrambled control vector (Scrambled) invading towards 10% serum (*, $P < 0.05$). shβ-arr-2 cells displayed significantly inhibited invasion towards media with 10% serum when compared to Kp-10-treated Scrambled cells (**, $P < 0.05$), but did not display significantly different invasion when compared to untreated Scrambled control cells invading towards 10% serum ($P > 0.05$). Columns represent mean percentage of cells invaded as compared to control cells invaded towards media with 10% serum ± SEM from at least three independent experiments.

(ii) Western blot analysis of MDA-MB-231 cells stably transfected with shRNA for β-arrestin 2. MDA-MB-231 cells stably expressing a scrambled control shRNA sequence were used as a control.

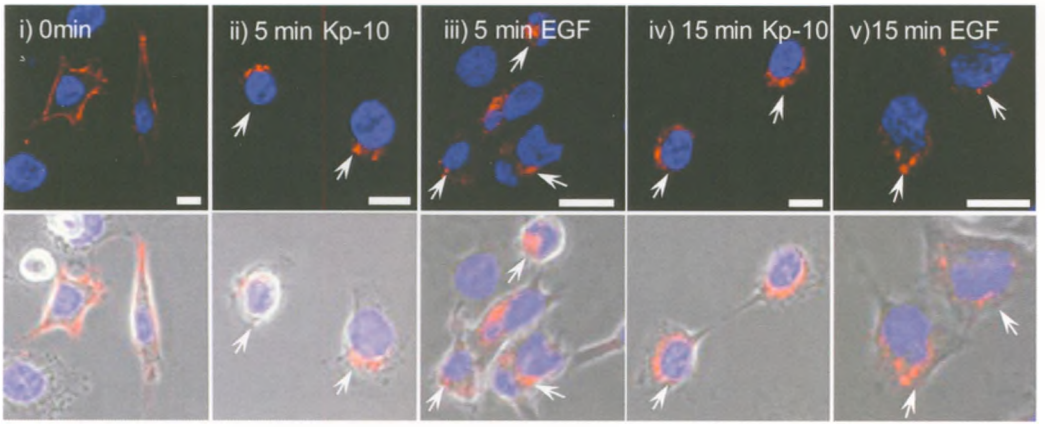

Fig. 2.17. Kp-10 and EGF induce internalization of FLAG-GPR54.

MDA-MB-231 FLAG-GPR54 cells were grown on glass coverslips and incubated in the absence (0 min) or presence of Kp-10 (10 nM) or EGF (10 ng/mL) for 5 or 15 min. Cells were then subjected to immunofluorescence staining for FLAG (red) and nuclei staining with 0.1% Hoechst 33258 dye (blue). Strong presence of FLAG-GPR54 may be seen at the cell membrane at 0 min (i). With treatment at 5 minutes by 10 nM Kp-10 (ii) and 10ng/mL EGF (iii), FLAG-GPR54 may be seen internalizing into the cells to peri-nuclear regions, also visible with Kp-10 and EGF treatment at 15 minutes (iv and v, respectively). Representative confocal micrographs from 5 independent experiments are shown. *Scale bar, 10 μm.*

GPR54 co-internalizes with EGFR in intracellular vesicles

To determine whether or not GPR54 and EGFR were localized to the same endocytic vesicles upon internalization, we co-expressed FLAG-GPR54 and EGFR-GFP in MDA-MB-231 and HEK 293 cells. In unstimulated MDA-MB-231 cells, FLAG-GPR54 was primarily localized to the cell membrane and EGFR-GFP at the cell membrane (Fig. 2.18-i). Treatment with 10 nM Kp-10 (Fig. 2.18-ii) or 10 ng/mL EGF (Fig. 2.18-iii) resulted in internalization of both FLAG-GPR54 and EGFR-GFP, with partial co-localization of the two receptors upon Kp-10 treatment. Similar results were obtained in HEK 293 cells, where treatment of cells with either Kp-10 (Fig. 2.19-ii, iv) or EGF (Fig. 2.19-iii, v) resulted in partial co-localization of FLAG-GPR54 and EGFR-GFP in endocytic vesicles.

GPR54 associates with EGFR

We determined whether GPR54 interacts with EGFR using co-immunoprecipitation studies. We observed that FLAG-GPR54 interacts with EGFR in the absence of Kp-10 in HEK 293 cells (Fig. 2.20). A significant difference in the association of GPR54 and EGFR upon treatment with Kp-10 was not observed.

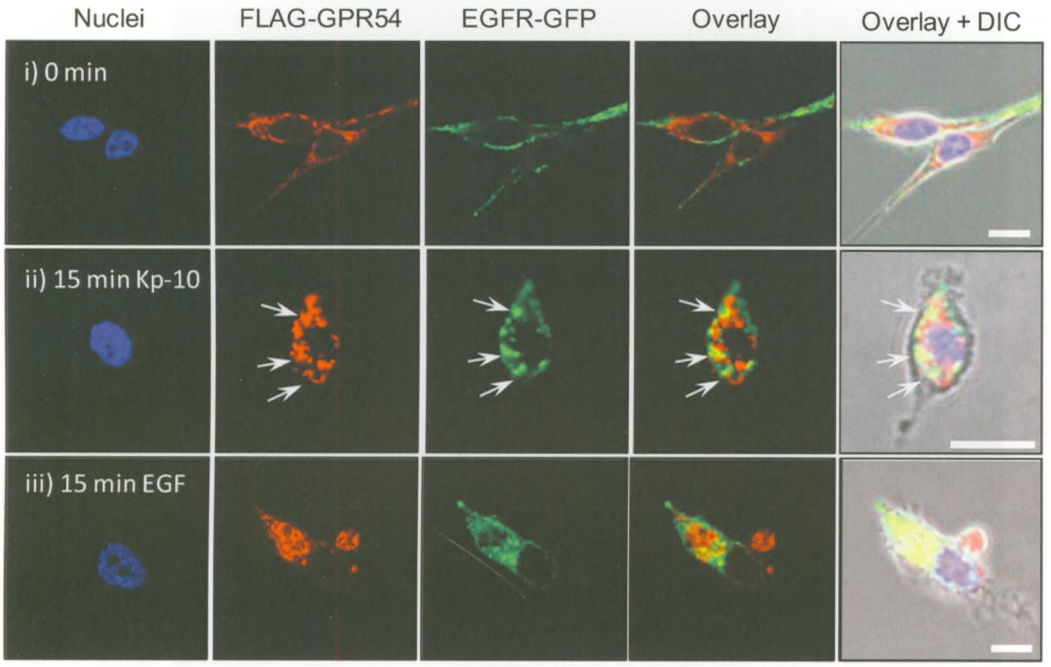

Fig. 2.18. FLAG-GPR54 and EGFR-GFP show co-localization in MDA-MB-231 cells.

MDA-MB-231 cells transiently transfected with FLAG-GPR54 and EGFR-GFP were plated onto glass slides and incubated in the absence (0 min, i) or presence of Kp-10 (10 nM, ii) or EGF (10 ng/mL, iii) for 15 min. After 15 minutes of Kp-10 stimulation (ii), both FLAG-GPR54 and EGFR-GFP may be seen internalizing into the cells with partial co-localization (*yellow, see arrows*). Treatment with 10 ng/mL EGF for 15 minutes (iii) also induces FLAG-GPR54 and EGFR-GFP internalization, but shows only partial co-localization. Representative confocal micrographs from three independent experiment are shown. *Scale bar, 10 μm.*

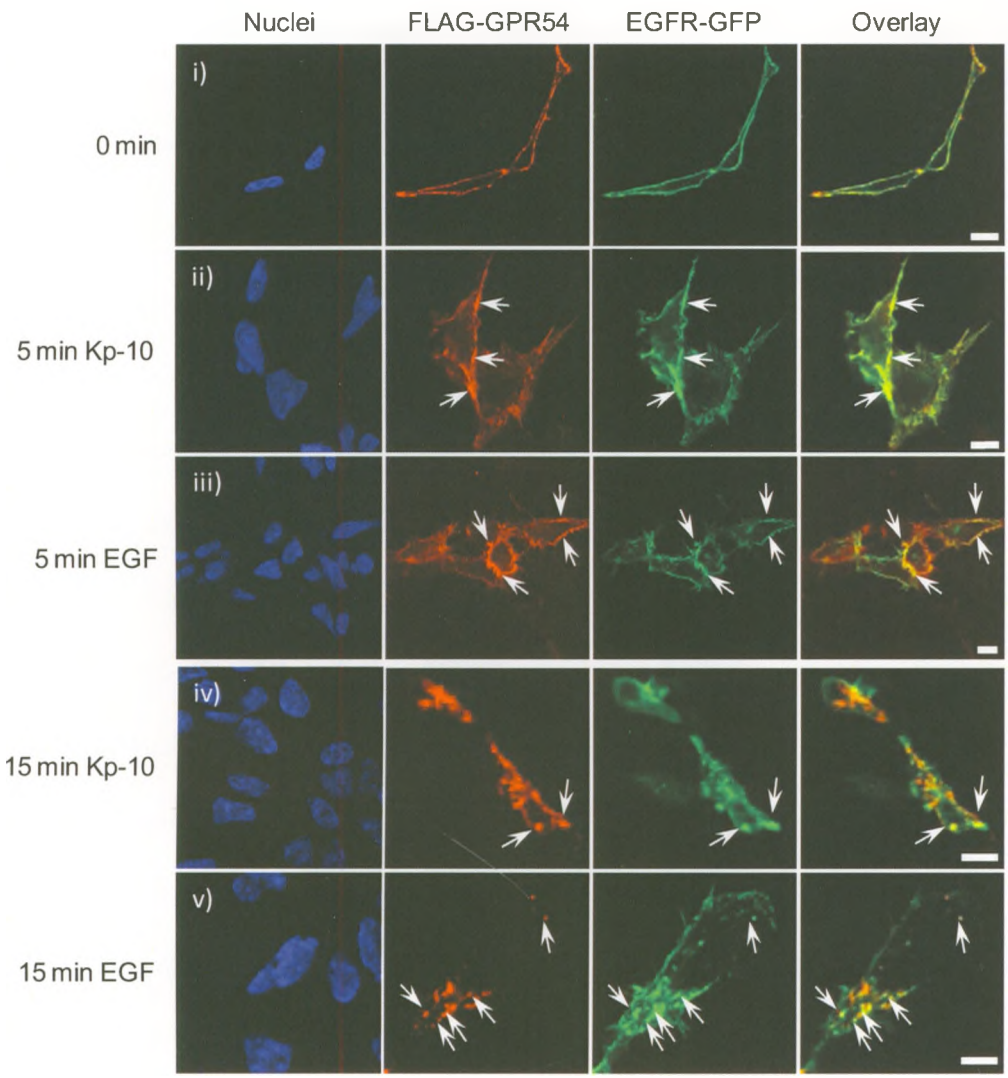

Fig. 2.19. Co-localization of FLAG-GPR54 and EGFR-GFP in HEK 293 cells.

HEK 293 cells were transiently transfected with FLAG-GPR54 and EGFR-GFP and allowed to grow for 48 hours on glass coverslips. Cells were then incubated in the absence (0 min, i) or presence of Kp-10 (10 nM, ii, iv) or EGF (10 ng/mL, iii, v). Cells were then subjected to immunofluorescence staining for FLAG (red) and nuclei staining with 0.1% Hoechst 33258 dye (blue). EGFR-GFP shows as green. (i) HEK 293 cells display strong cell membrane localization of GPR54 (*see arrows*). (ii, iv) Treatment of cells with 10 nM Kp-10 within 5 and 15 minutes induces FLAG-GPR54 and EGFR-GFP internalization, with visible colocalization (*yellow, see arrows*). (iii, v) Treatment of cells with 10 ng/mL EGF for 5 and 15 minutes results in the internalization of both FLAG-GPR54 and EGFR-GFP, with visible colocalization (*yellow, see arrows*). Representative micrographs from three independent experiments. *Scale bar, 10μm.*

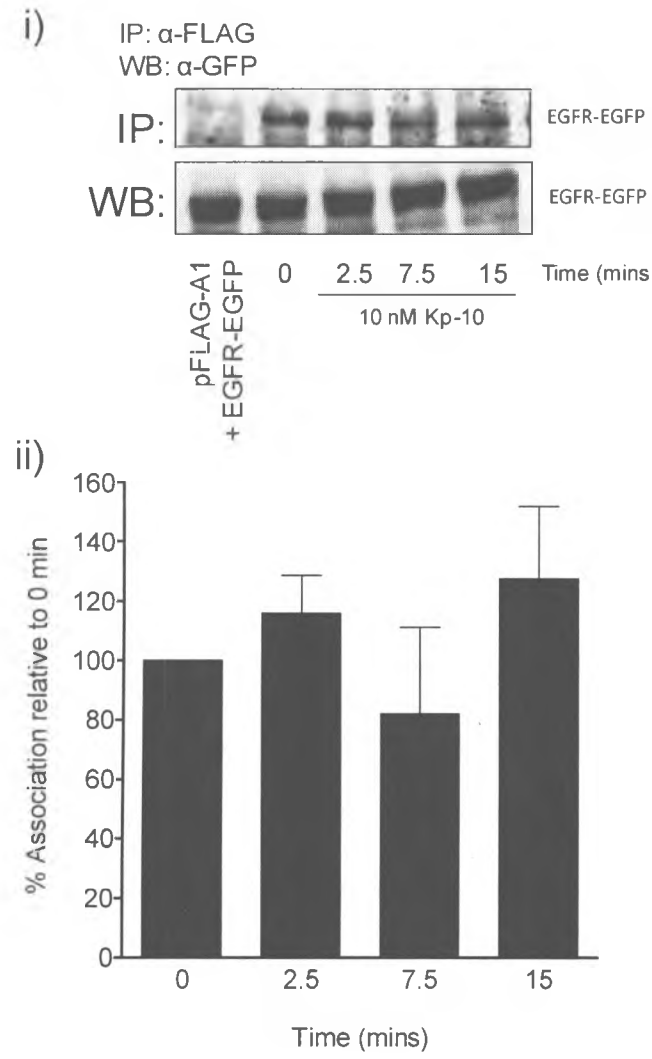

Fig. 2.20. GPR54 associates with EGFR.

i) FLAG-GPR54 co-immunoprecipitates EGFR in HEK 293 cells transiently expressing FLAG-GPR54 and EGFR-GFP. EGFR was detected even in untreated immunoprecipitates and appears to respond to Kp-10 treatment.

ii) Densitometric analysis of Western blots for EGFR protein expression using VersaDoc gel documentation system. Columns represent percent association relative to 0 min (100%) ± SD from at least three independent experiments.

2.4. Discussion

Our data suggest that kisspeptin/GPR54 signaling is pro-migratory and pro-invasive in breast cancer cells, supporting recent studies that kisspeptin signaling may correlate positively with breast tumor progression and metastatic potential (5, 6). We show for the first time Kp-10 results in the transactivation of EGFR in a Src-dependent manner and stimulates MMP-9 secretion and activity. Furthermore, we have identified that GPR54 complexes with EGFR under basal conditions, and this interaction is modulated by Kp-10.

Significant progress has been made in understanding the complexity of GPCR-receptor tyrosine kinase signaling over the last decade. Once seen as isolated receptors connecting extracellular signals to the activation of G proteins, GPCRs are now regarded as complex receptors capable of initiating a vast array of signaling pathways, including G protein-dependent and -independent signaling, binding various scaffolding molecules, and interacting both directly and indirectly with other receptor families. Intensely studied are the transactivation mechanisms of EGFR by GPCRs due to their involvement and important roles in not only normal physiological functions, but also in pathophysiology.

Elevated expression of EGFR is associated with highly aggressive and metastatic cancers, including cancer of the breast (28). A member of the ErbB family of receptor tyrosine kinases, EGFR has been characterized as a proto-oncogene. Upregulation of EGFR, the other ErB receptors, and their cognate ligands, mutations in structure, and changes in downstream signaling partners all contribute to resistance mechanisms employed by cancer cells. This ability of

EGFR, to function despite treatment with tyrosine kinase inhibitors and targeting antibodies, poses a major problem for cancer therapy (28). An understanding, therefore, of EGFR signaling cascades is vital in the production of specific therapies targeting metastasis.

Our findings reveal that unlike their role in other cancer cell types, in human breast cancer MDA-MB-231 and Hs578T cells, Kp-10 positively regulates breast cancer cell motility and invasion. Kp-10 does not act as a chemoattractant but potentiates migration on Matrigel-invasion towards 10% serum. Additionally, inhibition of EGFR through the use of tyrphostin AG1478 resulted in significant inhibition of invasiveness which was not abrogated when cells were treated with Kp-10, suggesting that this process is dependent on EGFR activation. MDA-MB-231 FLAG-GPR54 cells exhibited strong invasion towards EGF, that was blocked by AG1478, as has been previously reported with the parental MDA-MB-231 line (29). Interestingly, Kp-10 was able to significantly increase the invasion of AG1478-pretreated cells towards EGF, suggesting that Kp-10 treatment is able to overcome at least some of the AG1478-mediated EGFR inhibition. However, AG1478 treatment of Hs578T cells did not decrease their invasion towards 10% serum, although we did observe that Kp-10 transactivates EGFR robustly in this cell line. Hs578T cells are derived originally from primary sarcoma (in contrast to MDA-MB-231, which are derived from invasive ductal carcinoma), and are less metastatic than MDA-MB-231 cells (30). In addition, we and others have shown that in comparison to human cancer cell lines like the MDA-MB-231 and MDA-MB-435, Hs578T express significantly less EGFR (31). Thus, it appears that

GPR54 may function differently in these cells and GPR54 may regulate migration and invasion independently of EGFR. Additionally, some cells exhibit innate resistance to tyrphostins, like AG1478, which may be a contributing factor in the Hs578T cells (32).

We found that kisspeptin stimulated MDA-MB-231 invasion *via* a β-arrestin 2-dependent mechanism. Knockdown of endogenous β-arrestin 2 in MDA-MB-231 cells significantly inhibited Kp-10-induced invasion. We have previously shown that the β-arrestins play important roles in regulating breast cancer cell invasion towards the bioactive lipid lysophosphatidic acid (LPA) (17).

In highly aggressive cancer cells such as MDA-MB-231 and MDA-MB-435, RhoA plays a dominant role in regulating cell migration (33). Kp-10 has been shown to stimulate Rho-dependent stress fiber formation in Chinese hamster ovary cells expressing GPR54 (3). Constitutively active Rho mutant V14 was shown in MDA-MB-231 cells to not only increase basal migration, but also to enhance basal EGFR activation (34). Activation of RhoA and dephosphorylation of FAK, processes responsible for EGF-induced cell retractile morphological changes, occurs upon EGF treatment in EGFR-overexpressing cells (35). Thus, kisspeptin treatment of MDA-MB-231 cells may promote migration by stimulating RhoA activation and FAK dephosphorylation *via* EGFR.

A number of GPCRs and their various ligands have been shown to increase the migration and invasion of cancer cells through a mechanism involving transactivation of EGFR. Protease-activated receptor-1 (PAR-1) (36), LPA_1-R (18), bombesin (37), CXCR4 (38), VPAC1 and VPAC2 (39) are just

several in which ligand-binding may result in EGFR activation and enhanced migration and invasion. EGFR activation can stimulate cancer invasion and cytoskeletal rearrangement signaling *via* the small GTPase Arf6 to recruit cortactin, paxillin, and integrin (40).

We further show that kisspeptin-mediated activation of GPR54 enhances the invasive abilities of MDA-MB-231, MDA-MB-231 FLAG-GPR54, and Hs578T cells in three-dimensional morphogenesis/invasion assays, in what could be a metalloprotease-involved mechanism. Our data reveals that Kp-10 can stimulate invasiveness by stimulating the production of matrix metalloproteases. Using gelatin zymography we show that treatment of MDA-MB-231 cells with Kp-10 results in an increase in MMP-9 secretion into the surrounding media. To date, this is the first demonstration that kisspeptins stimulate MMPs. Conversely, it has been reported that expression of *KISS1* in HT-1080 human fibrosarcoma cells where kisspeptin signaling is anti-invasive results in the negative regulation of MMP-9 production (41). Other studies have implicated a role for ADAM17 in regulating the invasive potential of MDA-MB-231 cells in three-dimensional invasion/morphogenesis assays, *via* a EGFR-PI3K-AKT pathway (42). Transactivation of EGFR by GPCRs has been shown to enhance migration and invasion of several cancer cell lines, including breast, glioblastoma, bladder and kidney (11, 43, 44). Furthermore, studies using the spontaneously immortalized human breast epithelial cell line MCF-10A overexpressing ErbB2 revealed enhanced invasion resulting from an increase in MMP-9 expression (45). However, knock-down of MMP-9 significantly inhibited cell invasion, suggesting

that it was not the overexpressed ErbB2, but MMP-9 that was responsible for their enhanced invasion (45).

We demonstrate that Kp-10 signaling through endogenous GPR54 in MDA-MB-231 and Hs578T cells transactivates endogenous EGFR. In MDA-MB-231 cells we show that this transactivation works *via* mechanisms involving Src as inhibition of Src abrogates EGFR transactivation. MDA-MB-231 cells exhibited a peak in phosphorylation at 5 min post-treatment, followed by a re-activation of the receptor. Hs578T cells, however, displayed a steady rise in pEGFR, peaking between 5 and 15 minutes. Previously, it has been shown that c-Src may lie upstream of EGFR and directly regulate its activation (46). Thus, the cyclic nature of EGFR activation in the MDA-MB-231 parental cells may partly be due to Src-mediated activation of EGFR, followed later by EGFR pro-ligand release. Interestingly, we have also reported that stimulation of MDA-MB-231 cells with Kp-10 results in cyclic pattern of ERK1/2 activation (21). Furthermore, depletion of β-arrestin 2 resulted in the abrogation of Kp-10-mediated ERK1/2 activation in MDA-MB-231 cells (21).

We also show, for the first time, that GPR54 interacts with EGFR by co-immunoprecipitation studies. To date, a limited number of GPCRs have been shown to interact with EGFR. Recently, it was discovered that the vasopressin 1A receptor, a G_q–coupled GPCR also interacts with EGFR by co-immunoprecipitation studies, although how this interaction affects signaling of either the vasopressin receptor or GPR54 is currently unknown (47). More thoroughly studied is the interaction of the β1-adrenergic receptor (β1-AR) with

EGFR. Under basal conditions, FLAG-β1-AR was found to associate with EGFR by co-immunoprecipitation (48). However, over-expression of the G protein-coupled angiotensin receptor ($AT_{1A}R$), which can also transactivate EGFR, does not cause it to associate with EGFR, suggesting that this interaction is unique to a subset of GPCR (48). In this same study, fluorescence resonance energy transfer (FRET) was then performed to determine if ligand stimulation could regulate association between the two receptors. Of interest, treatment with the β1-AR agonist, isoproterenol, did not seem to affect FRET efficiency when compared to untreated cells, but EGF stimulation resulted in five-fold greater loss in FRET when compared to isoproterenol treatment (48). Furthermore, we found that EGF treatment promotes the internalization of GPR54 in breast cancer cells and that GPR54 and EGFR co-localize partially upon treatment of Kp-10 or EGF. Similar observations have been reported with β2-adrenergic receptor where co-localization with EGFR was observed in response to isoproterenol stimulation (49). Interestingly, a previous study in HEK 293 cells showed that treatment with EGF causes significant internalization of the δ-opioid receptor (50).

Targeted therapies against EGFR in cancer treatment have been disappointing due to the robust nature of EGFR signaling. Elucidation of novel signaling pathways may uncover new drug targets. Our work provides the first evidence that GPR54 signaling positively regulates breast cancer cell invasiveness, *via* EGFR crosstalk that involves Src, MMPs and β-arrestins. Current ongoing studies are looking to identify the binding domains between both receptors, and further examine the nature of the crosstalk. These studies will

reveal whether or not combination therapy by targeting GPR54 as well as EGFR in breast cancer is worth considering.

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FOOTNOTES/ACKNOWLEDGEMENTS

The authors would like to acknowledge Mr. Timothy Li for his invaluable assistance.

The abbreviations used are: EGFR, epidermal growth factor receptors; ADAM, a disintegrin and metalloprotease; TIMP, tissue inhibitor of metalloproteases; GFP, green fluorescent protein.

CHAPTER 3: GENERAL DISCUSSION AND CONCLUSIONS

3.1. General discussion and conclusions

Metastasis of cancer cells to distant sites and the formation of secondary tumours is the leading cause of cancer-related deaths. It is now well-established that EGFR plays a critical role in breast cancer tumour progression and metastasis. We have identified a novel player, namely the G protein-coupled receptor GPR54, that transactivates EGFR to regulate breast cancer cell migration and invasion, processes implicated in metastasis.

Our data reveal that the actions of the kisspeptins on breast cancer cells are pro-migratory and pro-invasive, in contrast to their role in numerous other cancer cell lines, such as melanoma, pancreatic, renal, and thyroid, each of which showed a decrease in migration (1-4). We demonstrated that Kp-10 results in the transactivation of EGFR, necessary for Kp-10-stimulated breast cancer cell invasiveness. Furthermore, we have shown that EGF can stimulate GPR54 endocytosis in breast cancer cells, and have identified a novel interaction between EGFR and GPR54 that is modulated by Kp-10.

Significant progress has been made in understanding the complexity of GPCR-RTK signaling over the last decade. Once seen as isolated receptors connecting extracellular signals to the activation of G proteins, GPCRs are now regarded as complex receptors capable of initiating a vast array of signaling pathways, including G protein-dependent and -independent signaling, involvement with scaffolding molecules, and interacting both directly and indirectly with other receptor families. The transactivation of EGFR by GPCRs

plays important roles in both normal physiological functions and in pathophysiological conditions such as cancer.

Elevated expression of EGFR is associated with highly aggressive and metastatic behaviour in cancers, including cancer of the breast (5). A member of the ErbB family of receptor tyrosine kinases, EGFR has been characterized as a proto-oncogene. Upregulation of EGFR, the other ErB receptors, their cognate ligands, mutations in structure, and changes in downstream signaling partners all contribute to drug resistance mechanisms employed by cancer cells. This ability of EGFR and its other family members, to function despite treatment with tyrosine kinase inhibitors and targeting antibodies, poses a major problem for treatment against this receptor (5). Thus, a better understanding of EGFR function and signaling cascades is vital to the production of specific therapies targeting metastasis.

Our findings reveal that unlike their role in other cancer cell types, Kp-10 positively regulates breast cancer cell motility and invasion. We showed that Kp-10 does not act as a chemoattractant to stimulate breast cancer cell invasion, but acts *via* EGFR to promote this process. We used two well-established invasive human breast cancer cell lines, MDA-MB-231 and Hs578T. We had previously shown that MDA-MB-231 cells express GPR54 (6). Both MDA-MB-231 and Hs578T cell lines expressed EGFR, although a much higher protein level was detected in MDA-MB-231 cells. Inhibition of EGFR through the use of tyrphostin AG1478 significantly inhibited invasion of MDA-MB-231 cells stably expressing FLAG-tagged GPR54. This effect was not abrogated when cells were treated with

Kp-10, suggesting that invasion of these cells appears to be dependent on EGFR activation, or that the concentration of Kp-10 was too low to overcome EGFR inhibition.

Similar to our observations, a number of other GPCRs have been shown to increase the migration and invasion of cancer cells through a mechanism involving the transactivation of EGFR. These include the protease-activated receptor-1 (PAR-1) (7), LPA_1-R (8), bombesin (9), CXCR4 (10), VPAC1 and VPAC2 (11). Studies have shown that activation of EGFR results in the production of second messenger PIP2, which allows for changes in filamentous actin assembly and increases cell deformability. EGFR activation can stimulate cancer invasion signaling *via* the small GTPase Arf6. Upon EGFR activation, tyrosine 1068 and 1086 are phosphorylated and subsequently function as docking sites for GEP100, an Arf6 guanosine exchange factor. GEP100 is then able to activate GDP-Arf6 to GTP-Arf6, a mechanism that recruits AMAP1, an Arf6 effector protein. Active AMAP1 recruits cortactin, paxillin, and integrin, and through a process of cytoskeletal rearrangement, helps form invasive structures (12).

Our studies using EGF as a chemoattractant showed that MDA-MB-231 FLAG-GPR54 cells exhibited strong invasion towards EGF and AG1478 treatment resulted in the significant inhibition of this invasion. This has been previously demonstrated with non-transfected MDA-MB-231 cells (13). It is interesting to note, however, that Kp-10 was able to significantly increase the invasion of AG1478-pretreated cells towards EGF, suggesting that Kp-10

treatment is able to overcome the effects of the inhibitor. AG1478 treatment of Hs578T cells did not decrease their invasion towards 10% serum. This could be due to innate resistance these cells may have to tyrphostins, like AG1478, and thus resistance to the tyrosine kinase inhibitor may explain the lack of effect in these cells, as has been reported in other cancer cell lines (14).

Kisspeptins have previously been shown to stimulate cytoskeletal changes required for cell migration. Kp-10 stimulation of Chinese hamster ovar cells transfected with GPR54 resulted in stress fiber formation, an effect that was abolished through the use of C3 exoenzyme, suggesting the involvement of the Rho subfamily of G proteins (15). In renal carcinoma cell lines Caki-1 and ACHN, treatment with metastin (Kp-54) resulted in inhibited migration in wound healing and Matrigel invasion assay (16). Metastin treatment also resulted in the vast remodelling of the cytoskeleton, with excessive stress fiber formation and focal adhesions (16). Treatment of the cells with the ROCK inhibitor Y-27632 prominently reduced actin and stress fiber formation (16). This study revealed that although kisspeptins/GPR54 activated Rho and ROCK to generate stress fiber formation and adhesion, Rac1 is inhibited, resulting in the inhibition of lamellipodia and filopodia formation (16).

Activation of RhoA has generally been considered as linked to the formation of stress fibers and focal adhesions, while Cdc42 and Rac1 are viewed as mediators of filopodia and lamellipodia formation, respectively (17). However, the function of each RhoGTPase is not only a condition-dependent phenomenon, but also a cell-specific mechanism. Although in most cell types Rac1 inhibition

results in decreased migration, colon carcinoma, rat fibroblasts, and macrophages deficient in Rac1 showed no effect on migration (17). Interestingly, overexpression of Rac1 in Madin-Darby canine kidney (MDCK) epithelial cells results in the inhibition of migration and invasion. Recent studies using the Rac1 inhibitor NSC23766 or depleting Rac1 by siRNA, showed that inhibition of Rac1 results in significantly enhanced migration in highly metastatic breast cancer cell line MDA-MB-231 (17). Thus, it is possible that in MDA-MB-231 cells, activation of GPR54 by the kisspeptins may result in the inhibition of Rac1, promoting the migration of these cells (16). However, Rac1 depletion and inhibition experiments in Hs578T have shown a decrease in migration (17). Rac1 knock-down experiments in MDA-MB-231 showed a concurrent increase in RhoA activation, while no change was seen in Hs578T cells (17). It was proposed then, that since the Rho GTPases maintain a fine balance in a particular cell type, disturbances in Rho GTPase results in different outcomes (17).

Constitutively active Rho mutant V14 was shown in MDA-MB-231 cells to not only increase basal migration, but also to enhance basal EGFR activation (18). Activation of RhoA and dephosphorylation of FAK, processes responsible for EGF-induced cell retractile morphological changes, occurs upon EGF treatment in EGFR-overexpressing cells (19). Thus, kisspeptin may activate a two-fold mechanism that promotes MDA-MB-231 migration: through the inhibition of Rac1 and the subsequent upregulation of RhoA, and RhoA activation and FAK dephosphorylation *via* downstream EGFR pathways. Hs578T cells were derived originally from primary sarcoma (in contrast to MDA-MB-231, which

were derived from invasive ductal carcinoma), and are less tumourigenic and metastatic than MDA-MB-231 cells (17). The metastatic MDA-MB-231 and MDA-MB-435 cells express more EGFR compared to Hs578T cells (20). Hs578T cells also express very low levels of the other ErbB receptors, especially ErbB2 and ErbB3, which are known to promote EGFR activity and enhance the metastatic potential of cancer cells (20). Activation of GPR54 signaling in Hs578T cells thus may not share the same pathways as in the MDA-MB-231 cells to promote migration and invasion.

β-arrestins are well known for the role in GPCR regulation and as a multifunctional scaffolding proteins (21). Studies from our laboratory have shown that the expression of β-arrestins is high in metastatic cancer cells (MDA-MB-231 and MDA-MB-435) compared to non-malignant epithelial cells (22). Additionally, we found elevated levels of β-arrestin 2 in patient breast tumors from advanced stages of the disease (22). We have also reported that β-arrestin regulates MDA-MB-231 cell invasiveness in response to the bioactive lipid lysophosphatidic acid (22). In another recent study we reported that β-arrestin 2 regulates GPR54 signaling in MDA-MB-231 cells (6). Thus we examined whether or not β-arrestin 2 regulates Kp-10-mediated breast cancer cell invasion (6). We found that depletion of endogenous β-arrestin 2 decreases the ability of MDA-MB-231 cells to invade towards media with 10% serum, in the presence or absence of Kp-10. This indicates that β-arrestin 2 is implicated in regulating MDA-MB-231 cell invasiveness when serum is used as a chemoattractant, and addition of Kp-10 to the media with 10% serum has no further effect. Thus the

role of β-arrestin in regulating breast cancer cell invasion does not appear to be specific to Kp-10-mediated invasion. This is not surprising since β-arrestins associate with numerous proteins that are key regulators of cytoskeletal rearrangement necessary for cell migration such as actin filament-severing protein cofilin, MAPK, Rho GTPases (23, 24), and can regulate breast cancer invasion *via* Ral GTPases (22)

We also demonstrate that Kp-10 enhances the invasive ability of MDA-MB-231, MDA-MB-231 FLAG-GPR54, and Hs578T cells in three-dimensional invasion assays, in what could be a metalloprotease-involved mechanism. Previous studies have shown that MDA-MB-231 and Hs578T cells do not respond to the proliferative effects of EGF, suggesting that the observed increase in invasion was not a product of increased cell proliferation (13, 25). In concordance, previous work has shown that ADAM17-mediated shedding of TGF-α signaling through the EGFR-PI3K-Akt pathway contributes to the high invasive potential of MDA-MB-231 cells in three-dimensional invasion/morphogenesis assays (26).

We also show for the first time that treatment of MDA-MB-231 cells with 10 and 100 nM Kp-10 results in an increase in the secretion and activity of MMP-9 using gelatin zymography. MMP-9 has been shown to play a critical role in the degradation of the extracellular matrix and its expression is correlated with the aggressiveness of various tumours (3). Previous studies done using cancer cell lines where kisspeptins have an anti-metastatic effect have shown that Kp-10 decreases MMP-2 and MMP-9 mRNA, and increases tissue inhibitor of

metalloprotease-1 (TIMP-1) mRNA levels (27). Additionally, expression of *KISS1* in HT-1080 human fibrosarcoma cells resulted in the negative regulation of MMP-9 production (27). It is important, therefore, to avoid generalizations when studying the kisspeptins and GPR54, as their actions seem to differ depending on the cell context.

We demonstrate that Kp-10 signaling *via* GPR54 transactivates EGFR in a Src-dependent manner, as inhibition of Src abrogates EGFR transactivation. It is of interest to note that the dynamics of transactivation differ in the respective breast cancer cell lines. The highly metastatic human breast cancer MDA-MB-231 cells show transactivation of EGFR within 1 minute, with a progressive rise in phosphorylated EGFR, peaking at 5 and 30 minutes, with a fall at 15 minutes. Hs578T cells displayed a steady rise in phosphorylated EGFR, peaking between 5 and 15 minutes. We have previously reported a similar cyclic pattern of ERK activation in MDA-MB-231 cells (6). Upon stimulation with 100 nM Kp-10, an increase in pERK1/2 was seen at 2.5 minutes, followed by a decrease at 5 minutes, and a further increase at 10 minutes, peaking at 30 minutes post-treatment (28). The decrease in pEGFR 15 minutes post-treatment seen in MDA-MB-231 cells has been similarly demonstrated in studies of EGFR transactivation by the vasopressin and neurotensin receptors (29). In a study by Rozengurt *et al.*, it was demonstrated that EGFR transactivation by vasopressin or neurotensin (both G_q-coupled GPCRs) could be enhanced by the use of PKC inhibitors. The researchers demonstrated that a rapid dephosphorylation of EGFR occurs with treatment by neurotensin, but the use of GF109203X, a PKC inhibitor, resulted in

a pronounced EGFR transactivation at 2.5 and 10 minutes post-neurotensin treatment. After selectively inhibiting certain PKC isoforms, the authors concluded that PKCα is responsible for mediating feedback inhibition of G_q-coupled GPCR-induced EGFR transactivation (29). GPR54 has been shown to activate PKC (30). Therefore, it is feasible that the decrease in EGFR phosphorylation we have observed at 15 minutes in MDA-MB-231 cells may be the result of increased PKCα-mediated feedback inhibition of GPR54-stimulated EGFR (30).

A recent study had noted that the C-terminus of GPR54 is able to bind to protein phosphatase 2A (PP2A) (28). The regulatory B-type subunit of PP2A (PR130) has been noted to interact with EGFR (31). In COS-7 cells expressing PR130 siRNA, it was noted that EGF stimulation of EGFR resulted in enhanced desensitization and degradation of EGFR through increased proteosome-dependent EGFR degradation and increased interaction of EGFR with E3 ligase c-Cbl (31). In addition, in these knock-down cells, faster inactivation of downstream EGFR signaling partners Akt and ERK1/2 was seen (31). These effects could be rescued by the introduction of RNA interference-resistant Myc-PR130. Thus, the authors suggested that PR130, and in turn, PP2A holoenzyme $PP2A_{T130}$, helps prevent degradation of EGFR and promotes its recycling (31). Dephosphorylation of EGFR seen in MDA-MB-231 cells may, therefore, act to protect EGFR from receptor degradation, promoting its signaling in response to Kp-10 stimulation of GPR54.

Transactivation of EGFR by GPCRs is further complicated by the fact that the same GPCR ligand is able to induce activation of a different subtype of the ADAMs depending on cell type. In addition, the same ligand may activate a different ADAM subtype, which then may release a different EGFR ligand. For instance, LPA induces EGFR transactivation *via* HB-EGF shedding in several cancer cell lines through the activation of ADAM10 in ACHN cells, whereas ADAM17 is responsible in CaKi2 and A498 cells (32). Thus, although we have shown that Kp-10 enhances MMP-9 secretion, it is currently unknown which ADAM is implicated in GPR54-induced EGFR transactivation in breast cancer cells, and will be the subject of future studies.

We next investigated whether or not GPR54 interacts with EGFR and found that both receptors constitutively associate with each other in unstimulated cells, and that Kp-10 modulated this complex formation. Since specific GPR54 antibodies are lacking, we used FLAG-GPR54 receptor for the co-immunoprecipitation studies and detected the presence of EGFR in the immunoprecipitated complex, even under basal conditions. It is possible that β-arrestin or GRK2 scaffolds GPR54 as we have previously shown, resulting in receptor populations that may interact with other binding partners, like EGFR in unstimulated conditions (6).

To date, a limited number of GPCRs have been shown to associate with EGFR. Recently, the vasopressin 1A receptor, a G_q-coupled GPCR, was shown to interact with EGFR, although how this interaction affects signaling of either the vasopressin receptor or EGFR is currently unknown (33). More thoroughly

studied is the interaction of the β1-adrenergic receptor (β1-AR) with EGFR. Under basal conditions, immunoprecipitation of FLAG-tagged β1-AR showed presence of EGFR in the immunoprecipitate (34). In contrast, overexpression of the angiotensin receptor ($AT1_AR$), which is known to transactivate EGFR, was not sufficient to induce association between $AT1_AR$ and EGFR, suggesting that this interaction is unique to a subset of GPCRs (34).

We also found that EGF treatment promotes the internalization of GPR54 and that GPR54 and EGFR co-localize upon treatment with Kp-10 or EGF. This suggests that GPR54 and EGFR may exist in a signalosome, involved in an association that may promote the efficiency of their signaling as has been previously observed (35). Similar observations have been made with the β2-adrenergic receptor (β2-AR) and EGFR. Isoproterenol, a β2-AR agonist, mediated internalization of EGFR, with subsequent co-localization of the β2-AR and EGFR (36). In this study, the authors noted that both β2-AR and EGFR associate with GRK and this association is necessary not only for the formation of the β2-AR-EGFR complex, but efficiency in signaling to downstream partners like ERK1/2 (36). Additionally, it has been shown that GRK-mediated phosphorylation of the C-terminal residues of the β2-AR recruit β-arrestin 1/2 (34). Expressing a mutant form of the β1-AR unable to bind GRK (GRK^- β1-AR), and thus β-arrestin 1/2, showed no interaction with EGFR upon catecholamine stimulation. Furthermore, knockdown of β-arrestin 1/2 also prevented EGFR interaction with catecholamine treatment (34). It was also noted that β-arrestin 1/2 recruitment to the β1-AR-

EGFR complex is required not only for ERK1/2 targeting, but also activation (34).

Another study has shown that treatment with EGF causes significant internalization of the G protein-coupled μ- and δ-opioid receptors (37). This study revealed that the interaction of the μ- and δ-opioid receptors with EGFR is dependent on GRK2 phosphorylation by EGFR (37). We have previously shown that GPR54 constitutively interacts with GRK2 (6). Whether this type of mechanism is responsible for the EGF-induced internalization of GPR54 in HEK 293 and MDA-MB-231 cells is yet to be tested. Our future studies will determine whether or not EGFR activation by Kp-10 in breast cancer cells correlates with complex formation between these two receptors, and whether β-arrestins and GRK2 are part of the complex.

In vitro studies of kisspeptin/GPR54 signaling in cancer cells have generally shown anti-migratory and anti-invasive actions. Here we show for the first time that treatment of breast cancer cells with a kisspeptin (Kp-10) results in enhanced migration and invasion. We also propose a mechanism by which this is may be occurring, through the epidermal growth factor receptor. How signaling of the kisspeptins and GPR54 differs in breast cancer, compared to in cancers where kisspeptins show anti-migratory and anti-invasive effects (for example renal, pancreatic, thyroid, and melanoma) is still not fully known. A recent study showed that GPR54 is differentially spliced in Sengalese sole fish (38). Inclusions of Intron III results in a trunctated receptor isoform lacking the three later trans-membrane domains (38). Although this study did not look at human GPR54, they

did observe high conservation between human and teleost GPR54 amino acid sequences (> 72%) (38). Thus, this study points to a possible mechanism by which the observed differences in cell lines and tumors are occurring. Differential splicing of GPR54 in cancer cells may result in altered downstream signaling, helping to reconcile the variability seen in GPR54 activation amongst different cancer cells. Furthermore, naturally occurring mutations in GPR54 exist, and include missense (L148S), nonsense (R331X), nonstop (X399R) mutations (39). Our previous work demonstrated that the R331X mutant of GPR54 binds both GRK2 and β-arrestin 2 more strongly under basal conditions than the wild-type receptor, suggesting that some mutants of GPR54 may exhibit different signaling characteristics due to their interactions with downstream signaling components (6). Recently, our group showed that the β-arrestins are upregulated in human breast tumors (22). Thus, it is also possible that GPR54's downstream binding partners, like β-arrestin 2, are differentially expressed in certain cancers, promoting or abrogating its signaling, resulting in different components of the pathway to be preferentially activated.

Targeted therapies against EGFR in cancer treatment have been disappointing due to the robust nature of EGFR signaling. Elucidation of novel signaling pathways may uncover new drug targets. Our work provides the first evidence that GPR54 signaling positively regulates breast cancer cell invasiveness *via* EGFR transactivation that involves Src, MMPs and β-arrestins. Current ongoing studies are looking to identify the binding domains between both receptors, and will further examine the nature of the crosstalk. These studies will

reveal whether or not combination therapy by targeting GPR54 as well as EGFR in breast cancer is worth considering.

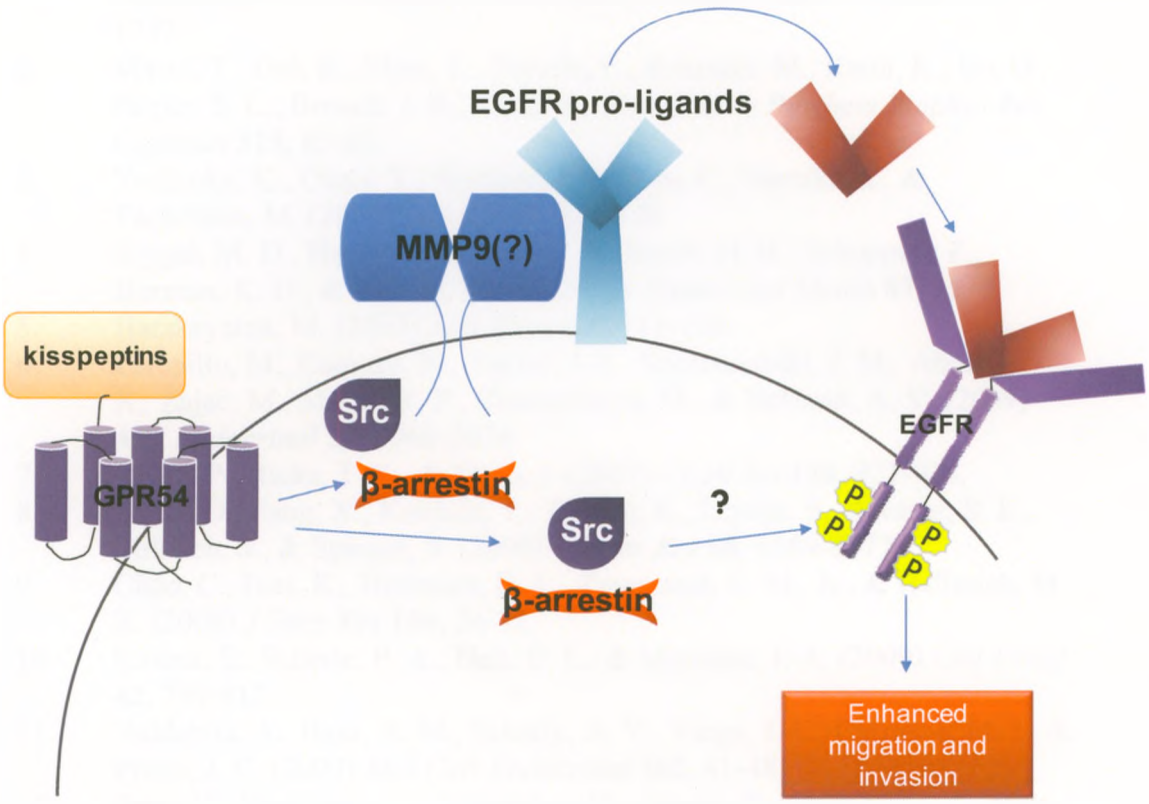

Fig. 3.1. Proposed GPR54-mediated transactivation pathway of EGFR in breast cancer. Activation of GPR54 by the kisspeptins results in the transactivation of EGFR, through a Src-mediated pathway. Release of EGFR pro-ligands by MMP-mediated shedding may be involved. GPR54-mediated activation of EGFR leads to enhanced migration and invasion of breast cancer cells. β-arrestin 2 is required for GPR54-enhanced invasion. It is not known, however, whether β-arrestin 2 is directly involved in Src or MMP activation.

3.2. References

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